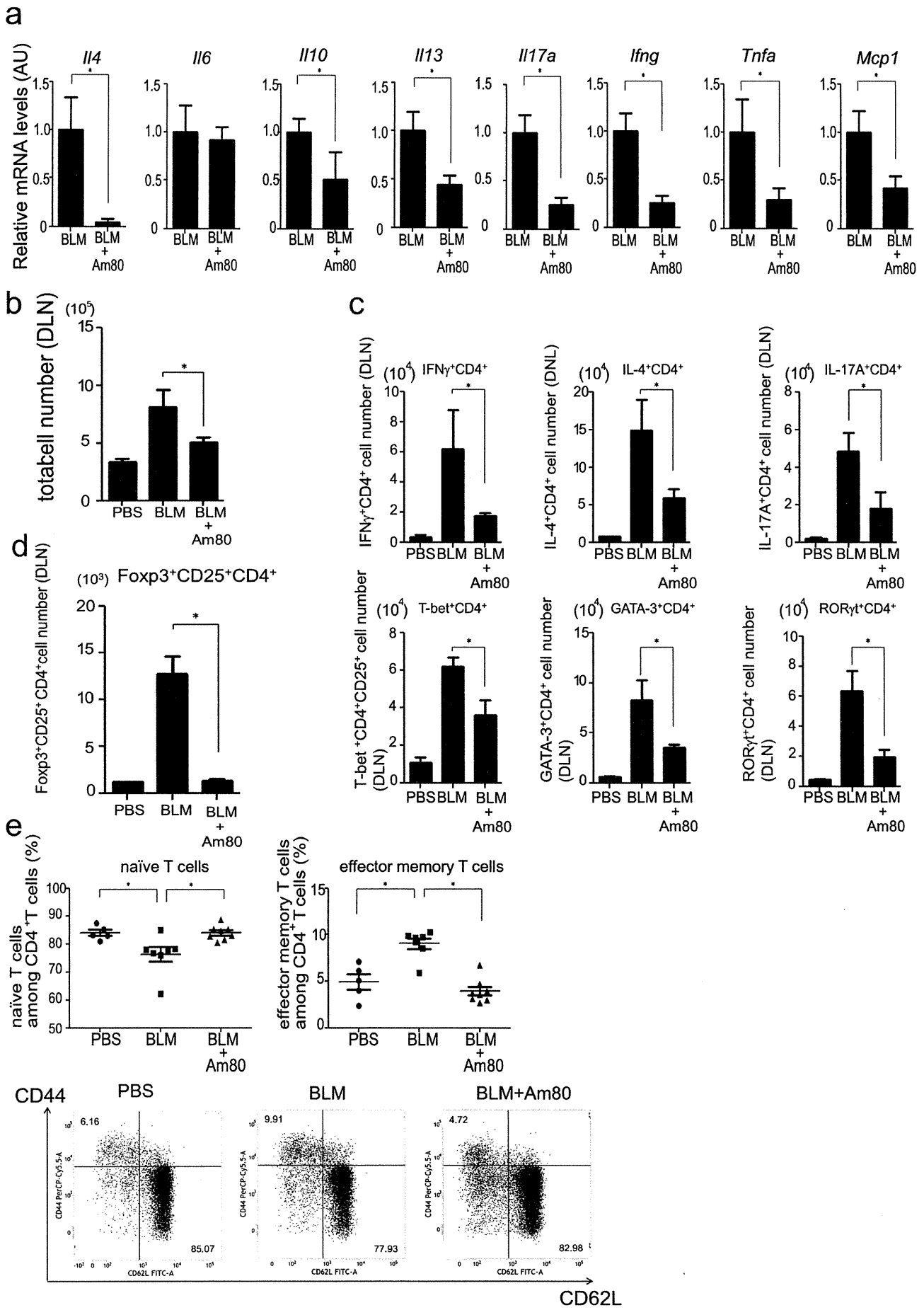
a
Il4
Il6
Il10
Il13
Il17a
Ifng
Tnfa
Mcp1
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BLM
BLM + Am80
b
totabell number (DLN)
PBS
BLM
BLM + Am80
c
IFNγ+CD4+ cell number (DLN)
IL-4+CD4+ cell number (DNL)
IL-17A+CD4+ cell number (DLN)
T-bet +CD4+CD25+ cell number (DLN)
GATA-3+CD4+ cell number (DLN)
RORγt+CD4+ cell number (DLN)
d
Foxp3+CD25+CD4+cell number (DLN)
Foxp3+CD25+CD4+
e
naïve T cells
effector memory T cells
naïve T cells among CD4+T cells (%)
effector memory T cells among CD4+T cells (%)
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CD62L
PBS
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85.07
9.91
77.93
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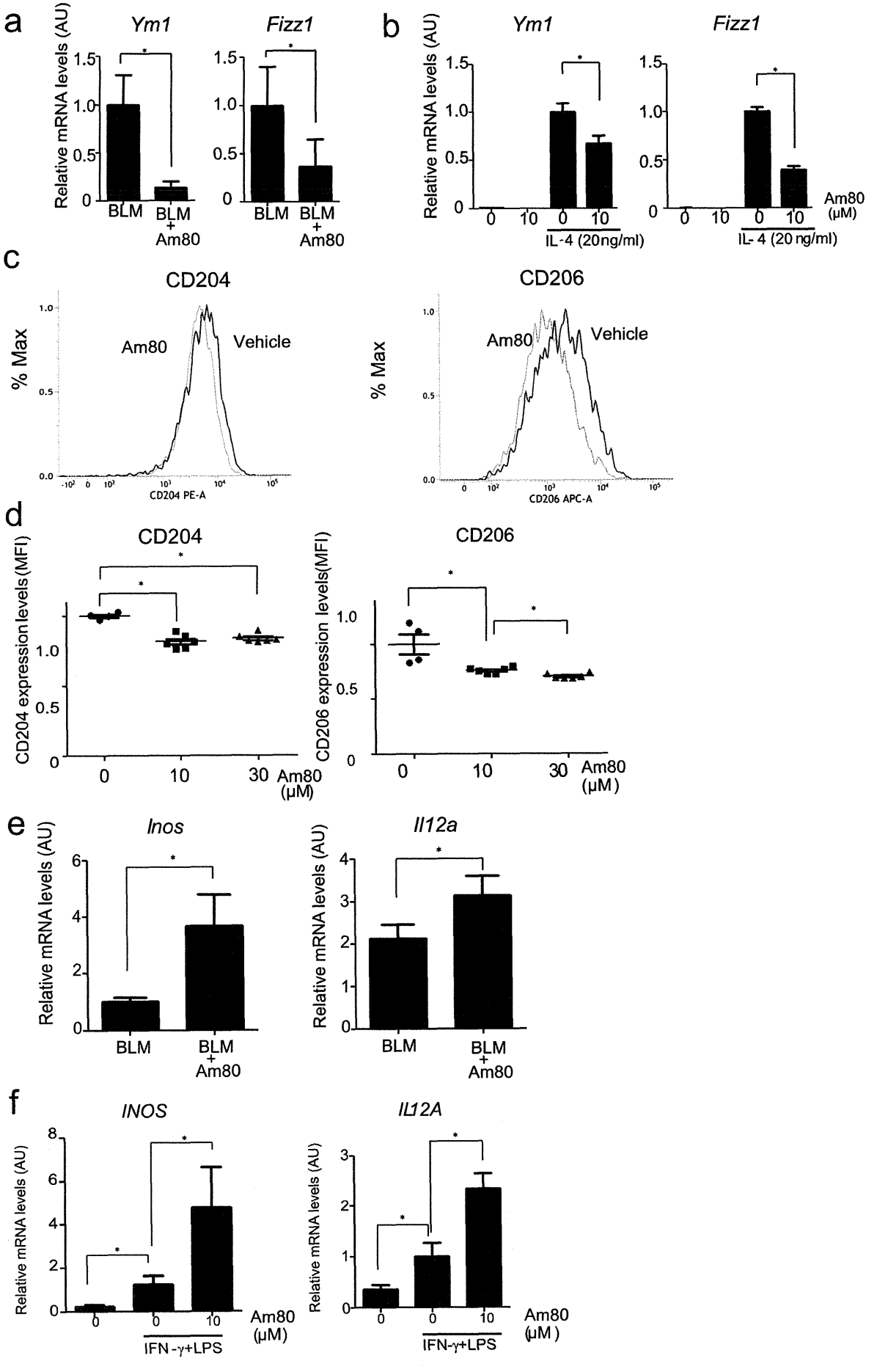
82.98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Fizz1
Relative mRNA levels (AU)
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BLM + Am80
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Ym1
Fizz1
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Am80 (μM)
c
CD204
CD206
% Max
Am80
Vehicle
CD204 PE-A
CD206 APC-A
d
CD204
CD206
CD204 expression levels (MFI)
CD206 expression levels(MFI)
Am80 (μM)
e
Inos
Il12a
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BLM
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f
INOS
IL12A
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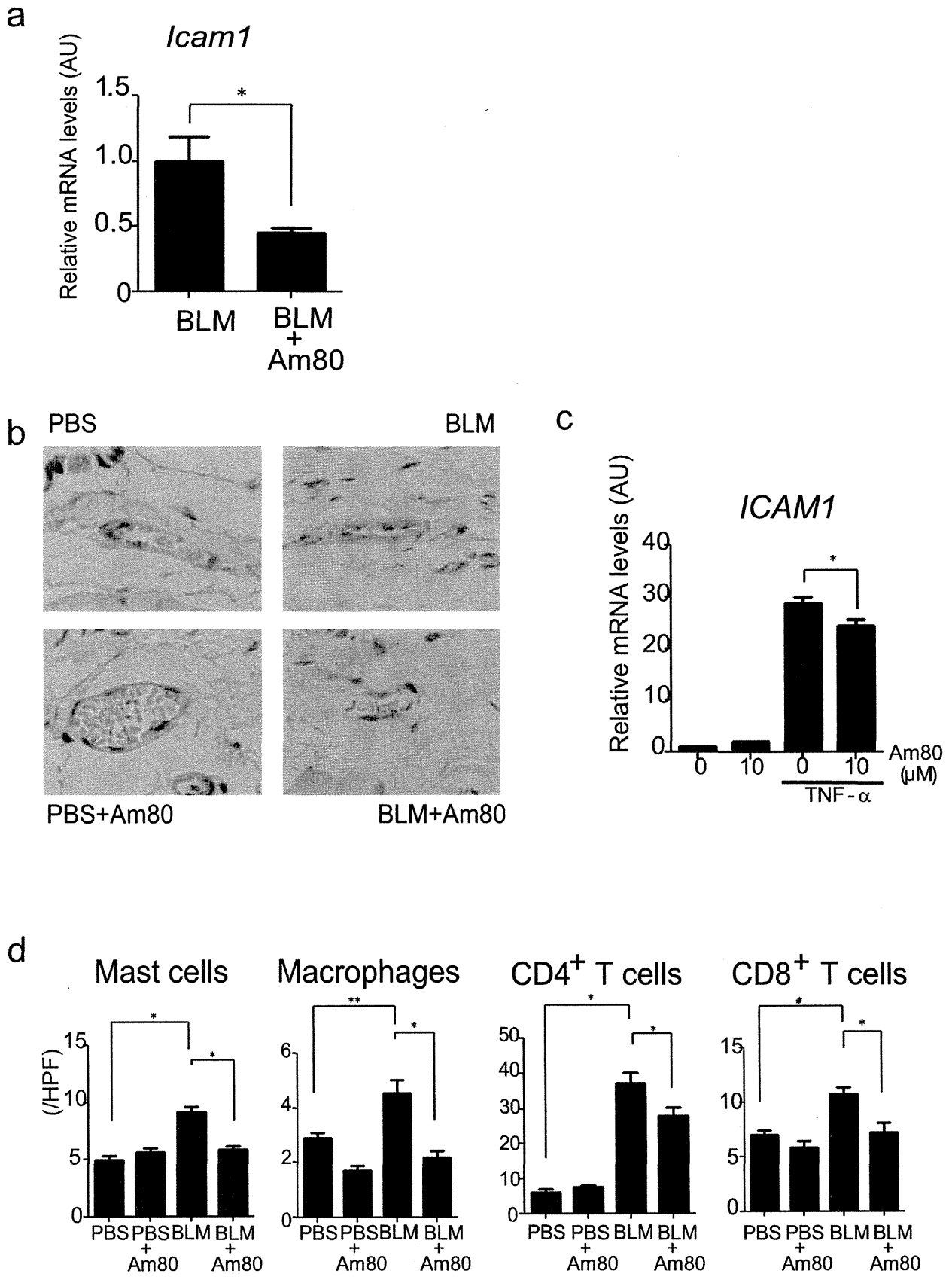
Am80 (μM)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PBS+Am80
BLM+Am80
c
ICAM1
Relative mRNA levels (AU)
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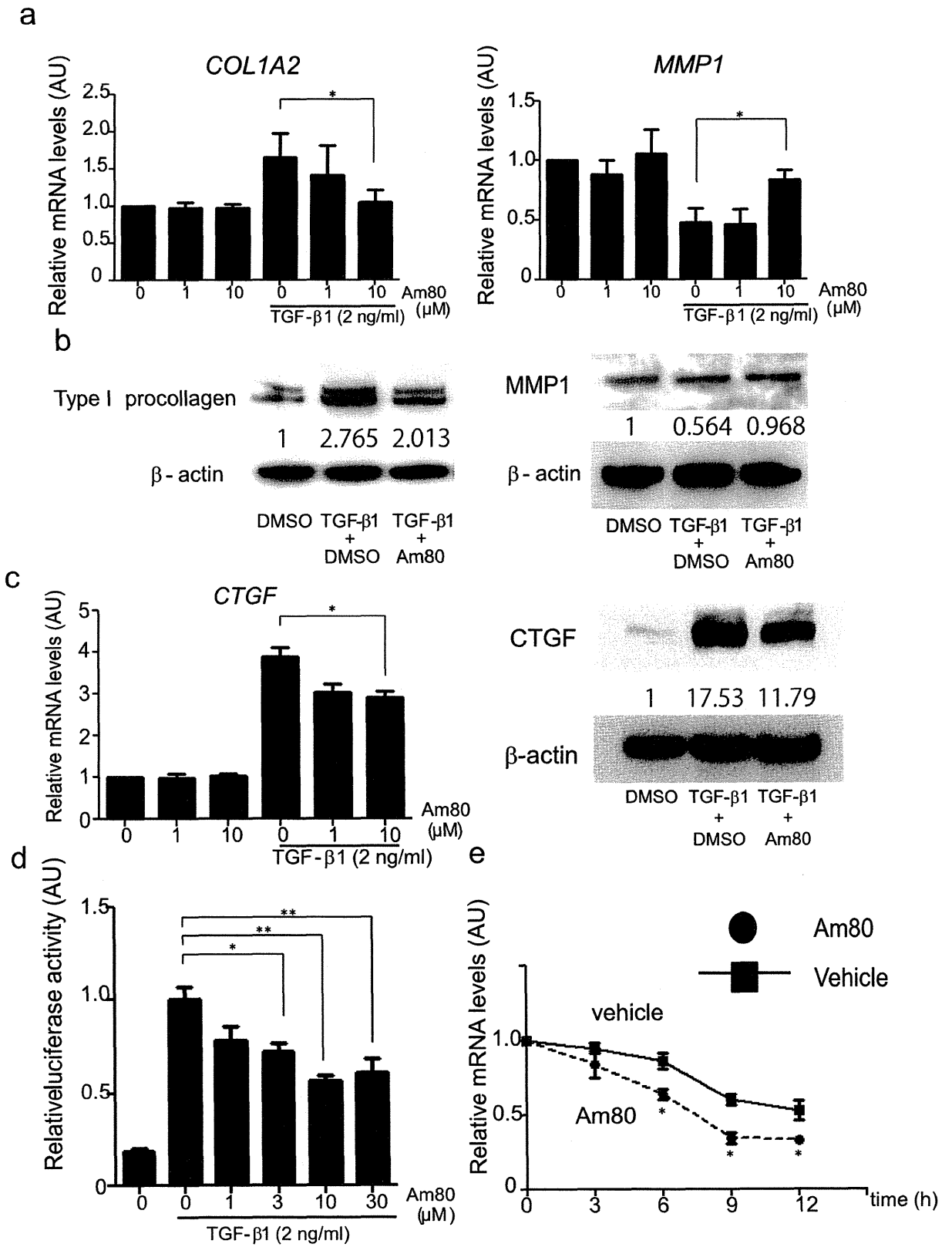
30
20
10
0
*
0
10
0
10
Am80 (μM)
TNF-α
d
Mast cells
Macrophages
CD4+ T cells
CD8+ T cells
(/HPF)
PBS
PBS + Am80
BLM
BLM + Am80


a
COL1A2
MMP1
Relative mRNA levels (AU)
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2.0
1.5
1.0
0.5
0
*
0 1 10 0 1 10 Am80 (μM)
TGF-β1 (2 ng/ml)
b
Type I procollagen
1 2.765 2.013
β-actin
DMSO TGF-β1 + DMSO TGF-β1 + Am80
MMP1
1 0.564 0.968
β-actin
DMSO TGF-β1 + DMSO TGF-β1 + Am80
c
CTGF
Relative mRNA levels (AU)
5
4
3
2
1
0
*
0 1 10 0 1 10 Am80 (μM)
TGF-β1 (2 ng/ml)
CTGF
1 17.53 11.79
β-actin
DMSO TGF-β1 + DMSO TGF-β1 + Am80
d
Relativeluciferase activity (AU)
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0 0 1 3 10 30 Am80 (μM)
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1.0
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EXTENDED REPORT

Nucleosome in patients with systemic sclerosis: possible association with immunological abnormalities via abnormal activation of T and B cells

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ABSTRACT

Objective To determine the serum levels of nucleosome in patients with systemic sclerosis (SSc) and relate the results to the clinical features of SSc.

Methods Serum nucleosome levels in 91 patients with SSc were examined by ELISA. The expression of Toll-like receptor (TLR) 9 in T and B cells was quantified by flow cytometric intracellular protein analysis. The effects of nucleosomes on lymphocytes were also analysed. Moreover, we assessed the effects of nucleosomes on fibrosis, using wild type and CD19-deficient bleomycin-treated mice, an experimental model for human SSc.

Results Serum nucleosome levels were elevated in SSc compared with healthy controls and correlated positively with the extent of skin and pulmonary fibrosis and immunological abnormalities. The retrospective longitudinal analysis showed the serum nucleosome levels to be attenuated during the follow-up period. TLR9, which can be stimulated by nucleosome expression was upregulated in the affected T and B cells of patients with SSc. Moreover, nucleosome stimulation strongly increased interleukin (IL)-4 and IL-17 expression of T cells, B-cell IgG production and proliferation of lymphocytes in SSc compared with those in healthy controls. In bleomycin-induced SSc model mice, serum nucleosome levels were elevated compared with control mice. Furthermore, nucleosomes increased IgG production and proliferation of mouse B cells. Although TLR9 expression was similar between wild type and CD19-deficient splenic B cells, CD19 deficiency reduced these nucleosome effects.

Conclusion These results suggest that nucleosomes and its signalling in B and T cells contribute to disease development in SSc via TLR9.

INTRODUCTION

Systemic sclerosis (SSc) is a multisystemic disorder of connective tissue characterised by excessive accumulation of extracellular matrix in the skin and various internal organs, such as lung, oesophagus, kidney and heart.[1] SSc has various immunological abnormalities, including autoantibody production, hyper-γ-globulinaemia and elevated levels of several cytokines.[2 3] Although these abnormalities were associated with several organ involvement and systemic vascular damage, the mechanism and pathogenesis of SSc remain unknown.[4]

Previous studies have revealed that serum levels of endogenous ligands for Toll-like receptors (TLRs), such as DNA, hyaluronan, high-mobility group box 1 protein and heat shock proteins, increased in SSc.[5–7] These endogenous ligands for TLRs are produced or released from damaged tissues and necrotic and apoptotic cells, which results in inducing immunological abnormalities in autoimmune diseases through TLR signalling.[5 7 8] Indeed, our previous studies have shown that serum hyaluronan and high-mobility group box 1 protein levels are associated with disease severity and immunological abnormalities in patients with SSc.[5 7] Furthermore, these endogenous ligands for TLRs induced several cytokine production from B cells and this B-cell reaction was regulated by one of the most important B-cell response regulators, CD19, which is a critical cell-surface signal transduction molecule of cells.[6] Our previous mouse study using CD19 knockout (CD19−/−) mice also indicated that CD19 deficiency suppresses fibrosis and autoantibody production by inhibiting TLR signals.[6] Nucleosomes are core particles composed of an octamer of two copies each of histones H2A, H2B, H3 and H4, around which helical DNA of 146 base pairs length is wrapped.[9 10] Nucleosomes can be found in low amounts in the serum and plasma of healthy individuals.[11] In addition, apoptotic and necrotic cell death during autoimmune systemic inflammation and infection has been identified as an important source of the nucleosomal release from cell.[12–14]

Actually, elevated levels of serum nucleosomes are found in various pathological conditions.[14 15] Although recent studies have shown that nucleosomes stimulate TLR9 as an endogenous ligand for TLR9 and thereby affect immunological abnormalities, any correlation of serum nucleosome levels with immunological measures and disease severity has not been previously investigated in patients with SSc.[16 17] It was therefore the objective of this study to investigate the clinical and immunological correlation of serum nucleosome levels in patients with SSc and a bleomycin (BLM)-induced mice model, which is well known and an established model for human SSc. In addition, we examined the responsiveness of T and B cells to nucleosome stimulation.

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PATIENTS AND METHODS

Serum samples from patients

Serum samples were obtained from 91 Japanese patients with SSc and signed informed consent was obtained from all patients. All patients fulfilled the criteria proposed by the American College of Rheumatology.[18] Patients were grouped into limited cutaneous SSc (lSSc) and diffuse cutaneous SSc (dSSc), according to the classification system proposed by LeRoy *et al.*[19] The duration of the disease was calculated from the time of onset of the first clinical event (other than Raynaud's phenomenon) that was a clear manifestation of SSc according to previous studies.[20–22] None of the patients with SSc was treated with oral corticosteroid, D-penicillamine or other immunosuppressive therapy at the evaluation. Twenty age-matched and sex-matched healthy Japanese individuals were used as normal controls. Plasma samples were also obtained from 38 patients. All samples were stored at −70°C prior to use. All studies were approved by the Committee on Ethics of University of Tokyo Graduate School of Medicine.

Clinical assessment

Complete medical histories, physical examinations and laboratory tests, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLco), were conducted for all patients. When the DLco and VC were <75% and <80%, respectively, of the predicted normal values, they were considered to be abnormal. Skin score was measured by scoring technique of the modified Rodnan total skin thickness score (TSS) as previously described.[23] Organ involvement was defined as described previously[20]: pulmonary fibrosis=bibasilar fibrosis on chest radiography and high-resolution CT; oesophagus=hypomotility shown by barium radiography and gastroesophageal reflux disease shown by gastrointestinal endoscopy; heart=pericarditis, congestive heart failure or arrhythmias requiring treatment; kidney=malignant hypertension and rapidly progressive renal failure with no other explanation; joints=inflammatory polyarthralgias or arthritis and muscle=proximal muscle weakness and elevated serum creatine kinase. These symptoms were assessed and confirmed by each specialist, including rheumatologists, chest physicians, gastroenterologists and cardiologists.

Mice

CD19−/− mice were generated as described[24] and backcrossed more than 15 generations onto the C57BL/6 background before use in this study. All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. The mice used in these experiments were 6 weeks of age.

BLM-induced SSc model mice

BLM (Nippon Kayaku, Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/mL and sterilised by filtration. BLM or PBS (300 μg) was injected subcutaneously into the shaved backs of the mice daily for 4 weeks with a 27-gauge needle, as described previously.[6] BLM-treated mice were also intravenously injected with 300 μg/kg of the TLR9 antagonist, ODN 2088 (Invivogen, San Diego, California, USA), or the negative control for ODN 2088 (Invivogen) weekly for 4 weeks. We confirmed that the negative control for ODN 2088 did not affect any analysis in this study (data not shown).

Histopathological assessment of dermal and lung fibrosis

Skin and lung sections obtained from mice were assessed under a light microscope. Sections were stained with H&E. We examined dermal thickness, which was defined as the thickness of skin from the top of the granular layer to the junction between the dermis and subcutaneous fat. The skin tissue hydroxyproline content was also measured as a quantitative measure of collagen deposition as previously described.[25] The severity of lung fibrosis was semi-quantitatively assessed according to the method of Ashcroft *et al.*[26] The grading criteria were as follows: grade 0=normal lung; grade 1=minimal fibrous thickening of alveolar or bronchiolar walls; grade 3=moderate thickening of walls without obvious damage to lung architecture; grade 5=increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; grade 7=severe distortion of structure and large fibrous areas and grade 8=total fibrous obliteration of fields. Grades 2, 4 and 6 were used as intermediate pictures between the aforementioned criteria.

Determination of hydroxyproline content in the skin tissue

Hydroxyproline is a modified amino acid uniquely found at a high percentage in collagen. Therefore, the skin and lung tissue hydroxyproline content was measured as a quantitative measure of collagen deposition as previously described.[25] The punch biopsy (6 mm) samples obtained from shaved dorsal skin and the harvested right lung of each mouse were analysed. A hydroxyproline standard solution of 0–6 mg/mL was used to generate a standard curve.

ELISA for serum nucleosomes, centromeric protein B, Ig, antinuclear antibodies and other autoantibodies

ELISA for serum levels of nucleosome in both human and mouse (Roche Applied Science, Indianapolis, Indiana, USA) was performed using specific ELISA kits, according to the manufacture's protocols. Serum IgG concentrations were assessed as described.[6] Antinuclear antibodies (Abs) were assessed by indirect immunofluorescence staining using HEp-2 substrate cells (Medical & Biological Laboratories, Nagoya, Japan) as described.[13] The specific ELISA kits were used to measure anti-nucleosome, anti-topoisomerase I (Medical & Biological Laboratories), anti-centromere (Medical & Biological Laboratories), anti-centromeric protein B (Funakoshi, Tokyo, Japan), anti-histone (Medical & Biological Laboratories), anti-single-stranded DNA Abs (Medical & Biological Laboratories) and centromeric protein B (Antibodies-online, Aachen, Germany). Each sample was tested in duplicate.

T-cell and B-cell purification and stimulation

Heparinised blood samples were obtained from 20 patients with dSSc and 10 healthy individuals. In addition, splenocytes were obtained from five wild type and five CD19−/− mice. These T or B cells are enriched with each isolation kit using AutoMACS isolator (Miltenyi Biotec, Bergisch Gladbach, Germany). A total of >99% of these cells were CD3 or CD19. To analyse mRNA expression of interleukin (IL)-4, IL-10, IL-17 or interferon (IFN)-γ in T cells, real-time PCR quantification was performed as described previously.[6] Expression of TLR9 was determined by flow cytometric intracellular protein analysis and real-time PCR. Abs used in this study included fluorescein isothiocyanate (FITC)-conjugated anti-human or mouse CD3 and CD19 as well as phycoerythrin (PE)-conjugated monoclonal Ab to TLR9 (all Abs from eBioscience, San Diego, California, USA). The cells (1×10^5) were stimulated with 1, 5 or 10 μg/mL of nucleosomes (Reaction Biology Corp, Malvern, Pennsylvania, USA) or 10 μg/mL of CpG (ODN 2006 for humans and ODN 1826 for mice), DNA (Sigma-Aldrich, St Louis, Mississippi, USA) and/or histone (Sigma-Aldrich). In addition, the nucleosomes were

isolated from plasma of patients with SSc, according to manufactures' protocol (Epigentek, Brooklyn, New York, USA). To assess whether patient nucleosomes stimulate lymphocyte stronger than commercial competent nucleosomes, T cells and B cells obtained from 10 patients with SSc were stimulated with 10 μg/mL of their own plasma nucleosomes. T cells were cultured for 72 h and expression levels of cytokines were measured by real-time PCR. In addition, B cells were cultured for 8 days and IgG concentrations in the culture medium were measured by ELISA (Bethyl Laboratories, Montgomery, Texas, USA). Proliferation of T and B cells was quantified by a colorimetric 5-bromo-2′-deoxyuridine (BrdU) cell proliferation ELISA kit (Roche Applied Science, Indianapolis, Indiana, USA). After 24 h incubation with or without nucleosomes, BrdU (10 μM) was added to each well and incubated for 24 h. ODN 2088 or the ODN 2088 negative control (Invivogen) was added 60 min before nucleosome stimulation at concentrations of 10 μg/mL. Each sample was performed in triplicate. We confirmed that the negative control for ODN 2088 did not affect any analysis in this study (data not shown).

Statistical analysis

Statistical analysis was performed using Mann–Whitney U test for determining the level of significance of differences between sample means, Fisher's exact probability test for comparison of frequencies and Bonferroni test for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. A p value <0.05 was considered statistically significant.

RESULTS

Serum nucleosome levels in SSc

Serum nucleosome levels were significantly elevated in patients with SSc compared with controls (figure 1A; $p<0.01$). There was no difference in nucleosome concentration when serum or plasma samples were studied ($r=0.94$; figure 1B). For the SSc subgroups, serum nucleosome levels were significantly elevated in patients with dSSc relative to those with lSSc ($p<0.05$). Values higher than the mean+2SD of the control serum samples were found in 62% of all patients with SSc, in 67% of patients with dSSc and in 56% of patients with lSSc. By contrast, only 5% of controls had elevated nucleosome levels. In addition, the retrospective longitudinal analysis in this study showed the serum nucleosome levels to be attenuated during the follow-up period (figure 1C).

Clinical features of patients with SSc having elevated serum nucleosome levels

We assessed the clinical features of patients with SSc having elevated nucleosome levels compared with the patients with SSc having normal nucleosome levels (table 1). The duration of disease from the onset of clinical manifestations of SSc, including Raynaud's phenomenon, was significantly shorter ($p<0.01$) in patients with SSc having increased nucleosome levels than normal nucleosome levels. Patients with SSc having elevated nucleosome levels had significantly higher frequency of dSSc ($p<0.01$), pulmonary fibrosis ($p<0.01$) and presence of anti-topoisomerase I Ab ($p<0.01$), higher modified Rodnan TSS points ($p<0.05$) and serum levels of IgG ($p<0.05$), decreased %DLco ($p<0.05$) and more frequent involvement of pitting scar/ulcer ($p<0.01$) and oesophagus ($p<0.01$) than those with normal levels. Consistent with these findings, nucleosome levels were significantly elevated in patients with SSc having pitting scar/ulcer, pulmonary fibrosis or oesophageal involvement

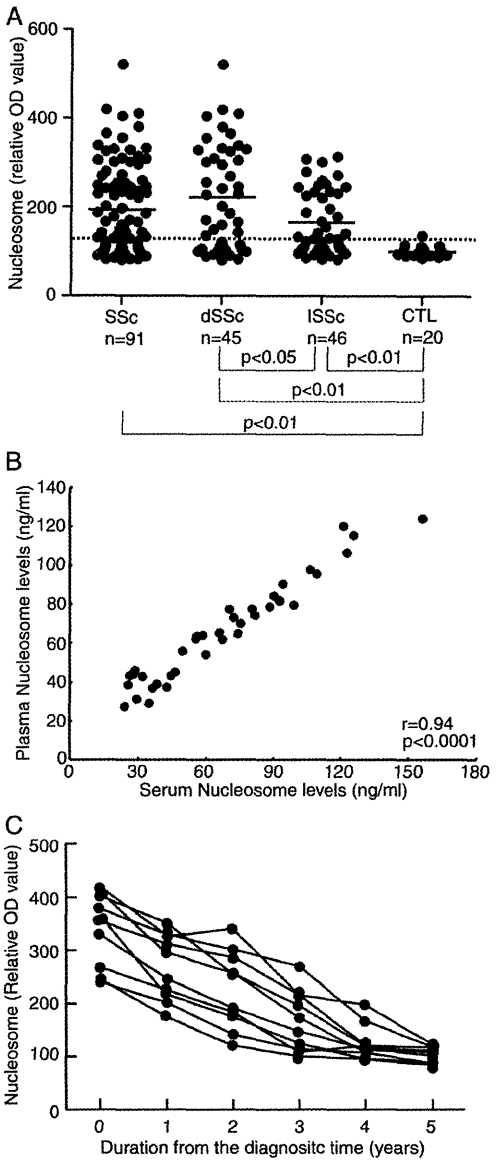

Figure 1 (A) Nucleosome levels in serum samples from patients with diffuse cutaneous systemic sclerosis (dSSc), those with limited cutaneous systemic sclerosis (lSSc) and healthy controls (CTL). Serum nucleosome levels were determined by ELISA. Horizontal lines show the mean values. A broken line indicates the cut-off value (mean+2SD of the healthy control samples). (B) The concentrations of nucleosomes were almost similar when serum or plasma samples were studied. (C) Serial changes in serum nucleosome levels during the follow-up period in 10 patients with systemic sclerosis (SSc). This retrospective longitudinal analysis showed the serum nucleosome levels to be attenuated during the follow-up period. OD, optical density.

compared with those without each clinical measure (figure 2). Serum nucleosome levels also weakly correlated inversely with %DLco ($r=-0.42$, $p<0.001$) or %VC ($r=-0.29$, $p<0.005$) and positively with modified Rodnan TSS ($r=0.29$, $p<0.005$; figure 3). Furthermore, serum nucleosome levels correlated positively with serum levels of IgG ($r=0.65$, $p<0.001$). However, serum nucleosome levels did not correlate with any other clinical symptoms, antinuclear Abs, autoantibodies, including anti-nucleosome and anti-centromeric protein-B Abs and centromeric protein-B.

Table 1 Clinical and laboratory features of patients with SSc

Characteristic	Elevated nucleosome n=56	Normal nucleosome n=35
Sex, no. of men/women	7/49	3/32
Age at onset, median (quartiles) years	52 (40–62)	52 (36–59)
Disease duration, median (quartiles) years	4 (1–6)**	8 (6–11)
Duration from the initiation of Raynaud's phenomenon, median (quartiles) years	5 (2–6)**	10 (6–12)
Disease pattern, no. with dSSc/lSSc	41/15**	15/20
Clinical features		
Modified Rodnan TSS, median (quartiles) points	18 (9–24)*	9 (3–15)
Pitting scar/ulcer	66**	34
Organ involvement		
Lungs		
Pulmonary fibrosis	71**	23
%VC, median (quartiles)	93 (76–107)	94 (74–113)
%DLco, median (quartiles)	55 (45–60)**	76 (70–83)
Oesophagus	77**	43
Joints	18	14
Muscles	20	17
Heart	14	14
Kidneys	11	9
Laboratory findings		
Positive for anti-nucleosome Ab	2	3
Positive for anti-topoisomerase I Ab	68**	14
Positive for anti-centromere Ab	18	29
Positive for anti-CENP-B Ab	14	20
Positive for anti-histone Ab	5	6
Positive for anti-ssDNA Ab	2	3
Serum IgG, median (quartiles)	1880 (1570–2284)**	1280 (1195–1500)
Serum IgM, median (quartiles)	191 (74–286)	184 (96–272)
C reactive protein, median (quartiles)	0.28 (0.06–0.55)	0.24 (0.06–0.51)

Unless noted otherwise, values are in percentage.
*p<0.05, **p<0.01 versus patients with SSc having normal nucleosome levels.
Ab, antibody; DLco, diffusion capacity for carbon monoxide; dSSc, diffuse cutaneous systemic sclerosis; lSSc, limited cutaneous systemic sclerosis; SSC, systemic sclerosis; ssDNA, single-stranded DNA; TSS, total skin thickness score; VC, vital capacity.

The responsiveness of SSc T and B cells to nucleosome stimulation

To determine the role of serum nucleosomes in the function of SSc T and B cells, first we assessed TLR9 expression levels on T and B cells from patients with SSc (figure 4A). The levels of TLR9 expression on SSc T and B cells were significantly higher than those observed in healthy controls ($p<0.01$, respectively). Similar results were obtained in real-time PCR analysis. Next, we assessed the responsiveness of T and B cells stimulated with 10 μg/mL of nucleosomes. Stimulation of SSc or healthy T cells with nucleosomes significantly increased expression of IFN-γ, IL-4, IL-17 and IL-10 compared with media alone, respectively ($p<0.01$, figure 4B). Although there was no significant difference in IFN-γ and IL-10 expression between patients with SSc and healthy controls, IL-4 and IL-17 mRNA levels of SSc T cells were higher than those of healthy T cells ($p<0.01$, respectively). Similarly, stimulation of B cells with nucleosomes increased IgG production compared with media alone ($p<0.01$, figure 4C). Furthermore, the production of IgG in SSc B cells treated with nucleosomes was higher than those in healthy B cells ($p<0.01$). Although the proliferative effect of CpG, a specific TLR9 ligand, was observed on both SSc and healthy lymphocytes, CpG significantly increased BrdU incorporation in SSc T and B cells compared with that in healthy controls ($p<0.01$, figure 4D). Similarly, the proliferative effect of nucleosomes was observed on both healthy and SSc lymphocytes in a dose-dependent manner. However, in SSc T and B cells, nucleosomes significantly increased BrdU incorporation compared with healthy controls ($p<0.05$). In addition, the mRNA expression levels of IL-4, IL-10, IL-17 and IFN-γ in SSc T cells and IgG production from SSc B cells stimulated with nucleosomes from patients with SSc were almost similar to those with commercial competent nucleosomes. We then assessed whether or not the TLR9 antagonist inhibited nucleosome-induced lymphocyte proliferation (figure 4D). In SSc and healthy control lymphocytes, treatment of both nucleosomes and the TLR9 antagonist inhibited the proliferative effect of nucleosomes compared with nucleosomes alone ($p<0.01$, respectively). The stimulation with cell-free DNA and/or histone could not increase cytokine mRNA expression and IgG production.

The TLR9 antagonist attenuated the development of skin and lung fibrosis induced by BLM

Skin fibrosis and lung fibrosis were assessed 4 weeks after the initiation of BLM treatment in wild type and CD19−/− mice (figure 5A), according to our previous studies.[6 27] Dermal thickness and hydroxyproline content which are highly sensitive to assess the collagen synthesis and skin sclerosis in BLM-treated

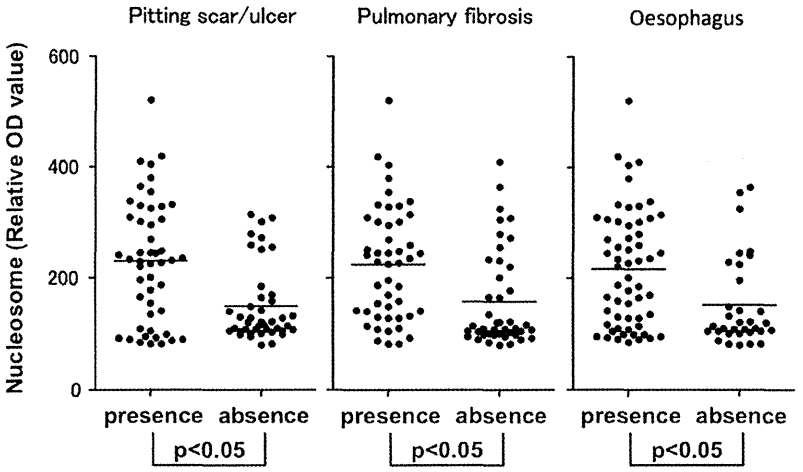

Figure 2 Serum nucleosome levels in the presence and absence of pitting scar/ulcer, pulmonary fibrosis, and oesophagus involvement in patients with systemic sclerosis. Serum nucleosome levels were determined by ELISA. OD, optical density.

wild type and CD19−/− mice were significantly higher than that in PBS-treated mice ($p<0.01$ and $p<0.05$, respectively). The TLR9 antagonist, ODN 2088, significantly reduced dermal fibrosis induced by BLM treatment in wild-type mice, though dermal thickness in mice treated with both BLM and ODN 2088 remained greater than that in only PBS-treated mice ($p<0.05$). However, in CD19−/− mice, ODN 2088 did not affect dermal thickness. Similar results were obtained for the lung fibrosis score (figure 5A). The histological changes were reduced by treatment with ODN 2088 in wild-type mice, whereas ODN 2088 did not change lung fibrosis in CD19 −/− mice. In addition, serum levels of nucleosomes were significantly increased in BLM-treated wild-type and CD19−/− mice, respectively ($p<0.01$, figure 5B). However, there was no significant difference between BLM-treated wild-type and CD19 −/− mice and ODN2088 did not affect the nucleosome levels.

Effects of nucleosomes on B cells in wild-type and CD19 −/− mice

The expression levels of TLR9 were similar in wild-type and CD19−/− B cells (figure 5C). Remarkably, nucleosome-stimulated wild-type B cells significantly increased BrdU incorporation compared with wild-type B cells cultured with only media ($p<0.01$, figure 5D). Similarly, wild-type B cells treated with nucleosomes significantly increased production of IgG compared with those treated with media alone ($p<0.01$). In contrast, there was no difference in BrdU incorporation and IgG production between CD19−/− B cells treated with nucleosome and CD19−/− B cells treated with media alone.

DISCUSSION

In this study, we showed that nucleosome levels were elevated in serum samples of patients with SSc, especially dSSc, relative to normal controls (figure 1A). Furthermore, the present study is the first to reveal that nucleosome levels correlated positively or negatively with several clinical and laboratory features (figures 2 and 3 and table 1). Furthermore, we showed that elevation of nucleosome levels was significantly related to the early stage of SSc (figure 1C and table 1). Collectively, these results suggest that the serum level of nucleosomes is a useful serological marker for evaluating the disease severity and disease activity.

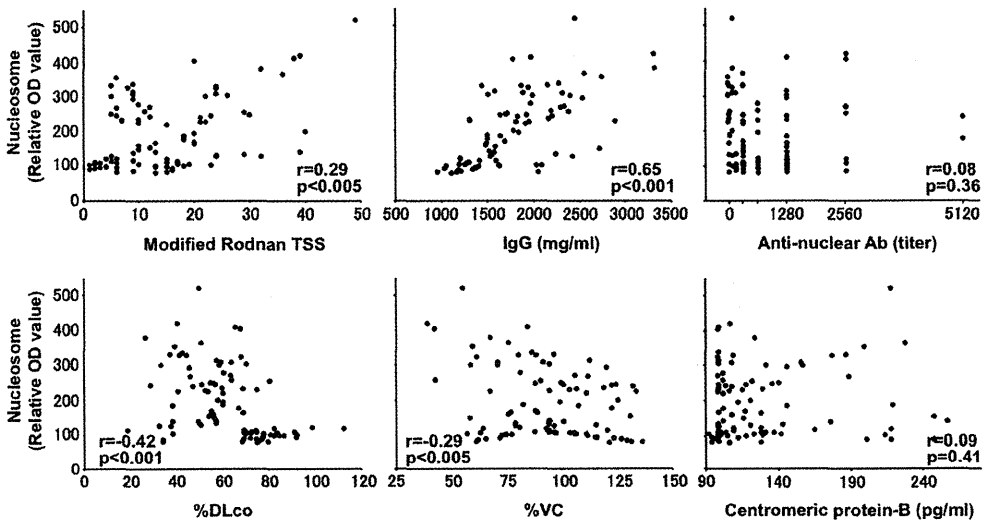

Figure 3 The correlations of serum nucleosome levels against modified Rodnan total skin thickness score (TSS), serum IgG levels, antinuclear antibody titres, % diffusion capacity for carbon monoxide (DLco), % vital capacity (VC), and centromeric protein-B levels in patients with systemic sclerosis. Serum nucleosome and centromeric protein-B levels were determined by ELISA.

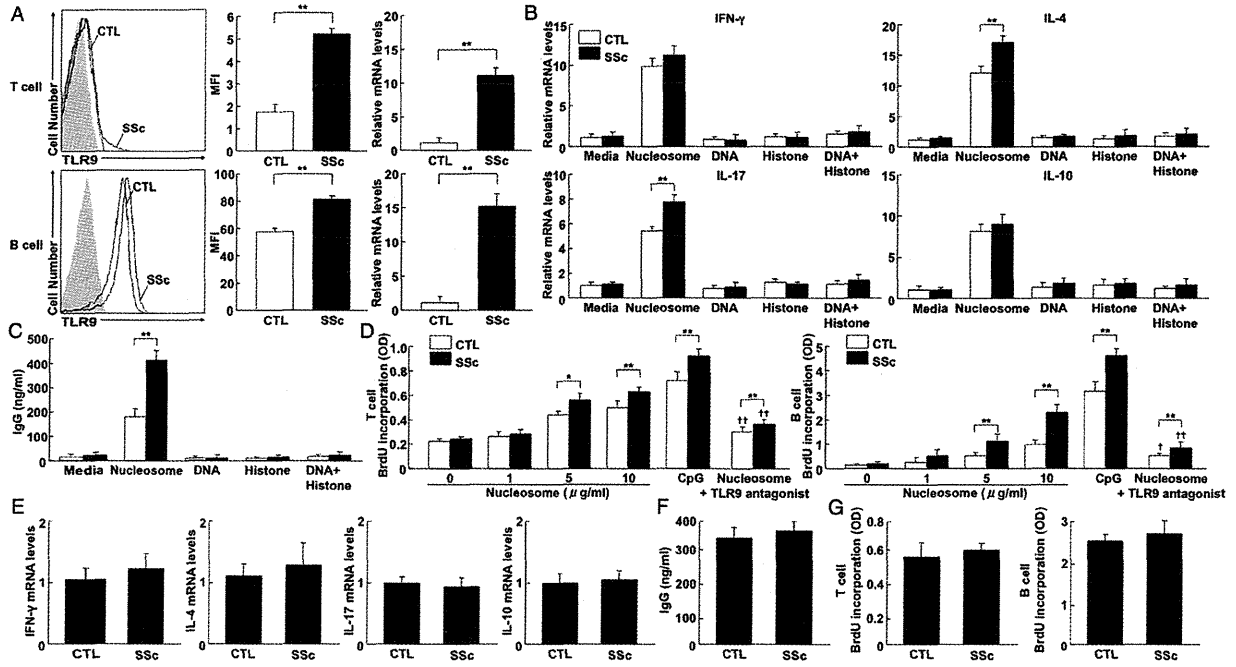

Figure 4 Blood samples were obtained from 20 patients with systemic sclerosis (SSc) and 10 healthy controls (CTL). (A) Toll-like receptor (TLR)9 expression in T cells (1×10^5) and B cells (1×10^5) was analysed by flow cytometry and real-time PCR. (B and C) For expression of interleukin (IL)-4, IL-10, IL-17 and interferon (IFN)-γ (B) and production of IgG (C), purified T cells (1×10^5) and B cells (1×10^5) were stimulated with nucleosomes, DNA, and/or histone. (D) For proliferation assay, after 24 h incubation, purified T cells (1×10^5) and B cells (1×10^5) were treated with nucleosomes and TLR9 antagonist. 5-Bromo-2′-deoxyuridine (BrdU) (10 μM) was also added to each well and incubated for 24 h. BrdU incorporation in proliferating cells was quantified by ELISA. (E–G) To assess whether nucleosomes from SSc stimulate lymphocyte stronger than commercial competent nucleosomes, T cells and B cells were stimulated with nucleosomes obtained from same SSc patient (n=10) or with competent nucleosomes. The mRNA expression levels of IL-4, IL-10, IL-17 and IFN-γ in SSc T cells (E) and IgG production from SSc B cells (F) were assessed. For proliferation assay, BrdU incorporation was measured by ELISA (G). Each histograms indicate mean (+2SD); *p<0.05, **p<0.01. †p<0.05, ††p<0.01 versus cells stimulated with 10 μg/mL nucleosomes. MFI, mean fluorescence intensity.

In this study, serum nucleosome levels correlated with immunological parameters (figure 3 and table 1). Nucleosomes which are released from damaged, necrotic and apoptotic cells interact with TLR9.[12–14 17] Nucleosomes appear to be the particles that provide DNA in vivo, possibly making the DNA immunogenic when not properly cleared.[28] Numbers of apoptotic or necrotic cells have been found to be increased in several autoimmune/inflammatory diseases.[6 14 15 29 30] Indeed, it is well established that TLR9 which binds bacterial DNA via unmethylated CpG sequences is thought to play a role in the T-cell and B-cell response to nucleosomes and the pathogenesis of systemic lupus erythematosus.[17 31 32] In this study, stronger expression of TLR9 was detected in SSc T and B lymphocytes than in healthy controls (figure 4A). Nucleosome stimulation enhances the lymphocyte proliferation, production of inflammatory cytokines and IgG production through TLR9 (figure 4B–D). Moreover, recent studies have reported that B-cell cytokine production is regulated by TLR9 signalling, in which the B-cell receptor pathway is not required.[33 34] Actually, in this study, the levels of nucleosomes were most positively correlated with serum IgG levels, which supposed that nucleosomes induced polyclonal B-cell activation (figure 3). Therefore, B cells respond to nucleosomes without B-cell receptor signalling, which may reflect that the serum levels of anti-nucleosome Abs do not increase in SSc. Furthermore, between the stimulation of patient nucleosomes and commercial competent nucleosomes, there were no significant differences in mRNA expression in SSc T cells, IgG production from SSc B cells and lymphocyte proliferation (figure 4E–G). These results suggested that nucleosome stimulation was independent to their structures, such as their DNA sequence and DNA methylation and acetylation. Previous studies have also indicated that TLR9 stimulation also increases T-cell activity and viability and induces differentiation directly via the MyD88-TLR9 pathway.[35–37] Although nucleosome levels correlated several disease manifestations, nucleosome levels did not correlate antinuclear Abs and nucleosome-related autoantibodies, including anti-nucleosome, anti-centromere, anti-centromeric B and anti-histone Abs (table 1 and figure 3). These results reflect that nucleosomes may play a direct role in immunological abnormalities associated with SSc, which is independent of the antigen-specific pathway.

Serum nucleosome elevation was also detected in BLM-induced SSc model mice (figure 5B). Moreover, TLR9 antagonist reduced dermal and lung fibrosis in BLM-induced SSc model mice, which were confirmed by hydroxyproline content measurement (figure 5A). In contrast, CD19 deficiency cancelled these inhibitory events of TLR9 antagonist, though the expression levels of TLR9 were similar between wild-type and CD19 −/− mice (figure 5A, C). Among B-cell response regulators, CD19, which is a critical cell-surface signal transduction molecule of B cells, is a most potent positive regulator.[6 38] Transgenic mice that overexpress CD19 by approximately threefold lose tolerance and generate autoantibodies spontaneously.[39 40] Actually, human patients with SSc exhibit a 20% increase in CD19 expression.[41] This CD19 overexpression may be related to autoantibody production and hyper-γ-globulinaemia in human SSc, because mice that overexpress CD19 to a similar extent as human SSc have hyper-γ-globulinaemia and elevated levels of various autoantibodies.[42] In previous study, we have showed that CD19

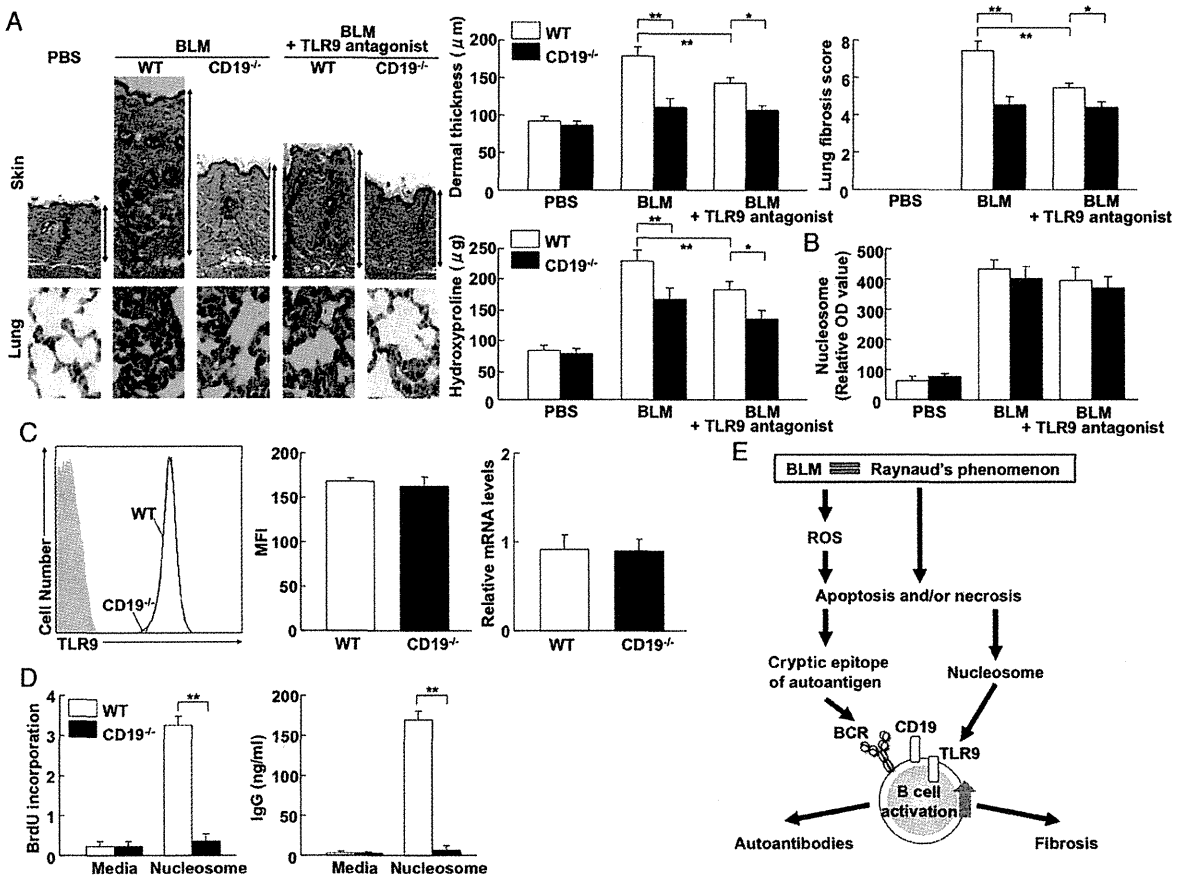

Figure 5 (A) Skin and lung fibrosis from wild type (WT) and CD19−/− mice treated with phosphate-buffered saline (PBS) or bleomycin (BLM) along with or without the Toll-like receptor (TLR)9 antagonist. Dermal fibrosis was assessed further by quantitatively measuring hydroxyproline content. (B) Nucleosome levels in serum samples from WT and CD19−/− mice treated with PBS or BLM along with or without the TLR9 antagonist. Serum nucleosome levels were determined by ELISA. (C) TLR9 expression in B cells was analysed by flow cytometry and real-time PCR. (D) 5-Bromo-2′-deoxyuridine (BrdU) incorporation and IgG production by B cells stimulated with nucleosomes. (E) A model linking systemic autoimmunity and tissue fibrosis in patients with SSc and BLM-induced SSc model mice. Patients with SSc exhibit enhanced reactive oxygen species (ROS) production, due to ischaemia and reperfusion injury following Raynaud's phenomenon. Similarly, BLM induces the production of ROS. Consequently, tissue damage, such as necrosis and apoptosis, induced by ROS increases nucleosomes which induce B-cell activation through the TLR9 signalling. In the surface blebs of apoptotic cells, autoantigens including topoisomerase I are concentrated, which may result in the antigen presentation of the cryptic epitopes. Remarkably, CD19 loss inhibits these B-cell activation and autoantibody production. Each histograms indicate mean (+2SD); *p<0.05, **p<0.01. The double-headed arrow indicates dermis. Original magnification, ×200. BCR, B-cell receptor; MFI, mean fluorescence intensity.

regulates fibrogenic cytokine production by B cells through TLR2 and TLR4 signalling, which was activated by BLM-induced endogenous TLR ligands.[6] Remarkably, in this study, CD19 deficiency inhibited nucleosome-induced B-cell proliferation and IgG production (figure 5D), suggesting that CD19 also influences TLR9 signalling. These results suggest that nucleosomes regulate fibrosis by inducing B-cell activation, which is primarily dependent on CD19 signalling.

It was previously hypothesised that immune responses to autoantigens are induced by cryptic self-epitopes that are generated by the modification of self-antigens during apoptosis.[43] In addition, apoptosis is detected in endothelial cells of early inflammatory lesions in patients with SSc, which is strongly associated with the prevalence of Raynaud's phenomenon.[44] Many previous studies have confirmed that production of free radicals is enhanced in human patients with SSc, due to ischaemia and reperfusion injury following Raynaud's phenomenon, an initial clinical manifestation of SSc.[45] Raynaud's phenomenon is accompanied by the presence of proinflammatory cytokines and the loss of redox control, leading to oxidative stress, tissue damage and endothelial cell apoptosis.[46] Previous studies have also shown that apoptotic cells secrete nucleosomes and are considered as source of extracellular nucleosomes.[47 48] Similarly, in the BLM-induced SSc model, apoptosis was prominently detected in the skin with upregulated expression of Fas and FasL and increased oxidative stress.[27 30 49] In this regard, it is considered that the elevation of nucleosome levels, which was observed in both patients with SSc and BLM-induced SSc model mice, reflects apoptosis and tissue damage. Nucleosomes released from apoptotic cells stimulate immune cells, including T and B cells through TLR9 as an endogenous ligand for TLR9. Taken together, we hypothesise that BLM and Raynaud's phenomenon induce fibrosis by enhancing nucleosome production, which activates B cells to produce mainly fibrogenic cytokines, including IL-6, via CD19 and TLR9 signaling[30 50] and induces autoantibody production (figure 5E). Although further studies are required to clarify the role of nucleosomes in the development of SSc, it may be a useful serological marker for the disease severity and a newly therapeutic target of SSc.

Contributors AY: study design, proceeding every study and analysis and preparing this manuscript. SS: preparing this manuscript. TFT: generating CD19-deficient mice and preparing this manuscript. TT, TF, SE, HN, KN, RS, TY, TT, TeT and YA: collecting patient samples and clinical data.

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Competing interests None declared.

Ethics approval We used patients' blood samples. All studies were approved by the Committee on Ethics of University of Tokyo Graduate School of Medicine.

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Fli1 deficiency contributes to the downregulation of endothelial protein C receptor in systemic sclerosis: a possible role in prothrombotic conditions

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Summary

Background Endothelial protein C receptor (EPCR), expressed predominantly on endothelial cells, plays a critical role in the regulation of the coagulation system and also mediates various cytoprotective effects by binding and activating protein C. So far, the role of EPCR has not been studied in systemic sclerosis (SSc).

Objectives To investigate the potential contribution of EPCR to the development of SSc.

Methods EPCR expression was examined in skin samples and cultivated dermal microvascular endothelial cells by immunostaining, immunoblotting and/or quantitative reverse-transcription polymerase chain reaction. Fli1, binding to the PROCR promoter, was assessed by chromatin immunoprecipitation. Serum EPCR levels were determined by enzyme-linked immunosorbent assay in 65 patients with SSc and 20 healthy subjects.

Results EPCR expression was decreased in dermal small vessels of SSc lesional skin compared with those of healthy control skin. Transcription factor Fli1, deficiency of which is implicated in SSc vasculopathy, occupied the PROCR promoter, and EPCR expression was suppressed in Fli1 small interfering RNA-treated endothelial cells and dermal small vessels of $Fli1^{+/-}$ mice. In patients with SSc, decreased serum EPCR levels were associated with diffuse skin involvement, interstitial lung disease and digital ulcers. Furthermore, serum EPCR levels inversely correlated with plasma levels of plasmin–α2-plasmin inhibitor complex (PIC). Importantly, bosentan significantly reversed circulating EPCR and PIC levels in patients with SSc, and the expression of Fli1 and EPCR in dermal small vessels was elevated in patients treated with bosentan compared with untreated patients.

Conclusions Endothelial EPCR downregulation due to Fli1 deficiency may contribute to hypercoagulation status leading to tissue fibrosis and impaired peripheral circulation in SSc.

What's already known about this topic?

- Endothelial protein C receptor (EPCR) plays a critical role in the coagulation system and mediates various cytoprotective effects by binding and activating protein C.
- The role of EPCR has not been studied in the impaired coagulation/fibrinolysis system of systemic sclerosis.

What does this study add?

- EPCR downregulation potentially contributes to the development of digital ulcers and tissue fibrosis in systemic sclerosis.
- This further supports the critical role of an impaired coagulation/fibrinolysis system in this disease.

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by fibrosis of the skin and certain internal organs following immune attacks and vascular injury.[1] As a part of its pathogenesis, a variable degree of luminal thrombosis following vascular injury may contribute to impaired peripheral circulation and the activation of vascular cells and fibroblasts.[2–4]

Endothelial protein C receptor (EPCR), a type I transmembrane glycoprotein in the CD1/major histocompatibility complex family, is expressed predominantly on endothelial cells and exerts various effects on the coagulation pathway and the behaviour of those cells through binding to protein C.[5] EPCR amplifies the activation status of protein C approximately 20-fold by binding and presenting it to the thrombin–thrombomodulin complex. Activated protein C is then released from EPCR, localizes on negatively charged phospholipid membranes of endothelial cells, and proteolytically inactivates factors Va and VIIIa under the aid of protein S. As factors Va and VIIIa are the key cofactors amplifying blood coagulation leading to thrombin generation, EPCR-dependent activation of protein C plays a critical role in the anticoagulation system. Indeed, EPCR loss results in embryonic lethality due to placental thrombosis in mice,[6] and anti-EPCR antibodies are independent risk factors for fetal death in humans.[7] On the other hand, activated protein C may also stay bound to EPCR, and this complex cleaves protease-activated receptor 1, initiating cell signalling with cytoprotective effects, such as anti-inflammatory and anti-apoptotic activities and protection of endothelial barrier integrity.[8,9] Thus, EPCR diversely operates endothelial cell homeostasis.

As coagulation, inflammation, apoptosis and endothelial barrier integrity are impaired in SSc, we here investigated the potential role of EPCR in this disease by using clinical samples and animal models.

Materials and methods

Ethics statement

This study was approved by the ethical committee and the committee on animal experimentation of the University of Tokyo Graduate School of Medicine, and was performed according to the Declaration of Helsinki. Written informed consent was obtained from all participants.

Immunohistochemistry

Immunohistochemistry with Vectastain ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.) was performed on formalin-fixed, paraffin-embedded skin sections using anti-EPCR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Skin samples were obtained from forearms of six patients with SSc and six healthy controls, and from the back skin of wild-type and Fli1-mutated mice.[10,11]

Cell cultures

Human dermal microvascular endothelial cells (HDMECs) were purchased from Takara Bio Inc. (Shiga, Japan) and cultured on collagen-coated tissue culture plates in EBM-2 (endothelial cell basal medium) supplemented with the EGM-2 (endothelial cell growth medium) BulletKit (Lonza, Walkersville, MD, U.S.A.). Murine dermal microvascular endothelial cells (MDMECs) were isolated as described previously.[12] Experiments were conducted with HDMECs and MDMECs in passages 1–3, in which a cobblestone appearance was maintained.

RNA isolation and quantitative reverse-transcription polymerase chain reaction in cultured cells and skin samples

Gene silencing of Fli1 in HDMECs, the generation of a bleomycin-induced murine SSc model, RNA isolation from cultivated cells and skin tissue, and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) were carried out as described previously.[13–15] The sequences of primers were as follows. Human PROCR (encoding EPCR): forward 5′-GTAGCCAAGACGC CTCAGAT-3′, reverse 5′-GATAGGGGTCGCGGAAGTA-3′; murine *Procr*: forward 5′-AGCGCAAGGAGAACGTGT-3′, reverse 5′-G GGTTCAGAGCCCTCCTC-3′; human *FLI1*: forward 5′-GGATGG-CAAGGAACTGTGTAA-3′, reverse 5′-GGTTGTATAGGCCAGCA G-3′; human *GAPDH*: forward 5′-ACCCACTCCTCCACCTTTGA-3′, reverse 5′-CATACCAGGAAATGAGCTTGACAA-3′; murine *Gapdh*: forward 5′-CGTGTTCCTACCCCCAATGT-3′, reverse 5′-TGTCATCATACTTGGCAGGTTTCT-3′.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out using EpiQuik ChIP kit (Epigentek, Farmingdale, NY, U.S.A.).[16] The putative Fli1 binding site in the PROCR promoter was predicted by Tfsitescan (http://www.ifti.org/Tfsitescan/). The primers that amplify a 209-bp fragment of the PROCR promoter (−200 bp to +9 bp) were as follows: PROCR ChIP: forward 5′-GAGAAGGGAAAAGGCAGGTC-3′, reverse 5′-CTGGCTGCAGTCTAGGGAAA-3′.

Immunoblotting

Whole-cell lysates were prepared from endothelial cells treated with endothelin-1 or bosentan, as described previously.[10] Samples were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis and immunoblotting with antibodies against EPCR or β-actin (Sigma-Aldrich, St Louis, MO, U.S.A.).

Patients enrolled in the measurement of serum endothelial protein C receptor levels

Serum samples, frozen at −80 °C until assayed, were obtained from 65 patients with SSc. These included 63 women and two

men, median age 59·5 years [interquartile range (IQR) 48·8–68·0] and disease duration 6·0 years (IQR 2·0–20·0). Samples were also obtained from 20 healthy individuals [20 women; median age 52 years (IQR 45·3–71·5)]. The numbers of patients positive for antibodies against topoisomerase I, centromere and RNA polymerase III antigens were 29, 21 and four, respectively, including one patient positive for both anti-topoisomerase I antibody and anticentromere antibody. Patients treated with corticosteroids or other immunosuppressants prior to their first visits were excluded. Patients were grouped by the LeRoy classification system:[17] 38 with diffuse cutaneous SSc (dcSSc) and 27 with limited cutaneous SSc (lcSSc). All patients fulfilled the new classification criteria for SSc.[18]

Measurement of serum endothelial protein C receptor levels

Specific enzyme-linked immunosorbent assay kits were used to measure serum EPCR levels (R&D Systems, Minneapolis, MN, U.S.A.). Briefly, polystyrene cups coated with anti-EPCR antibodies were incubated with 50 μL of assay diluent and 50 μL of 39-fold diluted serum, which was acidified beforehand, at room temperature for 2 h. Then, the cups were washed and incubated at room temperature for 2 h with horseradish peroxidase-conjugated anti-EPCR antibodies. Next, the wells were washed again, treated with tetramethylbenzidine, and incubated at room temperature for 30 min. Finally, sulfuric acid was added to terminate the reaction and the absorbance at 450 nm was measured. Serum EPCR levels were calculated using a standard curve.

Clinical assessment

Disease onset was defined as the first clinical event related to SSc other than Raynaud phenomenon. Disease duration was defined as the interval between the onset and the time of blood sampling. Elevated right ventricular systolic pressure was defined as ≥ 35 mmHg on echocardiogram. Scleroderma renal crisis was defined as malignant hypertension and/or rapidly progressive renal failure.

Statistical analysis

Statistical analysis was carried out with one-way ANOVA followed by Tukey *post hoc* test for multiple comparison; with the Mann–Whitney U-test to compare the distributions of two unmatched groups; with a paired t-test for the comparison of paired data after confirming the normal distribution of the data; with Spearman's rank correlation coefficient to evaluate the correlation with clinical data; with Wilcoxon matched-pairs signed-rank test for the comparison before and after treatment; and with the Shapiro–Wilk normality test to confirm a normal distribution. Statistical significance was defined as a P-value < 0·05.

Results

Endothelial protein C receptor expression is decreased in dermal microvascular endothelial cells of patients with SSc

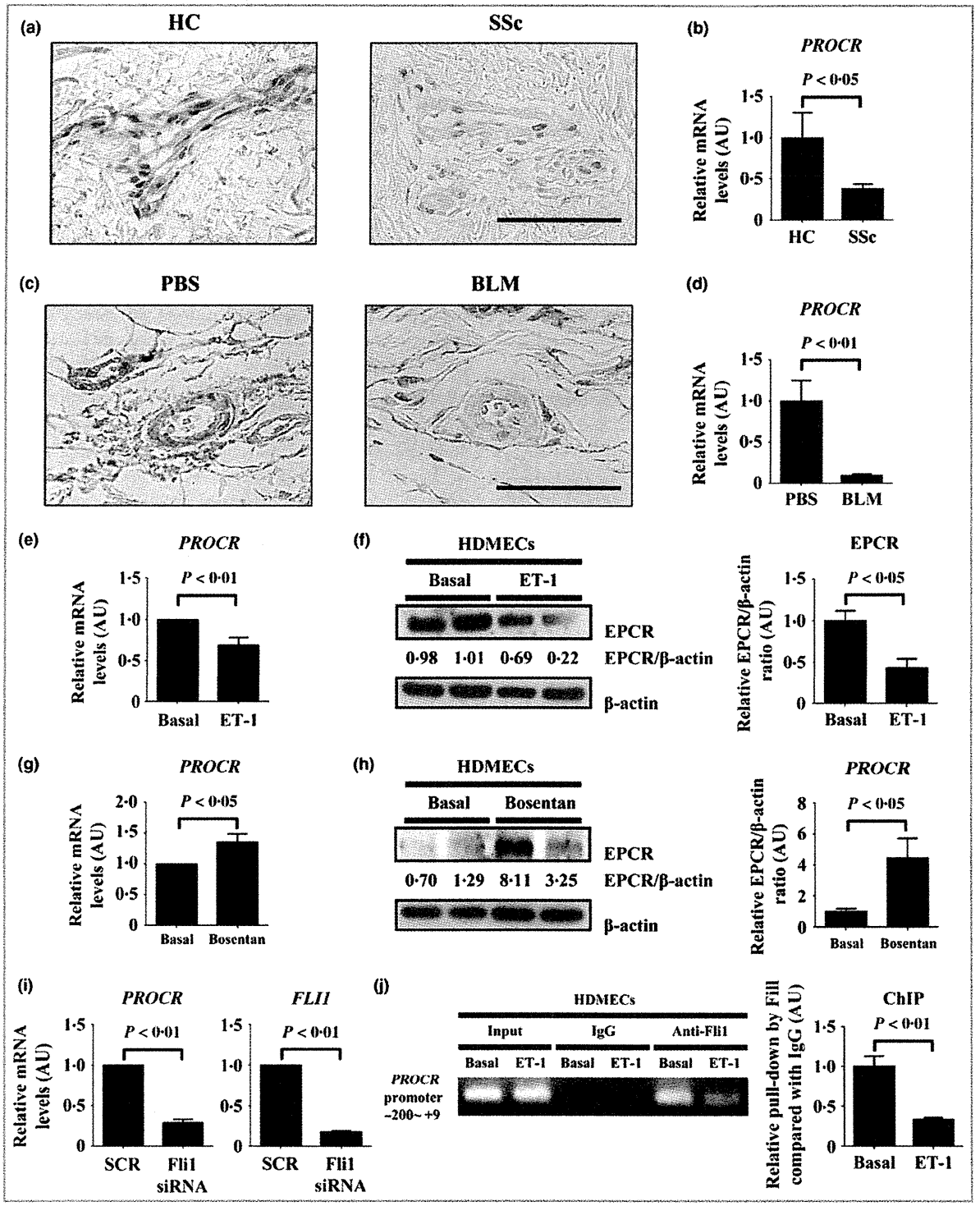
We initially investigated EPCR expression in dermal small vessels of patients with SSc and control subjects by immunohistochemistry (Fig. 1 and Table 1). In healthy controls, EPCR expression was detected abundantly in the nucleus and moderately in the cytoplasm of vascular cells, including endothelial cells and pericytes/vascular smooth muscle cells (Fig. 1a, left panel). On the other hand, in patients with SSc, EPCR was expressed weakly in the nucleus and marginally in the cytoplasm of those cells (Fig. 1a, right panel). Consistently with this, SSc lesional skin expressed PROCR mRNA at significantly lower levels than control skin (Fig. 1b). These results indicate that PROCR gene expression is decreased in dermal small blood vessels of patients with SSc.

Endothelial protein C receptor expression is decreased in dermal blood vessels of bleomycin-treated mice

As persistent fibrin deposition is involved in the pathological dermal fibrosis of animal models,[19] we next investigated EPCR expression in the skin of bleomycin-treated mice, an animal model of SSc.[20] As shown in Figure 1(c), 4 weeks of injection of bleomycin markedly reduced EPCR expression in the nucleus and cytoplasm of endothelial cells and pericytes/vascular smooth muscle cells. Accordingly, *Procr* mRNA levels in the treated skin were significantly lower in bleomycin-injected mice than in phosphate-buffered saline-injected (control) mice (Fig. 1d). Therefore, EPCR downregulation may be involved in the pathological dermal fibrosis of bleomycin-treated mice, possibly through persistent fibrin deposition.

Autocrine endothelin-1 stimulation reduces the expression of endothelial protein C receptor through transcription factor Fli1 in human dermal microvascular endothelial cells

As endothelin-1 is deeply associated with vascular activation and tissue fibrosis in SSc,[12,21] we next assessed its impact on EPCR expression in HDMECs by qRT-PCR and immunoblotting. As shown in Figure 1(e–h), endothelin-1 suppressed EPCR expression, while bosentan, a dual endothelin receptor antagonist, induced EPCR expression, indicating that autocrine endothelin-1 stimulation regulates EPCR expression in HDMECs. As endothelin-1 stimulation induces an SSc phenotype in endothelial cells at molecular levels at least partially by reducing the DNA binding ability of Fli1,[21] deficiency of which is implicated in SSc vasculopathy,[10,15,22–26] we next investigated whether Fli1 regulates endothelin-1-dependent EPCR expression in HDMECs. As shown in Figure 1(i), gene silencing of Fli1



resulted in the suppression of EPCR expression. Furthermore, Fli1 occupied the *PROCR* promoter and endothelin-1 stimulation decreased Fli1 binding to the *PROCR* promoter (Fig. 1j). These results indicate that endothelin-1 reduces *PROCR* gene expression through Fli1 in endothelial cells.

Fli1 deficiency contributes to the downregulation of endothelial protein C receptor in endothelial cells *in vivo*

As Fli1 deficiency is potentially involved in the activation of endothelial cells in SSc, and $Fli1^{+/-}$ mice show a vascular

Fig 1. Endothelial protein C receptor (EPCR) expression is decreased in systemic sclerosis (SSc) small dermal vessels, at least partially due to Fli1 deficiency. (a, c) Representative images of staining for EPCR (a) in human skin samples from healthy controls (HC) and patients with SSc and (c) in skin samples from C57BL/6 wild-type mice treated with phosphate-buffered saline (PBS) or bleomycin (BLM) for 4 weeks. Bar = 100 μm. (b, d) mRNA levels of the *PROCR* gene in the bulk skin from SSc and HC (b, n = 5 for each group) and those of the *Procr* gene in treated murine skin (d, n = 10 for each group) were assessed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). (e–h) EPCR expression levels were determined by qRT-PCR and immunoblotting in human dermal microvascular endothelial cells (HDMECs) untreated or treated with endothelin-1 (ET-1) for 24 h (e, qRT-PCR, n = 10; f, immunoblotting, n = 4) and in HDMECs untreated or treated with bosentan for 48 h (g, qRT-PCR, n = 8; h, immunoblotting, n = 4). In (f) and (h), representative blots are shown in the left-hand panels. The values below each blot represent relative expression levels of EPCR normalized to β-actin by densitometry. The results of densitometry are summarized in the right-hand panels (n = 4). (i) mRNA levels of the *PROCR* gene (left panel) and the *FLI1* gene (right panel) in HDMECs treated with scrambled nonsilencing RNA (SCR) or Fli1 small interfering (si)RNA were assessed by qRT-PCR (n = 6). (j) Chromatin was isolated from HDMECs untreated or treated with ET-1 (200 nmol L^{-1}) for 2 h, and immunoprecipitation was conducted with rabbit anti-Fli1 antibody or rabbit IgG. PCR amplification was carried out using *PROCR* promoter-specific primers. One representative of five independent experiments is shown (left panel). The occupancy of the target gene promoters by Fli1 was also quantified by qRT-PCR, with levels normalized to input values (graph on right). AU, arbitrary units.

activation status similar to that seen in SSc at molecular levels,[10,16,27] we next investigated whether Fli1 deficiency contributes to EPCR reduction in dermal microvascular endothelial cells *in vivo*. To this end, we carried out immunostaining for EPCR with skin samples from $Fli1^{+/-}$ and wild-type mice. Notably, EPCR expression was decreased in the nucleus and cytoplasm of vascular cells in $Fli1^{+/-}$ mice compared with wild-type mice (Fig. 2a). Consistent with this, *Procr* mRNA expression in the skin was significantly lower in $Fli1^{+/-}$ mice than in wild-type mice (Fig. 2b). These results indicate that Fli1 deficiency is enough to attenuate EPCR expression in dermal microvascular endothelial cells *in vivo*.

Bosentan reverses the Fli1 deficiency-dependent suppression of endothelial protein C receptor *in vivo* and *in vitro*

As bosentan reverses the Fli1 deficiency-dependent endothelial cell phenotype by increasing the DNA binding ability of Fli1 *in vivo* and *in vitro*,[21] we next asked whether bosentan reverses EPCR expression in *Fli1* ECKO mice. As shown in Figure 2(c), 4-week administration of bosentan increased EPCR expression in dermal microvascular endothelial cells and pericytes/vascular smooth muscle cells in Fli1 ECKO mice. Furthermore, bosentan increased EPCR expression in MDMECs isolated from *Fli1* ECKO mice (Fig. 2d), in which Fli1 expression was increased by bosentan.[12] These results indicate that bosentan reverses the Fli1 deficiency-dependent endothelial cell phenotype, including decreased EPCR expression *in vivo* and *in vitro*.

Serum endothelial protein C receptor levels in patients with systemic sclerosis

We next evaluated the clinical correlation of serum EPCR levels, because membrane-bound EPCR is released by metalloproteolytic cleavage.[28] In patients with SSc overall, serum EPCR levels were significantly decreased compared with healthy controls: median 305·8 ng mL^{-1} (IQR 259·2–352·7)

Table 1 Endothelial protein C receptor levels in skin sections from patients with SSc and HCs

					Vascular cells	
Sample	Age/sex[a]	Disease subtype	Autoantibody	Disease duration (years)	Nucleus	Cytoplasm
HC 1	77/F				+++	+
HC 2	42/F				++	+
HC 3	77/F				+++	+
HC 4	37/F				+++	+
HC 5	78/F				++	+
HC 6	78/F				++	+
SSc 1	68/F	dcSSc	Topo-I	3	+/++	–
SSc 2	48/F	dcSSc	Topo-I	1	–/+	+
SSc 3	50/F	dcSSc	Topo-I	4	–/+	–
SSc 4	25/M	dcSSc	Topo-I	1	+	–
SSc 5	63/F	lcSSc	ACA	2	+	–
SSc 6	71/F	lcSSc	ACA	10	++	+
SSc + Bos 1	60/F	dcSSc	Topo-I	4	+/++	+/++
SSc + Bos 2	63/F	dcSSc	Topo-I	3	++	+/++
SSc + Bos 3	54/F	dcSSc	Topo-I	3	+++	+/++

SSc, systemic sclerosis; HCs, healthy controls; Bos, bosentan; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; Topo-I, antitopoisomerase I antibody; ACA, anticentromere antibody. We used the following grading system: –, no staining; +, slight staining; ++, moderate staining; +++, strong staining. [a]Age in years: F, female; M, male.

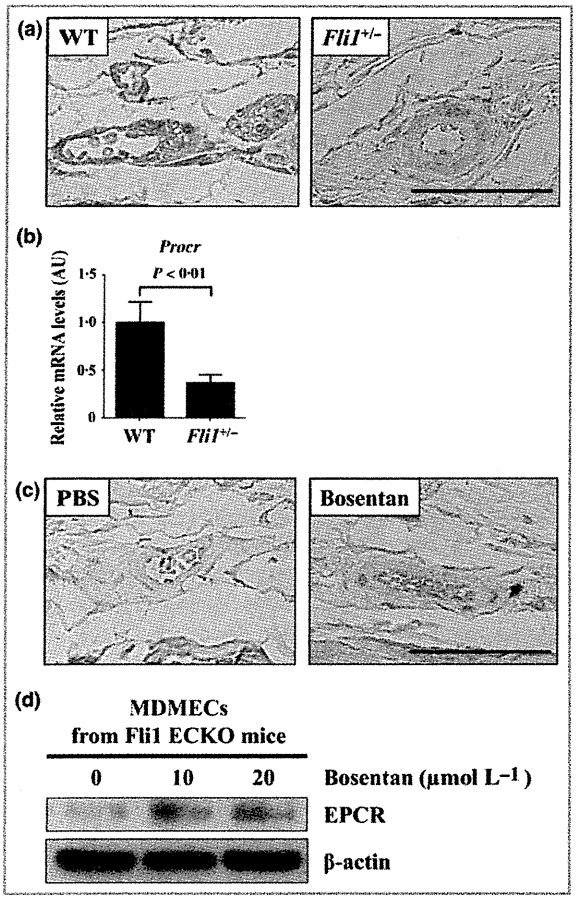


Fig 2. Bosentan reverses the decreased expression of endothelial protein C receptor (EPCR) in dermal small vessels of $Fli1^{+/-}$ mice. (a, c) Representative images of staining for EPCR in sections from the skin of wild-type (WT) mice and $Fli1^{+/-}$ mice (a) and in sections from the skin of Fli1 ECKO mice treated with phosphate-buffered saline (PBS) or bosentan for 4 weeks (c). Bar = 50 μm. (b) mRNA levels of the *Procr* gene in the back skin of wild-type and $Fli1^{+/-}$ mice were assessed by quantitative reverse-transcription polymerase chain reaction (n = 8 for WT mice, n = 7 for $Fli1^{+/-}$ mice). AU, arbitrary units. (d) Expression levels of EPCR and β-actin were determined by immunoblotting in murine dermal microvascular endothelial cells (MDMECs) untreated or treated with bosentan for 48 h. Representative blots of three independent experiments are shown.

vs. 330·8 ng mL^{-1} (IQR 295·2–374·7), P = 0·046. Evaluation in disease subsets showed that patients with dcSSc exhibited serum EPCR levels significantly lower than those seen in patients with lcSSc and healthy controls, while the levels were comparable between patients with lcSSc and healthy controls: 299·9 ng mL^{-1} (IQR 237·0–325·4) for dcSSc and 335·6 ng mL^{-1} (IQR 282·0–379·9) for lcSSc (Fig. 3a). In line with these findings, serum EPCR levels had a weak, but statistically significant, inverse correlation with modified Rodnan total skin-thickness score (MRSS) (r = −0·36, P < 0·01; Fig. 3b) in all patients with SSc, but there was no correlation with disease duration (r = 0·02, P = 0·87).

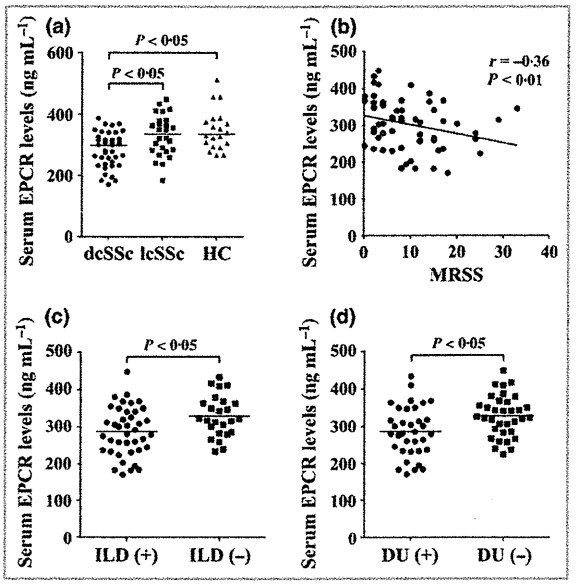


Fig 3. Clinical correlation of serum endothelial protein C receptor (EPCR) levels in patients with systemic sclerosis (SSc). Serum EPCR levels were measured by a specific enzyme-linked immunosorbent assay. (a) Serum EPCR levels were significantly decreased in patients with diffuse cutaneous (dc)SSc (n = 39) compared with patients with limited cutaneous (lc)SSc (n = 26) and healthy controls (HC, n = 20). (b) Serum EPCR levels were inversely correlated with modified Rodnan total skin-thickness score (MRSS) in patients with SSc (r = −0·36, P < 0·01). The solid line represents the regression line. (c) Serum EPCR levels were significantly lower in patients with SSc with interstitial lung disease (ILD) (n = 40) than in those without (n = 25). (d) Patients with SSc with a current or past history of digital ulcers (DUs, n = 35) had lower serum EPCR levels than patients with SSc without a history of DUs (n = 30). Horizontal bars represent the median of each group.

As dcSSc is characterized by extensive fibrosis of various internal organs, especially lung, we next compared serum EPCR levels in patients with or without fibrotic organ involvement, such as interstitial liver disease (ILD), oesophageal dysfunction and heart involvement. Of note, the presence of ILD was associated with a significant decrease in serum EPCR levels [291·6 ng mL^{-1} (IQR 237·0–348·8) vs. 318·9 ng mL^{-1} (IQR 282·0–364·4), P = 0·043; Fig. 3c], while the presence of other organ involvement did not affect serum EPCR levels (data not shown).

We also investigated the potential contribution of EPCR to the development of vascular symptoms (Table 2). As expected, serum EPCR levels were decreased in patients with SSc with a current or past history of digital ulcers compared with those with no history of digital ulcers (Fig. 3d), while the presence of other vascular symptoms, including Raynaud phenomenon, nailfold bleeding, telangiectasia, elevated right ventricular systolic pressure and scleroderma renal crisis, did not alter serum EPCR levels. Therefore, EPCR downregulation may be relevant to the development of tissue fibrosis in the skin and lung and impaired peripheral circulation leading to digital ulcers in patients with SSc.

Table 2 Correlation of serum endothelial protein C receptor (EPCR) levels with clinical features related to systemic sclerosis vasculopathy

	Serum EPCR levels (ng mL^{-1})		
Clinical symptoms	Patients with symptoms	Patients without symptoms	P-value
Raynaud phenomenon	304·6 (259·1–348·2)	380·8 (343·0–396·0)	0·09
Nailfold bleeding	311·8 (275·2–349·9)	279·0 (237·0–348·8)	0·30
Telangiectasia	312·9 (267·1–363·2)	294·0 (262·0–340·5)	0·44
Digital ulcers	281·1 (235·2–331·4)	321·8 (285·0–362·2)	0·014
Elevated RVSP	335·6 (274·2–373·2)	311·8 (265·9–346·4)	0·31
Scleroderma renal crisis	364·4 (315·7–406·6)	305·5 (259·1–348·2)	0·21

Values are the median (interquartile range) in each patient group. Elevated right ventricular systolic pressure (RVSP) was defined as ≥ 35 mmHg on echocardiogram. Scleroderma renal crisis was defined as malignant hypertension and/or rapidly progressive renal failure.

Inverse correlation of serum endothelial protein C receptor levels with plasma plasmin–α2-plasmin inhibitor complex levels

The association of decreased serum EPCR levels with digital ulcers suggests that endothelial EPCR downregulation contributes to impaired peripheral circulation through the hypercoagulation status in SSc. Therefore, we next looked at the correlation of serum EPCR levels with coagulation/fibrinolysis markers, such as plasma levels of thrombin–antithrombin complex, D-dimer and plasmin–α2-plasmin inhibitor complex (PIC). Among these, of note, there was a weak, but statistically significant, negative correlation between plasma PIC levels and serum EPCR levels (Fig. 4a). Given that PIC is a fibrinolytic marker directly reflecting the generation of plasmin *in vivo*, these results suggest that endothelial EPCR downregulation is involved in the induction of the prothrombotic condition in SSc.

Bosentan decreases serum plasmin–α2-plasmin inhibitor complex levels in patients with systemic sclerosis

As bosentan reversed EPCR expression in Fli1 ECKO mice, we finally examined the effect of bosentan on the EPCR-dependent coagulation system in patients with SSc. In 29 patients with SSc treated with bosentan, plasma PIC levels were significantly reduced after treatment [median 0·9 μg mL^{-1} (IQR 0·65–1·35) vs. 0·7 μg mL^{-1} (IQR 0·5–1·0), P = 0·0029; Fig. 4b]. In contrast, treatment with prostanoids did not alter plasma PIC levels in 14 patients with SSc [median 0·9 μg mL^{-1} (IQR 0·78–1·13) vs. 1·1 μg mL^{-1} (IQR 0·78–1·25), P = 0·10; Fig. 4c). Furthermore, bosentan, but not prostanoids, significantly increased serum EPCR levels (Fig. 4d, e). Moreover, three patients with SSc treated with bosentan showed much more elevated Fli1 and EPCR expression in dermal small vessels than three patients with SSc not treated with bosentan (Table 1 and Fig. 4f). These results suggest that bosentan improves the hypercoagulation status in patients with SSc at least partially through reversing EPCR expression in endothelial cells.

Discussion

This study was undertaken to investigate the mechanism underlying hypercoagulation status in SSc by focusing on EPCR. A series of clinical and experimental data indicates the potential contribution of EPCR downregulation due to Fli1 deficiency to the hypercoagulation status in SSc.

Fli1 deficiency is a potential predisposing factor of SSc reflecting environmental influences, because Fli1 is epigenetically suppressed in this disease.[29,30] The contribution of Fli1 deficiency to SSc vasculopathy has been well studied in Fli1-mutated mice in terms of their structural and functional abnormalities. Regarding the structural abnormalities, dermal small vessels of Fli1 ECKO mice are characterized by stenosis of arterioles, dilation of capillaries and vascular fragility, reminiscent of SSc vasculopathy. As for the functional abnormalities, Fli1 deficiency contributes to the altered expression of cell adhesion molecules in SSc endothelial cells; that is, the relatively higher expression of intercellular adhesion molecule-1 and glycosylation-dependent cell adhesion molecule-1 than E-selectin and P-selectin,[27] leading to the promotion of T helper cell (Th)2/Th17 infiltration in the lesional skin.[31] Further supporting the pivotal role of Fli1 deficiency in the functional abnormalities of SSc endothelial cells, we also demonstrated that EPCR downregulation in dermal small vasculature was also commonly seen in Fli1 ECKO mice and patients with SSc. Taking into account the inverse correlation of serum EPCR levels with plasma PIC levels in patients with SSc, EPCR downregulation is pathologically significant in this disease. Therefore, the current data further reinforce the notion that endothelial Fli1 deficiency is a key feature inducing SSc vasculopathy.

As shown in clinical trials, bosentan prevents the development of new digital ulcers in patients with SSc.[32,33] Accordingly, bosentan appears to exert a potential disease-modifying effect on SSc vasculopathy. For instance, 1-year administration of bosentan increases the number of nailfold capillaries with early and active patterns, while decreasing the number of those with late pattern.[34] Furthermore, the number of nailfold capillaries is increased by 3-year combination therapy with bosentan and iloprost, but decreased by iloprost alone.[35] Thus,

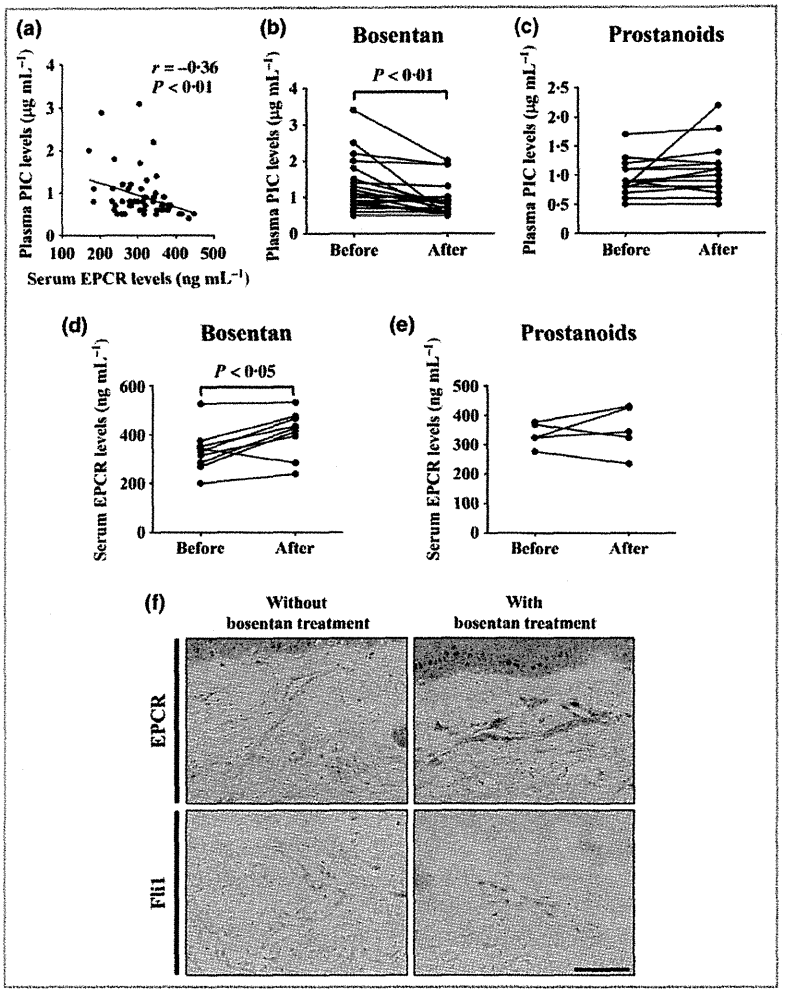


Fig 4. Circulating endothelial protein C receptor (EPCR) and plasma plasmin–α2-plasmin inhibitor complex (PIC) levels are reversed in parallel with the upregulation of Fli1 and EPCR in endothelial cells after treatment of patients with SSc with bosentan. (a) Serum EPCR levels inversely correlated with plasma PIC levels in patients with SSc ($r = -0{\cdot}36$, $P < 0{\cdot}01$; $n = 56$). The solid line represents the regression line. (b, c) Plasma PIC levels were significantly decreased after treatment with bosentan (b, $n = 29$), but not after treatment with prostanoids (c, $n = 14$). (d, e) Serum EPCR levels were significantly elevated after treatment with bosentan (d, $n = 9$), but not after treatment with prostanoids (e, $n = 5$). (f) Immunostaining for EPCR and Fli1 was carried out in three patients with SSc treated with bosentan and another three patients with SSc not treated with bosentan. Representative results are shown. Similar results were obtained in the remaining patients (Table 1). Bar = 50 μm.

bosentan appears to improve the peripheral circulation by normalizing neovascularization and vascular structural changes, but the impact of bosentan on the coagulation system has remained unstudied. Considering that bosentan increased serum EPCR levels in inverse parallel with plasma PIC levels, bosentan may normalize hypercoagulation status by increasing EPCR expression, subsequently contributing to the prevention of new digital ulcers in patients with SSc. Given that bosentan enhances Fli1 expression in endothelial cells by increasing its protein stability,[12] it is plausible that bosentan improves the impaired coagulation system characteristic of SSc, which is partly attributable to endothelial Fli1 deficiency.

In addition to impaired peripheral circulation, intravascular microthrombi induce and exacerbate inflammation. In the acute inflammatory stage of SSc, activated dermal fibroblasts are located predominantly around small blood vessels with mononuclear cells,[36] suggesting that these inflammatory cells are activated through their interaction with injured endothelium and subsequently stimulate fibroblasts. Intravascular fibrin deposits may be involved in this process as a trigger and/or amplifier of inflammatory reaction. Supporting this idea, serum EPCR levels were inversely correlated with MRSS, and the presence of ILD was associated with decreased serum EPCR levels in patients with SSc. Therefore, an impaired coagulation/fibrinolysis system is likely to be a part of the fibrotic mechanisms underlying SSc, as is the case with animal models of fibrotic disorders.[19,37–39]

Independently of regulation of the coagulation pathway, EPCR mediates cytoprotective effects, such as anti-inflammatory and antiapoptotic activities and protection of the endothelial