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. The 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.

The β transforming growth factors (TGFβ) (which exist in three isoforms, TGFβ1–3) are a family of closely related regulatory proteins with a potentially central role in both physiological and pathological fibrosis; they are chemotactic for fibroblasts, potent inducers of synthesis of collagen and other matrix proteins, and inhibitors of extracellular matrix breakdown. Subcutaneous injection of TGFβ induces local fibrosis and neutralisation of TGFβ activity inhibits the formation of fibrous tissue. TGFβ has the capacity to induce further synthesis of TGFβ, suggesting a potential positive feedback process in pathological fibrosis.
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).

TGFB1 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The 25 kDa form of TGFB1 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 TGFB (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 TGFB1 was detected with a chicken anti-TGFB1 (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 TGFB1 (R and D Systems). Data were processed using Grafit software (Microposoft). 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 TGFB1 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 TGFB1, the chemiluminescence signal obtained from TGFB1 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 TGFB1 and not the latent high molecular weight TGFB (TGFB bound to a carrier protein) which is present in large amounts in platelets. This has important implications for plasma and serum assay of TGFB1: assays capable of detecting latent TGFB would be prone to error because of detection of TGFB released from platelets during separation, storage, or, in the case of serum, during clotting. Although previous studies have shown that latent TGFB is present in normal plasma, no TGFB could be detected in normal plasma using our assay, even when blood was stored unseparated at room temperature for up to six hours. All TGFB1 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.

TGFB2 ELISA

TGFB2 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 TGFB1 assay; like that assay, the TGFB2 ELISA detects only the 25 kDa form of TGFB2, but not latent or complexed forms. The control group for the TGFB2 assay comprised 20 healthy controls randomly selected from the larger control group used in the TGFB1 study.

PROCOLLAGEN III AMINOPEPTIDE RADIOIMMUNOASSAY

Serum procollagen III aminopeptide (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 2.24–4.76 µg/ml) agrees with that provided by the kit manufacturer.

Results

TGFB1 was detected in plasma from six of 39 patients with systemic sclerosis (median 1590 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 TGFB1 in the plasma from six patients in the systemic sclerosis group by 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 TGFB1, but two of the
patients with increased concentrations had progressive diffuse disease (the table summarises their clinical details). The patient with disease of shortest duration had the greatest concentration of TGFβ1 and also a marked increase in PIIINP concentration (the greatest within the limited systemic sclerosis group). However, TGFβ1 was also detected in three patients with disease duration greater than the median of eight years. Three of the six patients with detectable TGFβ1 had evidence of interstitial pulmonary disease (and of the six patients with lung disease and no detectable TGFβ, five were receiving interferon alfa therapy or cyclophosphamide, or had received interferon alfa within the previous 12 months). However, the association between pulmonary disease and detectable TGFβ was not statistically significant (p = 0.13, Fisher’s exact test). TGFβ1 was not detected in any patient receiving interferon alfa, cyclophosphamide, D-penicillamine or corticosteroids.

No TGFβ2 was detected in plasma from any of the systemic sclerosis patients or the 20 healthy controls.

Concentrations of PIIINP were significantly greater in patients with systemic sclerosis compared with controls (fig 2), but no obvious relationship was detected between increased PIIINP and increased TGFβ (table).

**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 Coupes, 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 sclerodermatous 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

**Figure 2** Serum PIIINP concentrations in patients with diffuse systemic sclerosis (SS) (n = 11), limited systemic sclerosis (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 v controls (p = 0.0018) and Raynaud’s (p = 0.0045) and diffuse systemic sclerosis v 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.
high concentrations in certain forms of extra-
cellular matrix\(^9\). 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\(\beta1\) and \(\beta2\) 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 to
greater concentrations of TGF\(\beta\) in systemic
sclerosis (between 40 and 130 pg/ml).\(^{17}\)
Our findings are in conflict with those of Higley
et al.,\(^{18}\) who reported no significant difference in
plasma concentrations of TGF\(\beta1\) between
systemic sclerosis patients and controls, using an
ELISA assay with a limit of detection for the
active form of TGF\(\beta1\) of approximately 1 ng/
ml (10 times less sensitive than the assay used
here). However, they also reported detectable
(>1 ng/ml) TGF\(\beta1\) in 26% of healthy controls
and 75% of patients with Raynaud's
phenomenon. The presence of the active form
of TGF\(\beta1\) in healthy peripheral blood in
concentrations well within the biologically
active range would not be anticipated from
previous studies.\(^{17} 19 21\) 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\(\beta1\), but perhaps
also detected larger molecular weight forms of
TGF\(\beta1\) 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\(\beta\).\(^{18}\) Since
platelet physiology is disturbed in systemic
sclerosis,\(^{50}\) and endothelial damage (which
potentially upregulates TGF\(\beta\) activation)
occurs early in the disease,\(^{31}\) there would seem to
be a strong case for further examination of
platelet TGF\(\beta1\) storage and release in systemic
sclerosis.

As indicated above, microvascular abnor-
malities 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.\(^{16}\)

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 most 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 classic role which may be played by
rumour 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.\(^2\)

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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597–643.

2. Border WA, Ruoslahti E. Transforming growth factor-\(\beta\) in
disease; the dark side of tissue repair. J Clin Invest 1992;
90: 1–7.

3. Posthumaite A E, Keskı-Oja J, Moses H L, Kang A E. Stimula-
tion of the chemotactic migration of human fibroblasts by

4. Ignos R A, Massague J. TGF\(\beta\) stimulates the expression of
fibrocollagen and collagen and their incorporation into
the extracellular matrix. J Biol Chem 1986; 261:
4337–45.

modulates the expression of collagenase and metallopro-
teinase inhibitor. EMBO J 1987; 6:
1099–1104.

6. Roberts A B, Sporn M, Asooia R K, Smith J M, Roche N S,
Keller F M. Growth and differentiation factors in human
fibroblast differentiation and angiogenesis in vivo and stimula-
tion of collagen formation in vivo. Proc Nail Acad Sci USA

7. Shah M, Foreman D M, Ferguson W J. Coefficient of scarring
in adult wounds by neutralising antibody to TGF\(\beta\).

R M. A strategy for scleroderma: is TGF\(\beta\) the answer? Arth-

and type I procollagen gene expression in fibrotic skin

10. Deguchi Y. Spontaneous increase of TGF\(\beta\) production by
bronchoalveolar mononuclear cells of patients with
systemic autoimmune diseases affecting the lung. Ann Rheu-

levels of TGF\(\beta\) in patients with scleroderma. Arthritis

12. Falanga V, Julien J M. Observations in the potential role of
transforming growth factor \(\beta\) in cutaneous fibrosis
(systematic sclerosis). Ann NY Acad Sci 1990; 593:
161–71.

A role for TGF\(\beta\) in the transformation of dermal fibro-
blasts to myofibroblasts in skin diseases associated with the
collagen-induced hypersensitivity syndrome. J Invest
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