Plasma TGF β 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 (TGF β) can be detected in plasma from patients with systemic sclerosis and to examine the relationship between plasma TGF β 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. TGF β 1 and TGF β 2 were measured by enzyme linked immunosorbent assay (ELISA) (sensitivity 100 pg/ml) and PIIINP by radioimmunoassay.

Results—TGF β 1 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. TGF β 2 was not detected in plasma from patients or controls. No clear relationship was demonstrated between TGF β 1, clinical features or PIIINP concentrations.

Conclusions—The 25 kDa form of TGF β 1 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 TGF β 1 and disease activity.

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It has been suggested that increased TGF β production might play a role in the widespread subcutaneous and visceral fibrosis which characterises systemic sclerosis;⁸ cultured mononuclear cells from patients with systemic sclerosis produce TGF β in vitro⁹ and TGF β mRNA can be detected by in situ hybridisation in biopsy material.¹⁰ There have been conflicting reports of the concentrations of TGF β in the peripheral blood of patients with systemic sclerosis,^{11–13} but such studies have been restricted by the lack of a simple, reliable, sensitive assay for any of the TGF β family.

We have examined the hypothesis that TGF β is detectable in the peripheral blood of patients with systemic sclerosis, utilising immunoassays for the 25 kDa active forms of TGF β 1 and TGF β 2. We have further examined the hypothesis that abnormally increased concentrations of TGF β might contribute to the fibrotic process in systemic sclerosis, by investigating the relationship between TGF β and procollagen III aminopeptide, a serum marker of the fibrotic process.^{14–15}

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.¹⁶ 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.

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The β transforming growth factors (TGF β) (which exist in three isoforms, TGF β 1–3) are a family of closely related regulatory proteins with a potentially central role in both physiological and pathological fibrosis;^{1–2} they are chemotactic for fibroblasts,³ potent inducers of synthesis of collagen and other matrix proteins,⁴ and inhibitors of extracellular matrix breakdown.⁵ Subcutaneous injection of TGF β induces local fibrosis⁶ and neutralisation of TGF β activity inhibits the formation of fibrous tissue.⁷ TGF β has the capacity to induce further synthesis of TGF β , suggesting a potential positive feedback process in pathological fibrosis.²

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)¹⁷

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 isoforms 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 Graft 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.¹⁸ 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.¹⁹ 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 aminopeptide (PIIINP) was measured by competitive single antibody radioimmunoassay according to published methodology,²⁰ 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.¹⁵ 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

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 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 TGFβ1, but two of the

Figure 1 Plasma TGFβ concentrations in 39 patients with systemic sclerosis. Dotted line indicates limit of detection of the TGFβ1 assay (100 pg/ml); TG was undetectable (UD) in 33 of the patients.

Clinical features, plasma TGFβ1 concentrations and serum PIIINP in the systemic sclerosis patients with detectable TGFβ1

Sex/ Age	Disease classification	Disease duration (yr)	Visceral involvement	Autoantibodies	TGFβ1 (pg/ml)	PIIINP (µg/ml)
F/59	Limited (forearm involvement)	28	Oesophagus	Centromere	600	2.7
F/46	Limited (sclerodactyly only)	8	Oesophagus Pulmonary	Nil	600	3.1
F/39	Diffuse	16	Oesophagus	Scl-70	2500	2.5
M/48	Diffuse	2	Oesophagus Pulmonary	ANF	1100	7.1
F/42	Limited (leg involvement)	9	Oesophagus	Centromere	2000	2.9
F/62	Limited (forearm involvement)	9 months	Oesophagus Pulmonary	ANF	2800	8.2

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 ($p = 0.0027$) and Raynaud's ($p = 0.0049$). Differences between diffuse and limited systemic sclerosis and Raynaud's and controls did not achieve statistical significance.

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 TGF β 1 and also a marked increase in PIIINP concentration (the greatest within the limited systemic sclerosis group). However, TGF β 1 was also detected in three patients with disease duration greater than the median of eight years. Three of the six patients with detectable TGF β 1 had evidence of interstitial pulmonary disease (and of the six patients with lung disease and no detectable TGF β , 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 TGF β was not statistically significant ($p = 0.13$, Fisher's exact test). TGF β 1 was not detected in any patient receiving interferon alfa, cyclophosphamide, D-penicillamine or corticosteroids.

No TGF β 2 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 TGF β (table).

Discussion

We have demonstrated that the biologically active 25 kDa form of TGF β 1 is present in the plasma of a minority of patients with systemic sclerosis. These findings complement recent demonstrations of circulating TGF β in other disease processes associated with abnormal deposition of fibrous tissue: lung and liver fibrosis after marrow transplantation,²¹ chronic

vascular rejection of renal allografts¹⁷ and diabetes mellitus (B Coupes, P Brechley, unpublished data). There is, however, no simple relationship between the presence of circulating TGF β 1 and the extent of tissue fibrosis in the patients with systemic sclerosis (although there are indications that TGF β 1 is perhaps more likely to be detected in patients with interstitial lung disease). This superficially argues against a direct role for TGF β 1 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 sclerosis²²). It is also possible that active TGF β 1 may be only intermittently present in the circulation in systemic sclerosis; the plasma half life of the active form of TGF β is approximately three minutes.²³ Active TGF β is thought to be generated from latent TGF β at sites of tissue injury by a combination of proteolysis and low pH.²⁴ Active TGF β is then rapidly cleared by mechanisms which include binding to α_2 macroglobulin, the small proteoglycan, decorin, and possibly soluble TGF β receptors,^{19, 25} followed by clearance via the liver.²³ TGF β 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 TGF β would be required to clarify this question.

Difficulties in assessing active fibrosis in systemic sclerosis and possible fluctuations in plasma TGF β imply that the relationship between circulating TGF β 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 TGF β suppressing drugs: interferon alfa reduces TGF β expression in fibrotic liver disease²⁶ and corticosteroids inhibit transcription of TGF β genes.²⁷ The effects of cyclophosphamide and penicillamine on TGF β production are unknown, although the myelo-suppressive action of cyclophosphamide might be expected indirectly to reduce TGF β production by monocytes. Future prospective studies might usefully concentrate upon the subgroup of patients with early or rapidly progressive diffuse disease.

Circulating TGF β 2 was not detected in plasma from any patient or control. This is perhaps surprising, as there is some evidence to suggest that TGF β 2 may be more important in the pathogenesis of systemic sclerosis than TGF β 1: it has been reported that expression of TGF β 2 mRNA and protein is increased in most sclerodermatous skin biopsies, whereas TGF β 1 is found in only a minority.²⁸ It should be noted, however, that tissue expression of TGF β does not necessarily indicate local biological activity; tissue TGF β may be substantially in latent form (there is some evidence to suggest that TGF β may be sequestered in

high concentrations in certain forms of extracellular matrix²⁹). Tissue analysis of the co-distribution of TGF β , the TGF β latency associated peptide (cleaved from TGF β to create active TGF) and matrix molecules such as decorin (which binds and inactivates TGF β) should clarify this question.

The TGF β 1 and β 2 assays used in this study have a limit of detection of 60–100 pg/ml. It is possible that significant increases in TGF β 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 β in systemic sclerosis (between 40 and 130 pg/ml).¹² Our findings are in conflict with those of Higley *et al.*,¹³ who reported no significant difference in plasma concentrations of TGF β 1 between systemic sclerosis patients and controls, using an ELISA assay with a limit of detection for the active form of TGF β 1 of approximately 1 ng/ml (10 times less sensitive than the assay used here). However, they also reported detectable (>1 ng/ml) TGF β 1 in 26% of healthy controls and 75% of patients with Raynaud's phenomenon. The presence of the active form of TGF β 1 in healthy peripheral blood in concentrations well within the biologically active range would not be anticipated from previous studies.^{17 19 23} This perhaps suggests the possibility that the ELISA assay used by Higley's group¹³ was not truly specific for the active 25 kDa form of TGF β 1, but perhaps also detected larger molecular weight forms of TGF β 1 which are present in blood from healthy subjects in ng/ml concentrations.^{19 21}

The cellular origin of the TGF β detected in this study requires consideration. Previous studies have emphasised fibroblast TGF β production; less attention has been given to the platelet and macrophage, potentially greater sources of TGF β . Platelets contain large amounts of stored latent TGF β .^{1 18} Since platelet physiology is disturbed in systemic sclerosis,³⁰ and endothelial damage (which potentially upregulates TGF β activation) occurs early in the disease,³¹ there would seem to be a strong case for further examination of platelet TGF β 1 storage and release in systemic sclerosis.

As indicated above, microvascular abnormalities are prominent in systemic sclerosis.^{30 31} Although TGF β 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,³² a potent vasoconstrictor implicated in systemic sclerosis, and to reduce synthesis of nitric oxide, a potent endothelium derived vasodilator (A Roberts 1993, personal communication). Recent evidence suggests that reduced nitric oxide synthesis might contribute to the vasospasm of Raynaud's phenomenon.³³

It is unlikely that TGF β 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 β plays a central role in this network (analogous to the central role which may be played by tumour necrosis factor α in macrophage mediated inflammation;³⁵ neutralisation of TGF β activity seems to inhibit fibrosis potentially.^{7 36 37} TGF β also has the capacity to induce its own synthesis.³⁸ It has been suggested that an abnormal positive feedback mechanism for TGF β production might be of major importance in pathological tissue fibrosis.²

The effects of inhibiting TGF β activity have potential therapeutic implication in systemic sclerosis. It has been demonstrated in laboratory animals that neutralisation of TGF β activity inhibits tissue fibrosis in the lung, kidney and skin.^{7 36 37} Inhibition of TGF β 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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