EXTENDED REPORT

Up regulated expression of tumour necrosis factor α converting enzyme in peripheral monocytes of patients with early systemic sclerosis

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Background: Systemic sclerosis (SSc) is accompanied by abnormalities in humoral and cellular immune systems.
Objective: To determine the genes specifically expressed in the immune system in SSc by analysis of the gene expression profile of peripheral blood mononuclear cells (PBMC) from patients with SSc, including those treated with haematopoietic stem cell transplantation (HSCT). Additionally, to investigate the clinical significance of the up regulation of tumour necrosis factor α (TNFα) converting enzyme (TACE).
Methods: PBMC from patients with SSc (n = 23) and other autoimmune diseases (systemic lupus erythematosus (SLE, n = 16), rheumatoid arthritis (RA, n = 29)), and from disease-free controls (n = 36) were examined. Complementary DNA arrays were used to evaluate gene expression of PBMC, in combination with real time quantitative polymerase chain reactions. TACE protein expression in PBMC was examined by fluorescence activated cell sorter (FACS).
Results: In patients with SSc 118 genes were down regulated after HSCT. Subsequent comparative analysis of SSc without HSCT and healthy controls indicated SSc-specific up regulation for three genes: monocyte chemoattractant protein-3 (p = 0.0015), macrophage inflammatory protein 3α (p = 0.0339), and TACE (p = 0.0251). In the FACS analysis, TACE protein was mainly expressed on $CD14^+$ monocytes both in patients with SSc and controls. TACE expression on $CD14^+$ cells was significantly increased in patients with early SSc (p = 0.0096), but not in those with chronic SSc, SLE, or RA. TACE protein levels in SSc monocytes correlated with the intracellular CD68 levels (p = 0.0016).
Conclusions: Up regulation of TACE expression was a unique profile in early SSc, and may affect the function of TNFα and other immunoregulatory molecules.

Systemic sclerosis (SSc) is a multisystem disorder of connective tissue. Increased biosynthesis of multiple matrix proteins by interstitial fibroblasts is the hallmark of SSc, with development of skin sclerosis and involvement of visceral organs.[1] The pathogenesis of SSc includes vasculopathy associated with endothelial cell dysfunction, and extensive fibrosis secondary to fibroblast activation.[2 3] Functional abnormality in T and B lymphocytes has been considered in the pathogenesis, based on the presence of disease-specific autoantibodies and hypergammaglobulinaemia in SSc.[4 5] Growth factors and cytokines are also thought to play a part in the progression of connective tissue fibrosis in SSc. Among them, transforming growth factor β (TGFβ) and the Smad system have a central role in the SSc dermis.[6] However, the molecular basis of the pathogenesis of SSc has remained unclear.

The use of gene expression profiling, such as the complementary DNA (cDNA) array system, is increasingly being used for various diseases, and is used in the aetiological study of SSc.[7 8] Increased expression of several genes has been suggested, but a disease-specific gene profile of SSc has not yet been determined, possibly owing to the difficulty of achieving disease remission in SSc, which is necessary for a comparative analysis of the active and disease-free status. Recently, the efficacy of high dose chemotherapy after autologous haematopoietic stem cell transplantation (HSCT) for refractory autoimmune diseases has been reported.[9 10] HSCT has been performed in a number of cases of SSc, with good results.[11–13] When we performed autologous CD34 selected HSCT for our patients with SSc, we observed a prompt and persistent improvement of skin sclerosis and stabilisation of organ disease. Under this condition, it was possible to carry out a comparative analysis of gene expression profile between an active (pre-HSCT) and a remission status (post-HSCT) in the same patient with SSc.

We studied the gene expression profile in peripheral blood mononuclear cells (PBMC) from patients with SSc treated with HSCT, and found that expression of tumour necrosis factor α (TNFα) converting enzyme (TACE) was increased in circulating monocytes from patients with SSc. The correlation

Abbreviations: Ab, antibody; aCENP-B Ab, anticentromere protein-B Ab; ACR, American College of Rheumatology; ANA, antinuclear Ab; aRNP Ab, anti-ribonucleoprotein Ab; aTopo-I Ab, antitopoisomerase I Ab; CaMKIIβ, calcium/calmodulin dependent protein kinase II β; cDNA, complementary DNA; CRP, C reactive protein; CTGF, connective tissue growth factor; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; FSC, forward light scatter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSCT, haematopoietic stem cell transplantation; IL, interleukin; MCP, monocyte chemoattractant protein; MFI, mean fluorescence intensity; mIgG1, isotype matched control mouse IgG1; MIP, macrophage inflammatory protein; mRNA, messenger RNA; NF-κB, nuclear factor-κB; NIK, NF-κB inducing kinase; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; RA, rheumatoid arthritis; real time PCR, quantitative TaqMan real time polymerase chain reaction; RGS, regulators of G-protein signalling; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; SSC, side light scatter; TACE, tumour necrosis factor α converting enzyme; TGFβ, transforming growth factor β; TNFα, tumour necrosis factor α; TNF-R, TNF receptor

Table 1 Clinical features of the study subjects*

Characteristics	SSc (n = 20)	SSc treated with HSCT (n = 3)	RA (n = 29)	SLE (n = 16)	Controls (n = 36)
Sex (female/male)	17/3	2/1	23/6	14/2	25/11
Age (years), mean (SD)	51.0 (12.6)	43.3 (21.1)	58.9 (14.4)	40.5 (13.4)	40.0 (11.5)
Duration of disease (months), median (min–max)	41.0 (6–278)	18 (12–24)	36.0 (3–360)	144.0 (1–444)	
Prednisolone (mg/day), mean (min–max)	0.875 (0–10)	0.83 (0–2.5)	4.1 (0–25)	13.0 (0–60)	
Organ involvement, No (%)					
Lung	13 (65)	2 (67)	3 (10)	0 (0)	
Muscle	1 (5)	0 (0)	0 (0)	0 (0)	
Joint	8 (40)	0 (0)	29 (100)	3 (19)	
Renal	1 (5)	1 (33)	0 (0)	5 (31)	
Cardiac	2 (10)	0 (0)	0 (0)	0 (0)	
Serology, No (%)					
ANA	19 (95)	2 (67)	19 (66)	16 (100)	
aTopo-I Ab	11 (55)	2 (67)	N/A	N/A	
aCENP-B Ab	2 (10)	0 (0)	N/A	N/A	
aRNP Ab	1 (5)	0 (0)	N/A	11 (69)	

*The SSc groups consisted of 20 subjects, and three patients treated with HSCT. In these three patients with HSCT, RNA samples were obtained before (<1 month before mobilisation) and after HSCT.
Organ involvements in this study were defined as: lung (interstitial pneumonia proved by high resolution computed tomography), muscle (increased serum creatinine kinase or serum aldolase, or both, continuously), joint (arthralgia or arthritis, or both), renal (renal crisis in patients with SSc and nephritis in RA and SLE), cardiac (arrhythmia).[18–22]
N/A, not available.

of TACE expression with the clinical findings in patients with SSc was analysed and is discussed below.

PATIENTS AND METHODS

Patients and controls

Twenty three Japanese patients with SSc who fulfilled the 1980 criteria of the American College of Rheumatology (ACR) were assessed in this study.[14] These patients were categorised as those with diffuse cutaneous type disease characterised by generalised or widespread skin thickening.[15] Table 1 summarises their clinical features. Patients were classified as having early SSc (n = 12) if the disease duration after the appearance of the first non-Raynaud's phenomenon was within 3 years.[11] Other patients with SSc with a longer disease duration were classified as having chronic SSc (n = 11). Of the 12 patients with early SSc, three were treated with HSCT using autologous $CD34^+$ selected peripheral blood stem cells; blood samples were obtained throughout the clinical course. Other patients with autoimmune diseases had rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE). These patients fulfilled the criteria of the ACR, respectively.[16 17] As healthy controls, 36 disease-free Japanese volunteers, mean (SD) age 40.0 (11.5) years, were enrolled in the study.

Study design

To search for specific genes which changed between the active and remission status, before and after HSCT, we first analysed gene expression profiles of PBMC from patients treated with HSCT using cDNA array (n = 3). Next, specific up regulated genes in patients with SSc were explored using cDNA array by comparing mRNA levels in PBMC of patients with SSc who had not received HSCT (n = 6) and healthy controls (n = 5). Specific gene candidates were confirmed by real time polymerase chain reaction (PCR) in patients with SSc without HSCT (n = 9) and controls (n = 6). Finally, protein expression levels were analysed by a fluorescence activated cell sorter (FACS) analysis.

PBMC isolation

Blood sampling was carried out after obtaining written informed consent according to the guidelines of the ethical committee of Hokkaido University. PBMC were obtained from heparinised venous blood, using gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences Corp, NJ).

RNA extraction and cDNA array analysis

Total RNAs from PBMC were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Poly(A) RNA was isolated from total RNA using a MagExtractor (TOYOBO, Osaka, Japan), and poly(A) RNA (2 μg) was reverse transcribed by ReverTraAce (TOYOBO), in the presence of cDNA synthesis primers and biotin-16-deoxyuridine triphosphate (TOYOBO), according to the manufacturer's instructions. cDNA array analysis was performed using human cDNA expression filters (Human Immunology Filters (TOYOBO)), on which 621 species of human cDNA fragments and housekeeping genes are spotted in duplicate: (http://www.toyobo.co.jp/seihin/xr/product/genenavi/genenavigator.html, accessed 5 May 2005)). Hybridisation and subsequent cDNA array analyses were carried out as described previously,[23] with some modification. Briefly, after standard prehybridisation, cDNA array filters were hybridised with a biotin labelled cDNA probe in PerfectHyb solution (TOYOBO) overnight at 68℃. After washing under conditions of high stringency, specific signals on the filters were visualised using Phototope-Star Detection Kits (New England Biolabs, Beverly, MA). Fluorescence signals for mRNA expression levels were obtained using a Fluor-S Multiimager system (Nippon Bio-Rad Laboratories, Tokyo, Japan). The signal intensity among filters was compared in an E-Gene Navigator Analysis (GeneticLab, Sapporo, Japan), and was expressed as an mRNA expression index to the intensity of the internal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression.

Quantitative TaqMan real time polymerase chain reaction (real time PCR)

A two step PCR was carried out on serial dilutions of cDNA samples from PBMC from the patients with SSc and the controls. Real time PCR amplification and determination of cDNA transcripts were carried out with the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) and gene-specific sets of TaqMan Universal PCR master mix and assays-on-demand gene expression probes (Applied Biosystems).

Table 2 Down regulated genes expressed in PBMC from patients with SSc at 6 months after HSCT as indicated by a cDNA array

Gene name	Accession No	Gene name	Accession No	Gene name	Accession No
Bik	X89986	MCP-2	Y16645	GRP94	X15187
Caspase-8	U60520	MCP-3	X72308	HSP105β	AB003333
JunB	X51345	Neurophysin II	X03172	MMP3	J03209
AP-β	X95694	Delta	AF003522	TACE	U86755
AP-γ	X95693	Angiotensinogen	K02215	Cathepsin G	M16117
Erg-1	M21535	Gonadotropin α peptide	V00518	Pin1	U49070
GLI-3	M57609	Somatostatin	J00306	Calpastatin	U26724
IκBα	M69043	VIP	M36634	Moesin	M69066
IRF-2	X15949	Gastrin	V00511	Radixin	L02320
N-Myc	M13228	IP10	X02530	LAT	AF036905
Per2	AB002345	MIP-3α	U77035	Fyb	AF001862
Pax5	M96944	EphA5	L36644	Furin	X17094
LXRα	U22662	EphB6	D83492	PAI-2	M16006
RXRγ	U38480	Insulin receptor	M10051	CD3γ	X04145
RAC3	AF010227	MC1-R	X67594	CD3ε	X03884
PPARγ	L40904	MC4-R	L08603	CD3ζ	J04132
MCR	M16801	β2-AR	M15169	CD8β 1	X13444
MEK-5	U25265	FRP-3	U24163	CD5	X04391
MEK kinase-2	AF111105	Notch 2	AF097645	CD72	M54992
Raf-1	X03484	Thrombin R	M62424	CD6	X60992
Raf-B	M95712	IFNγR2	U05875	CD7	X06180
KKIAMRE	U35146	IL2Rγ	D11086	CD20	X12530
Rhotekin	AI970663	IL15Rα	U31628	CD27	M63928
IKKα	AF080157	c-Kit	X06182	CD28	J02988
JAK1	M64174	CXCR-4	AF025375	CD35	Y00816
MuSK	AF006464	Slap	D89077	CD38	M34461
TGFβ2	M19154	Shb	X75342	CD40L	L07414
GDF-8	AF019627	Sos1	L13858	CD42a	X52997
Inhibin α	M13981	Dbl	J03639	CD43	J04168
FGF-1	X51943	Ral GDS	U14417	CD46 BC1	M58050
FGF-5	M37825	RGS4	U27768	CD59	X16447
FGF-6	X63454	PI4-Kα	AJ011121	CD69	L07555
HGF β chain	E08541	FRP1	U49844	CD74	X03339
IGF-BP3	M35878	Sam68	M88108	TCRα	U36759
TNFβ	D12614	PPP1CA	X70848	TCRβ	L07294
IL2	U25676	CD45	Y00062	TCRγ	Y00790
IL10	M57627	p120	AF062343	CD138	J05392
IL15	AF031167	Ref-1	D90373	ICAM3	X69819
IL18	D49950	HSP60	M34664		
SCF	M59964	HSP90β	M16660		

One hundred and eighteen gene expressions decreased more than 20% in all three patients with SSc treated with HSCT at 6 months after HSCT compared with before HSCT (<1 month before mobilisation) by cDNA array analysis. Changes in white blood cell and monocyte counts in PBMC from three patients with SSc treated with HSCT were from 4867 (116)/μl to 5100 (917)/μl (p=0.5105*) and from 506 (157)/μl to 589 (44) /μl (p=0.7106*), respectively. *Paired *t* test.

FACS analysis

The following mouse monoclonal antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA): anti-human CD3-Cy-chrome, CD4-fluorescein isothiocyanate (FITC), CD8-phycoerythrin (PE) and FITC, CD56-FITC, CD19-FITC, CD68-PE, and CD69-PE. Monoclonal mouse antihuman-CD14-FITC, CD71-PE (Beckman Coulter Inc, Fullerton, CA) and antihuman TACE-PE (R&D systems, Abingdon, UK) were also used for surface immunostaining of the cells. The specificity of antihuman TACE has been characterised.[24] In the case of CD68, intracellular staining was done using Cytofix/Cytoperm Plus (BD Biosciences Pharmingen, San Diego, CA), according to the manufacturer's instructions. After washing twice with phosphate buffered saline (PBS), cells were subjected to FACS analysis of immunostained cells using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Statistical analysis and clinical significance

Calculations were made using the statistical software package StatView 5.0 (Abacus Concepts, Berkeley, CA). Comparisons of mRNA expression of PBMC were made using Mann-Whitney statistics. Group mean comparisons of the TACE protein expression levels, represented by mean fluorescence intensity (MFI), and TACE positive cells were based on Kruskal-Wallis H statistics. A paired *t* test was used to analyse the difference in blood cell counts of patients with SSc before and after HSCT. The data are presented as the means (SD). Differences were examined based on analysis of variance, and p values <0.05 were considered significant.

RESULTS

Comprehensive analysis of up regulated genes in PBMC from patients with SSc using cDNA array and real time PCR

We first analysed mRNA expression in PBMC from three patients with SSc treated with HSCT, in order to search for genes with expression levels down regulated after this treatment. In these patients, skin involvement, as expressed by the modified Rodnan total thickness skin score improved significantly by 54% (from 30.3 (6.8) to 12.6 (13.2)) and the modified Health Assessment Questionnaire improved by 22.8% (from 1.67 (0.88) to 1.29 (1.04)) at 6 months after effective HSCT. This improvement persisted even 3 years after this treatment. PBMC specimens were obtained from these patients before (<1 month before mobilisation) and 6 months after HSCT, and were processed for mRNA extraction followed by cDNA array analyses.

Table 3 Up regulated genes expressed in PBMC from patients with SSc without HSCT as indicated by a cDNA array

Classification	Up regulated genes	GenBank accession No	Ratio (fold increase)
Regulatory transcription factors	Per1	AB002107	7.61
	Erg-3	S40832	4.50
	Gfi-1	U67369	4.44
Protein kinases	CaMKIIβ	AF078803	4.90
Growth factors and hormones	IL1β*	X02532	9.70
	MIP-1β*	J04130	6.58
	TARC	D43767	5.56
	IL12p35	AF180562	4.27
	MIP-3α*	U77035	4.25
	MCP-3*	X72308	3.88
Membrane receptors	IL15Rα	U31628	3.46
Signalling intermediates	Gab1	U43885	6.09
	RGS-1*	X73427	5.21
	Shb	X75342	4.32
	TACE*	U86755	3.70
	Dbl	J03639	3.38
Lymphocyte signalling	CD34	M81104	3.40

The ratio of the gene expression index (see ''Patients and methods'') of patients with SSc without HSCT (n = 6) to healthy controls (n = 5) was calculated, and the list of up regulated genes using cDNA array (with an SSc/control ratio >3.0) is displayed. *Gene expression was confirmed by real time PCR.
CaMKIIβ, calcium/calmodulin dependent protein kinase II β; MIP-1β, macrophage inflammatory protein β; MCP-3, monocyte chemoattractant protein-3; RGS-1, regulators of G-protein signalling-1.

At 6 months after this treatment, down regulation of mRNA expression levels was seen in 118 genes using cDNA array (table 2). In patients with SSc without HSCT, 17 genes were specifically up regulated compared with controls (table 3). In addition, the profile of mRNA expression between baseline and after 6 months in patients with SSc without HSCT was very similar, thus a ''natural state for 6 months'' did not modify the mRNA levels examined in this study (data not shown). Real time PCR showed that only four gene expression levels had statistical significance as disease-specific genes in 17 patients with SSc who had not received HSCT (fig 1). As a result, gene expression levels of monocyte chemoattractant protein (MCP)-3, macrophage inflammatory protein (MIP)-3α, and TACE were down regulated after HSCT in the cDNA array and up regulated specifically in patients with SSc without HSCT by real time PCR (fig 2). In this study, we further investigated the expression of TACE, which has a crucial role in the immune system. A role for chemokines, including MCP and MIP families, in the pathogenesis of scleroderma has been suggested (reviewed by Atamas and White[25]).

Cell surface TACE expression

We first examined PBMC from healthy controls for the expression of TACE protein. In PBMC from healthy subjects, a small population was brightly stained by an antihuman TACE monoclonal antibody (fig 3A). Multicolour FACS analyses showed that surface TACE expression was barely detectable on $CD4^+CD3^+$, $CD8^+CD3^+$, $CD19^+$, and $CD56^+$ populations. In contrast, surface TACE expression was detected on the $CD14^+$ population (fig 3B). It was confirmed that these $CD14^+$ populations were monocytes, by profiles of forward and side light scatter (SSC), as well as by intracellular CD68 protein expression. Surface TACE expression levels on monocytes were not affected by the age and sex of the controls (data not shown).

We next investigated expression levels of TACE protein in subsets of PBMC from patients with SSc. In these patients, surface TACE expression was also detected on monocytes but not on $CD4^+CD3^+$, $CD8^+CD3^+$, $CD19^+$, and $CD56^+$ populations, respectively (data not shown). Figure 3C shows representative FACS profiles of TACE protein expression for $CD14^+$

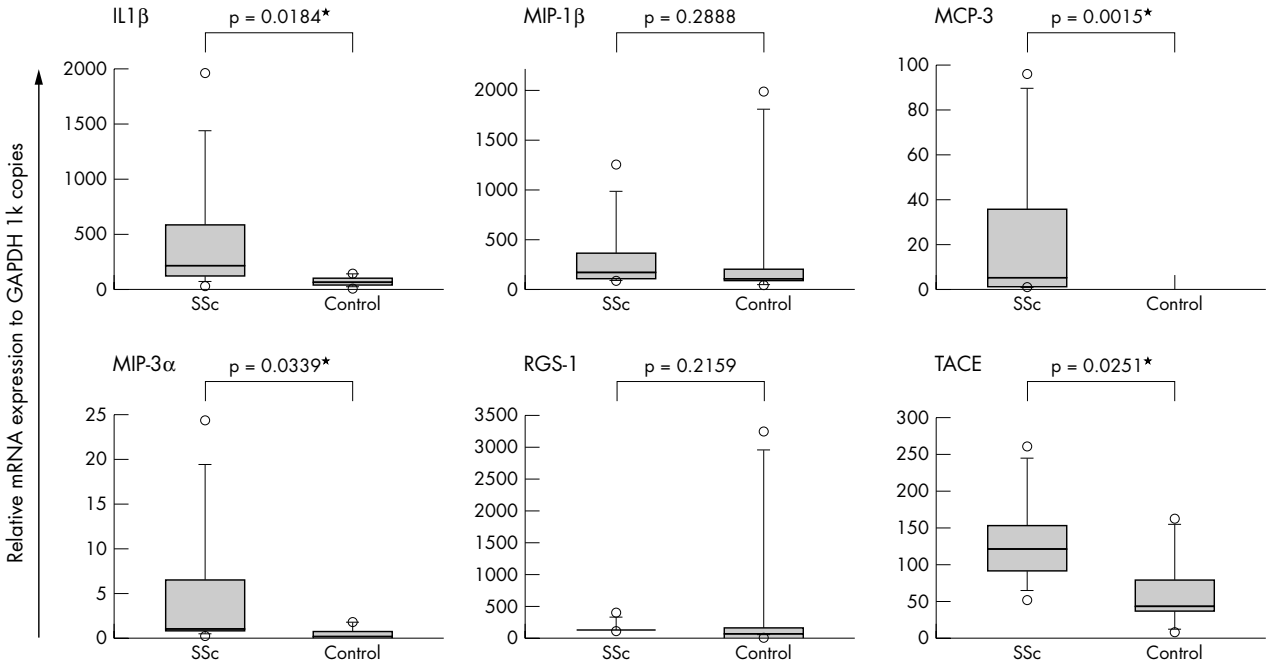

Figure 1 Quantitative analysis of up regulated genes in PBMC from patients with SSc, assessed using real time PCR. cDNA specimen from patients with SSc (n = 9) and disease-free volunteers (n = 6) were analysed for six genes species (TACE, interleukin (IL) 1β, MIP-3α, MIP-1β, MCP-3, and a regulator of G-protein signalling (RGS)-1), indicated from the cDNA array study (table 3). *$p<0.05$.

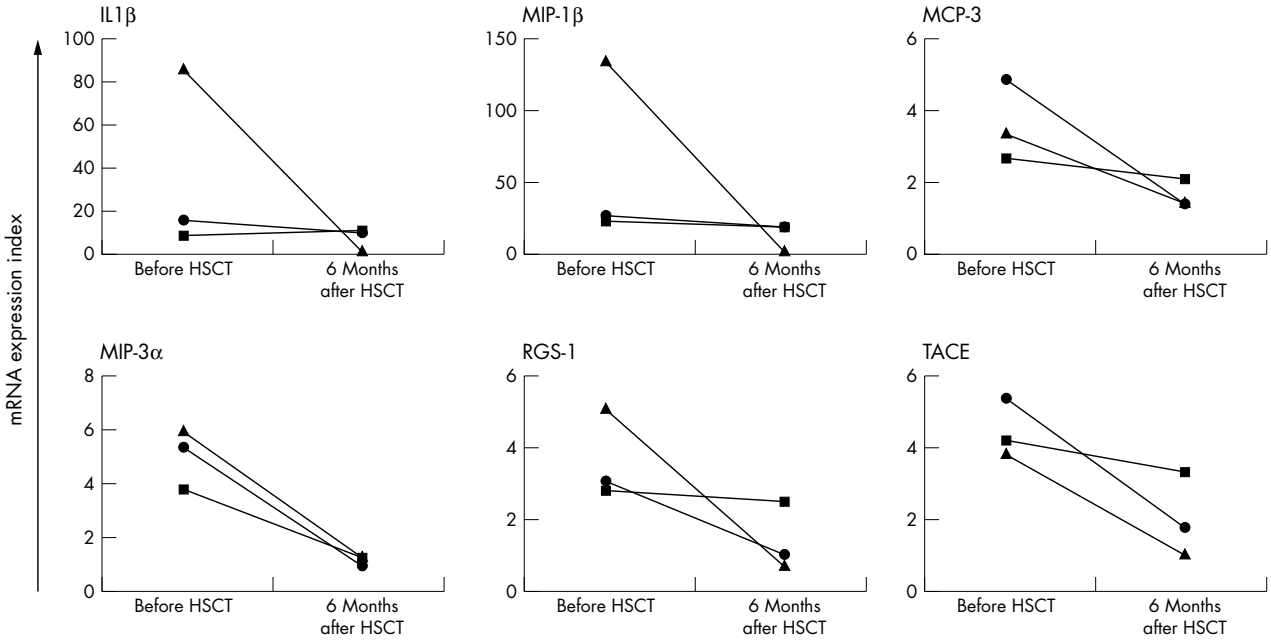

Figure 2 Changes of mRNA expression levels of IL1β, MIP-1β, MCP-3, MIP3-α, RGS-1, and TACE in patients with SSc before and after HSCT using cDNA array. RNA specimens were obtained from individual patients before (<1 month before mobilisation) and 6 months after HSCT. Relative expression levels of mRNA were determined using cDNA arrays performed simultaneously. The expression levels of each cDNA transcript were displayed as a relative mRNA expression index compared with the levels of internal GAPDH gene expression, as described in ''Patients and methods''.

monocytes. In the patients with SSc, TACE expression in monocytes was significantly increased in comparison with findings in healthy controls, and in patients with RA and SLE (fig 3C). When analysed statistically, cell surface TACE protein levels of monocytes and TACE positive cells in peripheral blood were significantly increased in patients with SSc, especially in patients with early SSc with disease duration of <3 years, in comparison with controls and patients with non-SSc autoimmune diseases, as well as those with chronic SSc (fig 4). In addition, TACE protein levels correlated with mRNA expression levels by real time PCR ($r = 0.640$, $p = 0.0462$). Thus, we concluded that up regulated expression of TACE protein by monocytes was a unique profile of early SSc.

Relationship between cell surface TACE expression and maturation/activation markers of monocytes

To better understand the mechanism of TACE up regulation in monocytes in SSc, we next investigated correlations between TACE and activation/differentiation markers of monocytes from patients with SSc. Coexpression of surface CD69, CD71, and intracellular CD68 with TACE was evaluated using FACS analysis (fig 5). These proteins were variously expressed on SSc monocytes, but only intracellular CD68 protein levels showed a significant correlation with cell surface TACE protein expression levels ($r = 0.671$, $p = 0.0016$), while cell surface CD69 and CD71 proteins did not correlate.

Association of cell surface TACE expression levels with clinical features of SSc

We then analysed the correlation of cell surface TACE protein levels of monocytes from patients with SSc (including patients with early and chronic disease) with clinical features of the disease. The expression levels of TACE protein on monocytes in patients with SSc, however, did not significantly correlate with titres of autoantibodies, including antinuclear Ab (ANA), antitopoisomerase I Ab (aTopo-I Ab), anticentromere protein-B Ab (aCENP-B Ab), anti-ribonucleoprotein Ab (aRNP Ab), as well as levels of serum immunoglobulins. The TACE protein levels did not correlate either with CRP in patients with SSc (fig 4, $r = -0.216$, $p = 0.3599$). The expression levels of TACE protein did not differ significantly between patients with SSc with or without visceral organ disease, including interstitial pneumonia and gastrointestinal complications (data not shown).

DISCUSSION

HSCT can be an effective treatment for subjects with severe autoimmune diseases, including SSc.[11–13] In our three patients with SSc who received HSCT, significant improvement of skin sclerosis was promptly achieved and persisted without any immunosuppressant drug treatment. In such patients, the gene expression profile can be studied comparatively between the active and remission state of SSc, with a minimum background of therapeutic reagents. In this study we performed a cDNA analysis of PBMC from patients with SSc who had undergone HSCT. After extensive analyses, up regulation of MCP-3, MIP-3α, and TACE in PBMC from patients with SSc who had not had HSCT was evident.

It was notable that these genes are commonly expressed by monocytes/macrophages. In SSc, although the earliest pathological events include dysfunction of microvascular systems and dermal fibroblasts,[26 27] cells and factors that mediate such abnormalities have not been defined. Histological studies of early SSc showed that cells infiltrating the skin of patients with early stage SSc are mainly $CD14^+$ monocytes/macrophages,[28 29] indicating the crucial role of these cells.

A role for chemokines has been suggested in the pathogenesis of SSc.[25 30–32] Our observation about MCP-3 and MIP-3α in PBMC from patients with SSc in this study is consistent with previous findings.[33–35]

In addition, overexpression of TACE in PBMC appeared to be a new hallmark of early stage SSc. In PBMC subpopulations, TACE protein expression was almost limited to

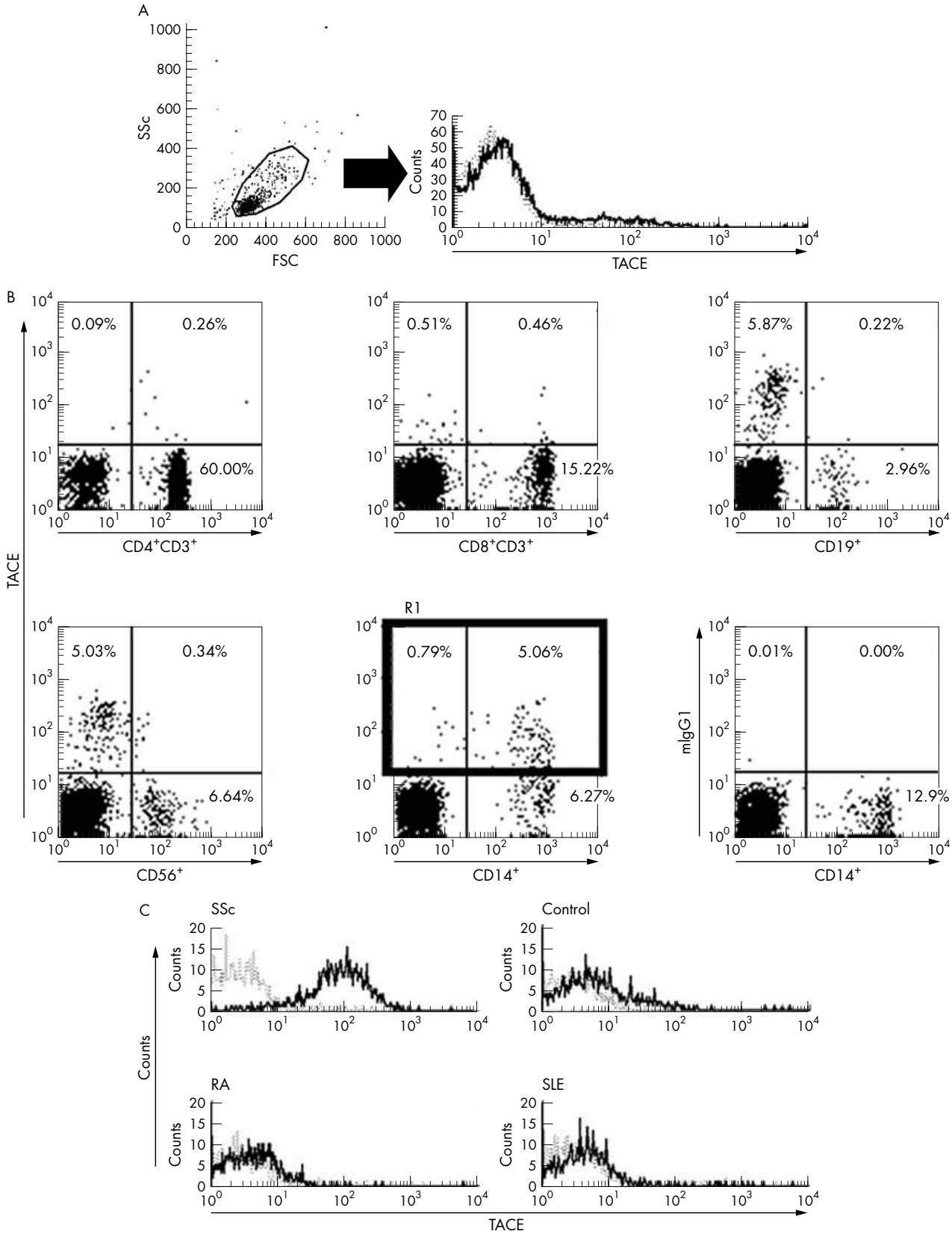

Figure 3 Expression of TACE protein in PBMC of healthy controls and patients. (A, left panel) A small population was detected in a high fluorescence intensity. (A, right panel) Solid line, cells stained with anti-TACE monoclonal Ab; dotted line, cells stained with isotype matched control mouse IgG1 Ab. (B) Expression of TACE protein in PBMC subsets from a healthy control. (C) Representative cell surface expression of TACE protein on monocytes from patients with autoimmune diseases and controls. Solid line, cells stained with anti-TACE monoclonal Ab; dotted line, cells stained with mIgG1 Ab. Results are representative of three independent experiments.

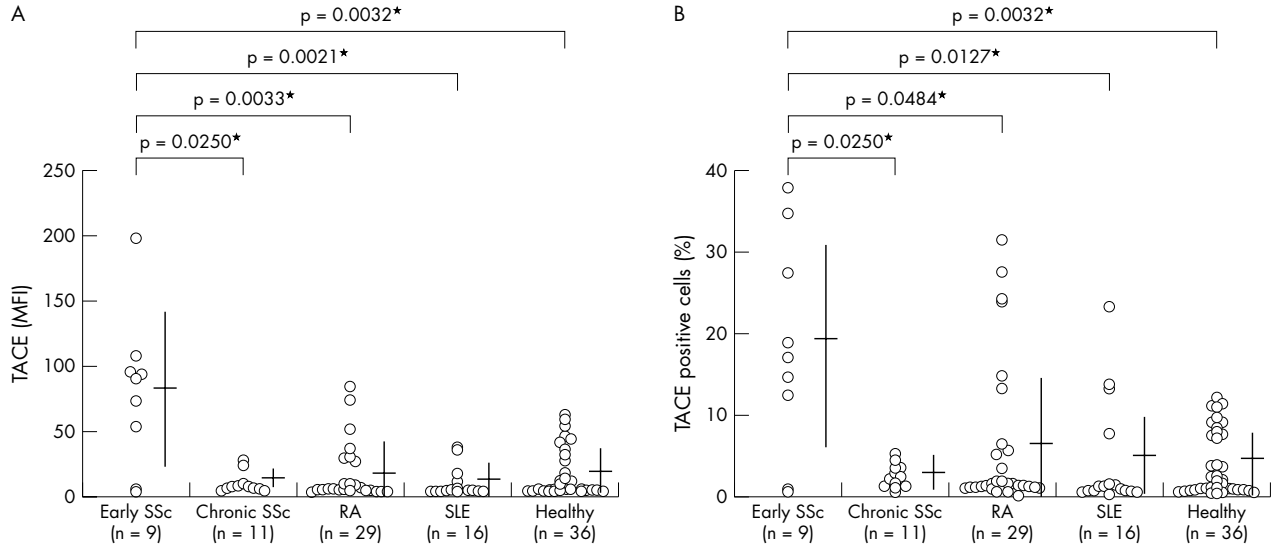

Figure 4 (A) Comparison of cell surface TACE expression by monocytes of patients with early SSc, chronic SSc, RA, SLE, and healthy controls. Cell surface expression levels of TACE were evaluated using FACS, gating on monocytes by forward and side light scatter and on $CD14^+$ cells. The levels of TACE protein expression were represented by the MFI. mIgG1 Ab-PE staining was performed in all measurements, and showed identical levels of background staining. *$p<0.05$, ($p=0.0065$ (Kruskal-Wallis H statistics)). (B) Comparison of TACE positive cells in PBMC of patients with early SSc, chronic SSc, RA, SLE, and healthy controls evaluated using FACS. TACE positive cells were defined on gate R1 in fig 3B. *$p<0.05$.

monocytes (fig 3B), whereas widespread distribution of TACE mRNA in tissue was suggested and its expression of protein levels in blood cells has not been yet described in detail.[36 37] TACE protein levels on monocytes were significantly higher in SSc than in controls (figs 3C and 4), suggesting a possible association between TACE and the function of TACE-expressing monocytes with the pathoaetiology of SSc. In autoimmune diseases, up regulation of TACE in inflammatory synovial tissue from patients with RA and increased TACE mRNA expression in PBMC of patients with multiple sclerosis have been suggested, but the disease specificity has been unclear.[38–40] Our report provides the first

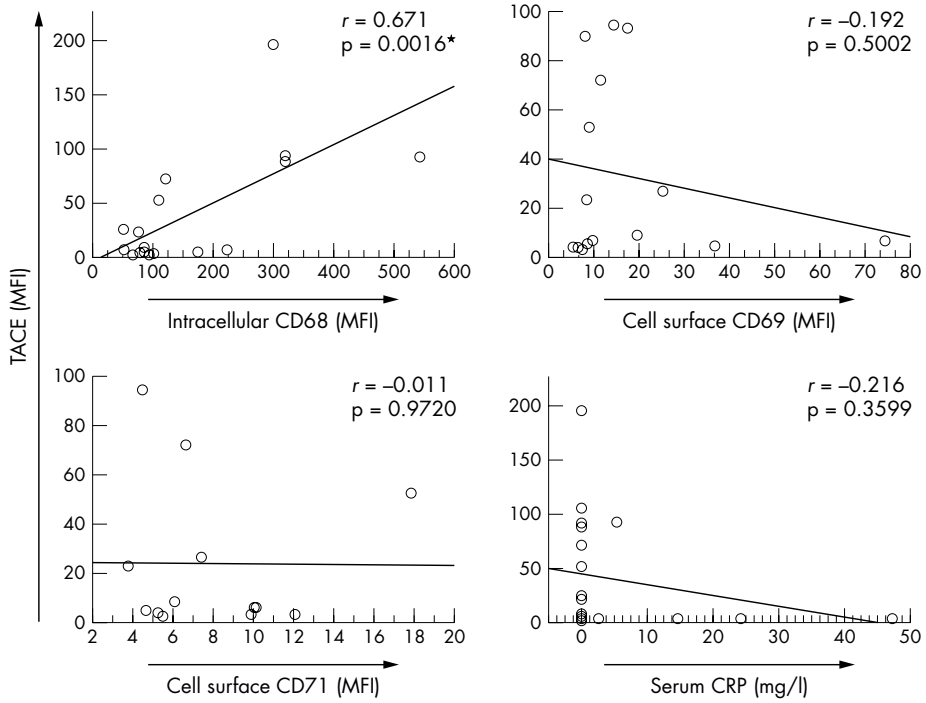

Figure 5 Correlation of TACE protein expression levels and maturation/activation markers on monocytes, and serum CRP levels in patients with SSc. Correlation of TACE protein levels with intracellular CD68 expression, cell surface CD69 expression, cell surface CD71 expression, and serum C reactive protein (CRP) levels are displayed. Cells were stained with anti-TACE-PE monoclonal antibody, or with FITC-anti-CD68, CD69, and CD71, in the presence of anti-CD14-FITC. Intracellular or cell surface levels of each marker were measured by FACS, gating on the monocytes as described above. The expression levels of individual proteins, represented by the MFI, were plotted on the horizontal axis (CD68, CD69, and CD71) and on the vertical axis (TACE), respectively. Serum CRP levels were measured by a latex agglutination test. *$p<0.05$.

evidence of disease-specific up regulation of TACE in peripheral monocytes in SSc, both at mRNA and protein expression levels.

TACE is a metalloproteinase of the ADAM (a disintegrin and metalloproteinase) family, and was initially described as a protease responsible for processing the membrane anchored TNFα precursor to the mature secreted form.[36 37] It is now accepted that substrates of TACE also include various cell surface proteins other than pro-TNFα; TNF receptors type I (TNF-RI) and type II (TNF-RII), L-selectin, IL6 receptor α chain, TNF related activation induced cytokine, and fractalkine (reviewed by Mezyk *et al*[41]). TACE mRNA is expressed by various types of cells, and can be induced by various extracellular stimuli.[41 42] We found that expression of TACE increased at mRNA levels in PBMC from patients with SSc (table 3, fig 1). Moreover, expression levels of TACE protein correlated with that of intracellular CD68, a lysosomal antigen expressed during differentiation of monocytes to macrophages, and did not correlate with CD69, CD71, activation markers in blood cell, and CRP levels (fig 5).[43] In patients with RA with positive CRP, surface TACE protein levels in peripheral monocytes also had no correlation ($r = -0.277$, $p = 0.1459$). Monocytes from patients with early stage SSc were likely to be activated in vivo, expressing TACE, independently of general acute reactants. Expression of TACE mRNA is regulated by transcription factors, including AP-2 and SP-1.[44] Analysis of these factors may help in understanding the mechanism of TACE up regulation.

It was notable that TACE up regulation in monocytes was not seen in patients with chronic SSc with diffuse skin sclerosis (fig 4). Hence, up regulation of TACE in circulating monocytes was probably not a secondary outcome from a persistent fibrotic condition. In the pathogenesis of SSc, TGFβ and connective tissue growth factor (CTGF) dysfunction has been considered.[8 45] In addition, the up regulated TNFα/TNF-R system has also been suggested in recent studies. It has been reported that the serum soluble TNF-R levels significantly correlated with the activity and disease progression of SSc.[46 47] Furthermore, Ellman and MacDonald reported a significant improvement in the skin score of patients with SSc after administration of recombinant anti-TNFα antibody.[48] These findings are consistent with involvement of an aberrant function of the TNFα/TNF-R system in SSc. The up regulation of TACE may be involved in such abnormality in TNF signalling in SSc through the cleaving function of TACE.

The effect of TACE on the function of the TNFα/TNF-R system in vivo is not fully understood, but the soluble form of TNFα can interact with both TNF-RI and TNF-RII.[49–51] Ligation of TNF-RI leads to recruitment of intracellular signalling proteins, resulting in the bifurcation of TNF-RI signalling: one is the apoptotic signal delivered through caspase-8, and the other is activation of proinflammatory properties delivered through nuclear factor-κB (NF-κB) inducing kinase (NIK) and NF-κB.[52] Unlike TNF-RI, signalling through TNF-RII largely leads to activation of NIK dependent and NF-κB dependent signalling.[53] Thus, increased activity of TACE can shift the balance of signals mediated by distinct types of TNF receptors and downstream events, including activation or death of target cells. Interestingly, mice with a targeted mutation in TACE were perinatally lethal and had morphological defects in the skin, indicating the essential role of TACE in normal skin development.[54]

Thus, it is intriguing to consider that TACE may participate in the progression of skin involvement in SSc, especially at the early stage. However, TNFα can also down regulate the expression of CTGF and indirectly modulate expression of type II TGFβ receptor in human fibroblasts.[55 56] In addition, TACE can also cleave other functional molecules as well as TNFα and the receptors.[41] Such pleiotrophic action of TACE makes it difficult to predict a bona fide role of TACE up regulation in SSc. Further investigation of the function of TACE, including analysis of laboratory animals that overexpress TACE, may provide information about the significance of TACE in SSc.

In summary, this study described for the first time the aberrant expression of TACE on monocytes from patients with early SSc. Recently, several chemical compounds have been reported to inhibit the activities of metalloproteinases, including TACE.[57] Such reagents may have a role in cases of TACE associated chronic inflammatory disease, including SSc. In addition, expression of TACE levels may also be a useful clinical measure of SSc, reflecting disease stages and the clinical prognosis of individual patients. Analysis of TACE function in SSc monocytes may not only provide insight into the pathogenesis but may also be a new diagnostic and therapeutic target for SSc.

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