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Enhanced production of transforming growth factor-beta (TGF-β) during autologous mixed lymphocyte reaction of systemic sclerosis patients

H. OTA, S. KUMAGAI*, A. MORINOBU, H. YANAGIDA & K. NAKAO *Second Department of Internal Medicine, School of Medicine, and *College of Medical Technology, Kyoto University, Kyoto, Japan*

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SUMMARY

Systemic sclerosis (SSc) is characterized by systemic fibrosis and microvascular lesions. As TGF-β is suggested to be related to skin fibrosis, we examined the production of TGF-β from peripheral mononuclear cells (MNC) of SSc patients. Since anti-TGF-β neutralizing antibody improved the defective proliferative response in autologous mixed lymphocyte reaction (AMLR) of SSc patients, TGF-β was thought to participate in the decreased AMLR of SSc patients. Greater amounts of TGF-β in the active as well as in the latent forms were produced during AMLR of SSc patients than that of normal subjects. It was suggested that TGF-β excessively produced from the MNC of SSc patients might play a major role in the fibrosis of the patients during AMLR-like *in vivo* responses.

Keywords autologous mixed lymphocyte reaction systemic sclerosis transforming growth factor-beta

INTRODUCTION

Systemic sclerosis (SSc) is characterized by systemic fibrosis and microvascular lesions. Mononuclear cells (MNC), mainly activated T cells and monocytes, infiltrate the skin of SSc patients [1,2]. The fibroblasts of SSc patients have been shown to produce excessive amounts of type I and III procollagen *in vivo* [3,4], as well as *in vitro* [5]. It was suggested that soluble mediators from MNC directly activate fibroblasts to produce excessive amounts of extracellular matrix [6]. Among them, cytokines such as IL-1 and tumour necrosis factor-alpha (TNF-α) which induce the proliferation of fibroblasts and/or the synthesis of collagens have been suggested to play a key role in the fibrotic mechanism of SSc. We previously reported the hyperproduction of IL-1 and TNF-α by monocytes of SSc patients [7]. Recently, TGF-β has been shown to induce fibroblasts to increase extracellular matrix production [8,9], and is also thought to be deeply involved in the fibrosis of SSc. It was demonstrated that TGF-β was strongly expressed in dermal and subcutaneous infiltrating cells in SSc [10–13]. TGF-β may be one of the soluble factors produced from the MNC infiltrated into the skin, which induce the production of extracellular matrix by fibroblasts.

We previously reported that peripheral T cells of SSc patients were hyperactive because of their hyperproduction of IL-2 [14]. In contrast, the autologous mixed lymphocyte reaction (AMLR) of SSc patients was unique, because their T cells showed early but decreased proliferative responses compared with those of normal controls [15]. We speculated that excessive production of TGF-β during the AMLR might participate in that unique proliferative response, because TGF-β has inhibitory effects on the proliferation and function of lymphocytes [16]. Here, we investigated TGF-β production during the AMLR of SSc patients. This is the first demonstration of *in vitro* hyperproduction of TGF-β by MNC of SSc patients.

Correspondence: Shunichi Kumagai MD, Professor, College of Medical Technology, Kyoto University, Kawaharacho 53, Syogoin, Sakyo-ku, Kyoto 606, Japan.

PATIENTS AND METHODS

Patients

The patients consisted of 10 Japanese patients (nine women and one man) ranging in age from 41 to 71 years (mean 58·8). All patients fulfilled the American Rheumatism Association criteria for the classification of systemic sclerosis (scleroderma) [17], and all had abnormal thickening and tightening of the skin, as well as Raynaud's phenomenon. Eight patients had widespread thickening of the skin (diffuse type), whereas two had limited type of sclerosis. The mean disease duration was 9·9 years (range 1–24 years). Six patients were not taking any immunosuppressive drugs at the time of this study. Of the remainder, one, one and two were taking or had taken prednisolone (2·5–10 mg/day), D-penicillamine (200–300 mg/day),

and both, respectively. The normal subjects were age- and sex-matched healthy volunteers.

AMLR

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by standard Ficoll–Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden). T cells and non-T cells were separated by rosetting method with sheep erythrocytes, as previously described [14]. The T cell preparations contained >94% $CD3^+$ cells in both normal subjects and SSc patients.

AMLR was performed in triplicate by culturing 5×10^4 T cells with an equal number of autologous non-T cells treated with mitomycin C (Sigma Chemical Co., St Louis, MO) in a final volume of 200 μl of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated normal human AB serum, in 96-well U-bottomed culture plates (Costar, Cambridge, MA). The culture was performed for 2–7 days at 37°C in a 5% CO_2 humidified atmosphere. Eight hours before termination of the culture, the cells were pulsed with 1 μCi of methyl-^{3}H-thymidine (Amersham, Arlington Heights, IL). The proliferative responses were measured by thymidine incorporation using a liquid scintillation counter. For TGF-β assay, the culture supernatants of AMLR performed in other plates were collected at days 3, 4 and 6 by centrifugation, and stored at −20°C before assay.

Neutralization of AMLR by anti-TGF-β antibody

The effects of anti-TGF-β neutralizing antibody on AMLR were investigated by adding anti-TGF-β1 neutralizing antibody or control antibody (normal chicken IgY) to the culture at the beginning of AMLR. Both antibodies were purchased from R&D System, (Minneapolis, MN), and were used at a final concentration of 10 μg/ml. These cultures were also incubated for 7 days, and the proliferative responses were measured by thymidine incorporation.

TGF-β assay

Mvl Lu mink lung epithelial cells (CCL64; American Type Culture Collection, Bethesda, MD) were used as the target cells to detect TGF-β activity in the supernatants from AMLR. The growth inhibition assay was performed with slight modification according to the original method described by Cheifetz *et al.* [18]. Briefly, 100-μl aliquots of CCL64 cells (1×10^5 cells/ml in RPMI 1640 containing 1% fetal calf serum (FCS)) were placed in 96-well flat-bottomed culture plates (Costar) and 100 μl of serially diluted standards and samples were applied to each well. The plates were incubated for 36 h at 37°C in a 5% CO_2 humidified atmosphere. Eighteen hours before the termination of culture, 1 μCi methyl-^{3}H-thymidine was added to each culture well, then the incorporated radioactivity was measured. The concentration of TGF-β in the supernatants was calculated according to the per cent inhibition of standard recombinant human TGF-β1 (King Brewing Co. Ltd., Kakogawa, Japan). In this bioassay the limit of detection was about 10 pg/ml.

The total amount of TGF-β in the supernatants of AMLR was measured as follows. The supernatants were acidified with 1 N HCl for 40 min at room temperature to activate the latent form of TGF-β, then were neutralized with 1 N NaOH.

To detect the active form of TGF-β, AMLR was performed using serum-free AIM-V medium (Gibco). The supernatants were collected on day 4 by centrifugation, and TGF-β activity was assayed without acid treatment.

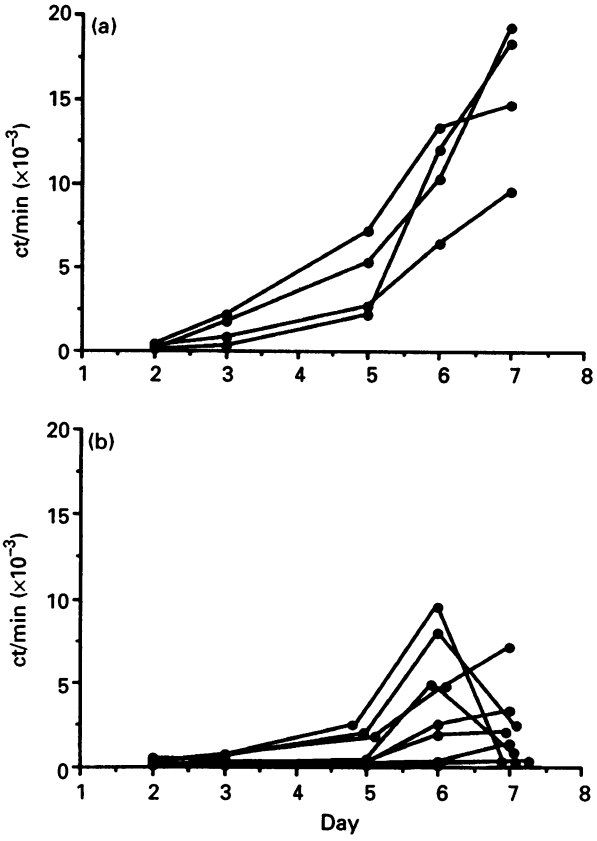

Fig. 1. The time course of autologous mixed lymphocyte reaction (AMLR) of four normal subjects (a) and of nine systemic sclerosis (SSc) patients (b). T cells (5×10^4) were cocultured with mitomycin C-treated non-T cells (5×10^4), and their proliferative responses were measured by thymidine incorporation. Each circle represents the mean of triplicates.

RESULTS

AMLR of SSc patients

As shown in Fig. 1, T cell proliferation on day 7 of AMLR was significantly decreased in SSc patients (mean ct/min ± s.e.m. 1737·4 ± 627·8) compared with that of normal subjects (14 149·4 ± 1656·3, $P < 0{\cdot}001$). The decreased T cell proliferation of SSc patients was observed through the course of AMLR. Three of the nine SSc patients showed unique proliferative responses, a peak of T cell proliferation on day 6 of AMLR, but it decreased rapidly on day 7. Five of the remaining six SSc patients showed significantly decreased proliferative responses, less than 3000 ct/min of thymidine incorporation throughout the AMLR. The kinetics of AMLR of the last one did not differ from that of normal subjects, but the peak proliferative response was lower.

Viabilities of the cells were measured by dye-exclusion test during AMLR. Roughly 30% of the cells were dead at day 4 of the culture, but the absolute number of dead cells was not different between normal subjects and SSc patients through the

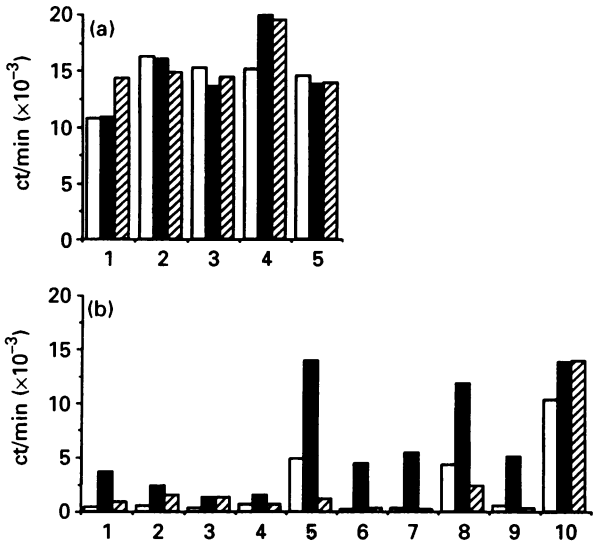

Fig. 2. Effects of an anti-TGF-β-neutralizing antibody on the proliferation of T cells from normal subjects (a) and from systemic sclerosis (SSc) patients (b) in autologous mixed lymphocyte reaction (AMLR). AMLR was performed in the absence of antibodies (□), and in the presence of 10 μg/ml of anti-TGF-β1-neutralizing antibody (■), or of control antibody (▨). Each bar represents the mean of triplicate.

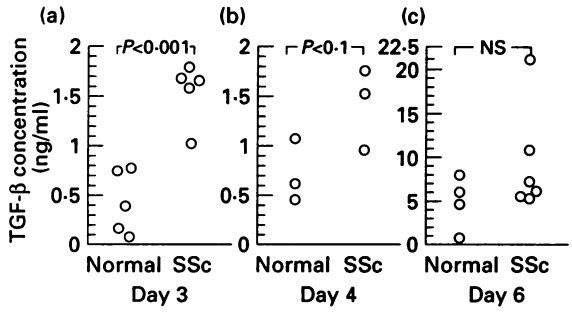

Fig. 3. TGF-β activity in the supernatants in autologous mixed lymphocyte reaction (AMLR) of normal subjects and systemic sclerosis (SSc) patients. T cells (5×10^4) were cocultured with mitomycin C-treated non-T cells (5×10^4), and supernatants were collected on days 3, 4 and 6. As the supernatants were assayed after acid treatment, the concentration is the total activity of latent and active TGF-β produced from mononuclear cells in AMLR.

culture. Hence, the low proliferation of SSc T cells in AMLR could not be due to cell death.

Anti-TGF-β neutralizing antibody improves the decreased AMLR of SSc patients

To define whether the excessive production of TGF-β participates in the decreased AMLR of SSc patients, we initially investigated the effects of addition of anti-TGF-β1 neutralizing antibody on their AMLR. As shown in Fig. 2, anti-TGF-β1-neutralizing antibody had little effect on the AMLR of five normal subjects (Fig. 2a). In contrast, the AMLR of most of the 10 SSc patients were improved in part by the anti-TGF-β1-neutralizing antibody but not by the control antibody (Fig. 2b). These results suggested that TGF-β was involved in the decreased AMLR of SSc patients, although it was not necessarily improved to the levels of normal subjects.

Excessive production of TGF-β during AMLR of SSc patients

TGF-β activity in the supernatants of AMLR was measured by bioassay using CCL64 cells. As the TGF-β protein begins to be detected in the supernatants at 48–72 h after stimulation, the supernatants were collected on days 3, 4 and 6 of AMLR. To measure the total production of TGF-β in AMLR, the supernatants were assayed after acid treatment, which changed the TGF-β from the latent to the active form. As shown in Fig. 3, the production of TGF-β gradually increased during the course of AMLR of both SSc patients and normal subjects. SSc patients produced significantly more TGF-β than normal subjects on day 3 of AMLR.

TGF-β activity in AMLR supernatants was confirmed by anti-TGF-β1-neutralizing antibody. When the acidified supernatants of 3 day AMLR were incubated with neutralizing antibody, the inhibitory effects of the supernatants on thymidine incorporation into CCL64 cells were almost completely abrogated (Fig. 4).

Detection of active TGF-β in supernatants of AMLR in SSc patients

TGF-β is generally produced in the latent form, then activated after secretion. TGF-β in latent form has no effect on target cells because only the active form can bind to TGF-β receptors. As the active form of TGF-β was thought to suppress the proliferation of T cells in AMLR, we measured the level of the active form of TGF-β in the supernatants in AMLR. As shown in Fig. 5, TGF-β activity was detected in the supernatants of none of the five normal subjects (<10 pg/ml). In contrast, the active form of TGF-β could be detected at concentrations of 15–25 pg/ml in seven of the eight SSc patients.

When the amounts of total and active TGF-β were compared with the T cell proliferation in the AMLR, the proliferation, however, did not correlate with total TGF-β, nor with that of active form.

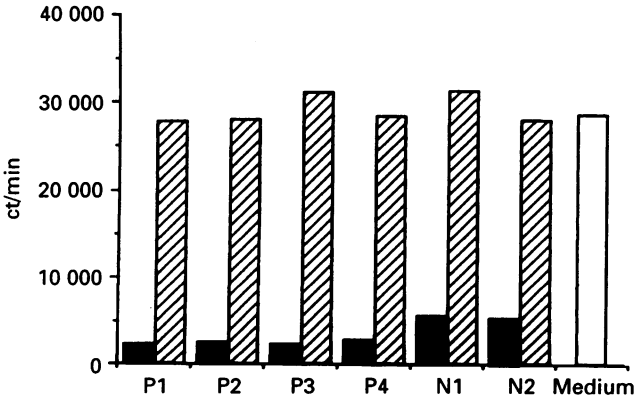

Fig. 4. Neutralization of TGF-β activity in the supernatants of autologous mixed lymphocyte reaction (AMLR) by anti-TGF-β antibody. When the acidified supernatants of 3 day AMLR of four systemic sclerosis (SSc) patients (P1–P4) and two normal subjects (N1, N2) were incubated with 20 μg/ml neutralizing antibody, the inhibitory effect of the supernatants on the growth of CCL64 cells was almost completely abrogated. ■, Non-treated supernatants; ▨, treated supernatants.

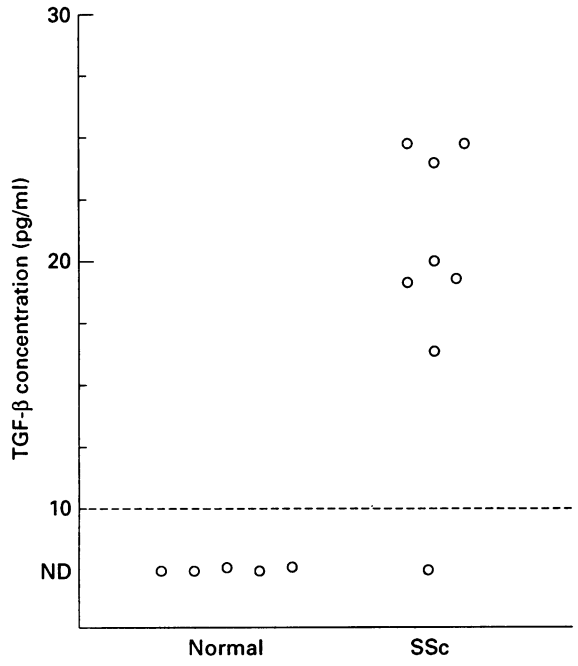

Fig. 5. The concentration of bioactive TGF-β in the supernatants of autologous mixed lymphocyte reaction (AMLR). To measure bioactive TGF-β in AMLR supernatants, AMLR was performed in serum free AIM-V medium, and the supernatants were collected on day 4. The active form of TGF-β was assayed without acid treatment. In this bioassay the limit of detection was about 10 pg/ml. SSc, Systemic sclerosis.

DISCUSSION

In this study, we demonstrated that TGF-β was at least in part involved in the early and defective AMLR in SSc patients, and that MNC of SSc patients during AMLR produced more TGF-β in the latent and active forms, whereas that of normal subjects was predominantly in the latent form. This was the first demonstration that MNC of SSc patients showed enhanced production of TGF-β upon *in vitro* stimulation, AMLR.

Alcocer-Varela *et al.* have reported that T cells of SSc patients proliferated early in AMLR, and the proliferation rapidly decreased [15]. In our experiments, AMLR of most of the SSc patients reached maximum earlier than that of normal subjects, but the level of the response was significantly decreased. Alcocer-Varela *et al.* suspected that T cells of SSc patients were already primed by autoantigens and showed a secondary response to such antigens in AMLR. The cause of the decreased proliferation in AMLR, however, remained unclear. Our data suggest that excessive production of active TGF-β is involved in the process, because an anti-TGF-β1-neutralizing antibody improved the AMLR.

AMLR of the SSc patients, however, was not completely normalized by anti-TGF-β1-neutralizing antibody. The total amounts of active TGF-β in the supernatants of AMLR, as well as that of active form, did not statistically correlate with T cell proliferation in AMLR, nor with the degree of improvement by anti-TGF-β1-neutralizing antibody treatment. When 20 and 100 pg/ml of recombinant TGF-β1 were added to the culture on day 3 of AMLR of normal subjects, T cell proliferation on day 7 was decreased to 25% and 31% of controls, respectively (data not shown). The decreased response of AMLR of SSc patients was not merely due to the excessive production of the active form of TGF-β1, but possibly to other inhibitory factors. Moreover, a relative lack of autoreactive T cells, or of stimulatory non-T cells, in the circulating lymphocyte pool could be another reason for defective AMLR of SSc patients, although we had no evidence on the frequency of these cells.

The mechanism of excessive production of TGF-β by MNC from SSc patients is uncertain. T cells have been shown to produce TGF-β upon various stimulations. Rosetting methods with sheep erythrocytes were shown to stimulate T cells and to induce their IL-6 production [19]. It was not possible that the rosetting method preferentially stimulated SSc T cells, because hyperproduction of TGF-β from SSc T cells was confirmed using the method whereby T cells are separated by negative selection (data not shown). Cyclosporin A and glucocorticoid decrease T cell proliferation and IL-2 secretion, but enhance the production of TGF-β [20,21]. This was not the case, because most of our SSc patients were not taking such drugs. Either IL-1 or concanavalin A (Con A) was reported to induce active TGF-β from purified T cells without the proliferative response, although a coexistence of both stimulations did not induce it [22]. The enhanced production of TGF-β from MNC of SSc might come from inadequate T cell stimulation during AMLR, because SSc monocytes were shown to produce higher levels of IL-1 [7].

Finally, the reason why MNC, T cells in particular, infiltrate the skin of SSc patients remains unclear. What autoantigens T cells recognize in AMLR also remains unknown. In this study, we have been shown that MNC of SSc patients produced excessive TGF-β both in latent and active form in AMLR, and that the latter was involved in their defective T cell proliferation in AMLR. We speculate that AMLR-like responses occur *in vivo*, especially in the skin of SSc patients where MNC infiltrated. If so, our results suggest that the TGF-β excessively produced by MNC during the *in vivo* AMLR-like response plays a major role in the fibrosis of SSc.

ACKNOWLEDGMENTS

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REFERENCES

1 Fleischmajer R, Perlish JS, Reeve JRT. Cellular infiltrates in scleroderma skin. Arthritis Rheum 1977; **20**:975–84.
2 Roumm AD, Whiteside TL, Medsger TA, Rodman GP. Lymphocytes in the skin of patients with progressive systemic sclerosis. Arthritis Rheum 1984; **27**:645–53.
3 Fleischmajer R, Gay S, Meigel WN, Perish JS. Collagen in the cellular and fibrotic stages of scleroderma. Arthritis Rheum 1978; **21**:418–28.
4 Kähäri V-M, Sandberg M, Kalimo H, Vuorio T, Vuorio E. Identification of fibroblasts responsible for increased collagen production in localized scleroderma by *in situ* hybridization. J Invest Dermatol 1988; **90**:664–70.
5 Gay RE, Buckingham RB, Prince RK, Gay S, Rodnan GP, Miller EJ. Collagen types synthesized in dermal fibroblast cultures from patients with early progressive systemic sclerosis. Arthritis Rheum 1980; **23**:190–6.
6 Worrall JG, Whiteside TL, Prince RK, Buckingham RB, Stachura I, Rodnan GP. Persistence of scleroderma-like phenotype in normal

fibroblasts after prolonged exposure to soluble mediators from mononuclear cells. Arthritis Rheum 1986; **29**:54–64.

7 Umehara H, Kumagai S, Murakami M *et al.* Enhanced production of interleukin-1 and tumor necrosis factor α by cultured peripheral blood monocytes from patients with scleroderma. Arthritis Rheum 1990; **33**:893–7.

8 Ignotz R, Massague J. Transforming growth factor beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 1986; **261**:251–6.

9 Roberts AB, Sporn MB, Assoian RK *et al.* Transforming growth factor type β: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. Proc Natl Acad Sci USA 1986; **83**:4167–71.

10 Scharffetter K, Lankata-Buttgereit B, Krieg T. Localization of collagen mRNA in normal and scleroderma skin by *in-situ* hybridization. Eur J Clin Invest 1988; **18**:9–17.

11 Gruschwitz M, Müller PU, Sepp N, Hofer E, Fontana A, Wick G. Transcription and expression of transforming growth factor type beta in the skin of progressive systemic sclerosis: a mediator of fibrosis? J Invest Dermatol 1990; **94**:197–203.

12 Kulozik M, Sharffetter K, Herrman K, Lankat-Bttgereit B, Heckmann M, Kreig T. Codistribution of collagen I and transforming growth factor beta 2 gene expression in systemic sclerosis. J Invest Dermatol 1989; **92**:465A.

13 Kulozik M, Hogg A, Lankat-Buttgereit B, Krieg T. Co-localization of transforming growth factor β2 with α1(I) procollagen mRNA in tissue sections of patients with systemic sclerosis. J Clin Invest 1990; **86**:917–22.

14 Umehara H, Kumagai S, Ishida H, Suginoshita T, Maeda M, Imura H. Enhanced production of interleukin-2 in patients with progressive systemic sclerosis; hyperactivity of CD4-positive T cells? Arthritis Rheum 1988; **31**:401–7.

15 Alcocer-Varela J, Laffón A, Alarcón-Segovia D, Ibañez de Kasep G. Early proliferative response in the human autologous mixed lymphocyte reaction in scleroderma. J Rheumatol 1984; **11**:48–52.

16 Kehrl JH, Wakefield LM, Roberts AB *et al.* Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med 1986; **163**:1037–50.

17 Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee: preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 1980; **23**:581–90.

18 Chiefetz S, Weatherbee JA, Tsang ML-S, Anderson JK, Mole JE, Lucas R, Massagué J. The transforming growth factor-β system, a complex pattern of cross-reactive ligands and receptors. Cell 1987; **48**:409–15.

19 Linardopoulos S, Corrigall V, Panayi GS. Activation of HLA-DR and interleukin-6 gene transcription in resting T cells via the CD2 molecule: relevance to chronic immune-mediated inflammation. Scan J Immunol 1992; **36**:469–77.

20 Li B, Sehajpal PK, Khanna A, Vlassara H, Cerami A, Stenzel KH, Suthanthiran M. Differential regulation of transforming growth factor β and interleukin 2 genes in human T cells: demonstration by usage of novel competitor DNA constructs in the quantative polymerase chain reaction. J Exp Med 1991; **174**:1259–62.

21 Ayanlar-Batuman O, Ferrero PA, Diaz A, Jimenez AS. Regulation of transforming growth factor-beta 1 gene expression by glucocorticoids in normal human T lymphocytes. J Clin Invest 1991; **88**:1574–80.

22 Bristol AL, Ruscetti WF, Brody TD, Durum KS. IL-1 α induces expression of active transforming growth factor-β in nonproliferating T cells via a post-transcriptional mechanism. J Immunol 1990; **145**:4108–14.