Table 1 Clinical and laboratory differences between juvenile and adult onset systemic sclerosis

Parameter	Juvenile onset SSc ($n = 11$)	Adult onset SSc ($n = 310$)
Age at onset	9.4 ± 5.0	48.7 ± 13.2
Male:female (n)	6:5*	49:261
Disease duration (years)	3.5 ± 3.2	6.4 ± 8.3
dcSSc:lcSSc (n)	8:03	127:183
Modified Rodnan total skin thickness score	14.9 ± 10.2	10.6 ± 9.9
Anti-topoisomerase I Ab (%)	90.9*	28.1
Anticentromere Ab (%)	0*	39.9
Anti-U1 ribonucleoprotein (RNP) Ab (%)	9.1	10
Pitting scar and/or digital ulcer (%)	90.9*	30
Skin calcification (%)	9.1	7.7
Interstitial lung diseases (%)	9.1*	45.8
Pulmonary arterial hypertension (%)	9.1	2.6
Esophagus (%)	36.7	67.7
Heart (%)	0	14.1
Muscle (%)	0	10.3
Joint (%)	27.3	25.8
Kidney (%)	0	1.3

* $p < 0.05$

Data are presented as the mean ± standard deviation (SD), unless otherwise indicated

SSc Systemic sclerosis, *dcSSc* diffuse cutaneous SSc, *lcSSc* limited cutaneous SSc, *Ab* antibody

the clinical features of jSSc and aSSc patients with anti-topoisomerase I Ab were compared (Table 2).

To determine if the differences in clinical courses were dependent on the onset-age among patients with same autoantibody, at the latest visit we compared the clinical features of jSSc and aSSc patients with anti-topoisomerase I Ab (Table 2). The follow-up period was shorter in the jSSc patient group than in the aSSc patient group (5.7 ± 3.0 vs. 11.7 ± 21.5 years), but the difference was not significant. No jSSc patient and ten aSSc patients (11.4 %) died as a consequence of SSc during the study period. Unlike the findings at their first visit, the frequency of pitting scar and/or digital ulcer and the frequency of ILD were comparable between jSSc patients and aSSc patients at their latest visit. Otherwise, there were no significant clinical differences between jSSc and aSSc patients.

The profile and the clinical course of each jSSc patient are shown in the Electronic Supplemental Material table. Nine patients were started on or continued to take oral prednisolone (PSL), and the mean dose of all patients was 10.6 ± 8.0 mg/day at their first visit. The mean modified Rodnan Skin score of these patients was 11.9 ± 7.3 at the time of their latest assessment compared to 14.9 ± 10.2 at their first visit. Case 4 had ILD at his first visit which was exacerbated 1 year later (5 years after his disease onset). In addition, five patients (cases 1, 3, 5, 10, and 11) developed ILD more than 3 years following the onset of SSc. Arrhythmia requiring treatment was detected in two patients (cases 5 and 10) after 3 years from disease onset. Renal crisis did not develop in any patients.

Table 2 Clinical features of juvenile and adult onset SSc with anti-topoisomerase I antibody at their first and latest assessments

Parameter	At the first assessment		At the latest assessment	
	Juvenile onset SSc ($n = 10$)	Adult onset SSc ($n = 88$)	Juvenile onset SSc ($n = 10$)	Adult onset SSc ($n = 88$)
Age at onset (years)	9.4 ± 5.2	47.0 ± 14.4		
Male:female (n)	6:4*	16:72		
Disease duration (years)	3.6 ± 3.4	4.3 ± 5.5		
Death from SSc (%)			0	11.4
Modified Rodnan total skin thickness score	13.7 ± 9.8	16.3 ± 10.2	11.0 ± 7.7	10.5 ± 9.4
Anticentromere Ab (%)	0	3.8	0	3.8
Anti-U1 ribonucleoprotein (RNP) Ab (%)	10	9.5	10	9.5
Pitting scar and/or digital ulcer (%)	90.0*	52.4	100	79.8
Interstitial lung diseases (%)	10.0*	85.2	60	88.6
Pulmonary arterial hypertension (%)	0	3.6	0	14.3
Esophagus (%)	40	70.6	40	72.9
Heart (%)	0	8.2	20	15.3
Muscle (%)	0	10.3	0	11.5
Joint (%)	30	27.9	30	31.3
Kidney (%)	0	2.3	0	4.5
Follow-up period (year)			5.7 ± 3.0	11.7 ± 21.5

* $p < 0.05$ vs. adult onset SSc at each time point

Data are presented as the mean ± SD, unless otherwise indicated

Journal of Dermatological Science 74 (2014) 214–221

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Blockade of Syk ameliorates the development of murine sclerodermatous chronic graft-versus-host disease

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ARTICLE INFO

Article history:
Received 21 February 2013
Received in revised form 18 February 2014
Accepted 24 February 2014

Keywords:
Syk
Chronic GVHD
Systemic sclerosis
CXCR4
Memory T cells

ABSTRACT

Background: Murine sclerodermatous chronic graft-versus-host disease (Scl-cGVHD) is a model for human Scl-cGVHD and systemic sclerosis (SSc). Syk is expressed in most of hematopoietic cells, fibroblasts, and endothelial cells. Syk is a protein tyrosine kinase that has an important role in transmitting signals from a variety of cell surface receptors.
Objective: This study aims to investigate the effect of R788 (fostamatinib sodium), an oral prodrug that is rapidly converted to a potent inhibitor of Syk, R406, on Scl-cGVHD.
Methods: R788 was orally administered twice a day to allogeneic recipients from day 14 to day 42 after bone marrow transplantation (BMT). *In vitro*, proliferation of GVHD-derived $CD4^+$ T cells and $CD11b^+$ cells was analyzed by R406.
Results: Allogeneic BMT increased Syk phosphorylation in T, B, and $CD11b^+$ cells. The administration of R788 attenuated severity and fibrosis of Scl-cGVHD. The elevated expressions of CXCR4 on T cells, B cells, and $CD11b^+$ cells were significantly down-regulated by R788 treatment. R788 reduced memory $CD4^+$ T cells ($CD44^{hi}CD62L^-CD4^+$). R406 inhibited proliferation of GVHD $CD4^+$ T cells and $CD11b^+$ cells *in vitro*. In addition, R788 treatment, inhibited proliferation of $CD11b^+$ cells in Scl-cGVHD mice. R788 treatment also reduced skin mRNA expressions of MCP-1, MIP-1α, IFN-γ, IL-13, IL-17A, and TGF-β1, but not influenced RANTES, CXCL12, and TFN-α.
Conclusion: Blockade of Syk suppressed migration factor of immune cells and antigen-specific memory $CD4^+$ T cells and proliferation and activation of GVHD $CD4^+$ T cells and $CD11b^+$ cells. The current studies suggested that Syk inhibitor is a potential candidate for use in treating patients with Scl-cGVHD and SSc.

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by excessive extracellular matrix deposition in the skin and visceral organs [1]. Three pathologic hallmarks characterize the SSc development: autoimmunity, vasculopathy, and progressive tissue fibrosis. While inflammatory, autoimmune processes and vasculopathy dominate early stages of SSc, progressive tissue fibrosis is the key feature of late-stage disease [2]. Chronic graft-versus-host disease (cGVHD) emerges from alloreactive reactions between donor-derived immune and host cell populations. Transplantation of B10.D2 bone marrow (BM) and splenocytes across minor histocompatibility loci into sublethally irradiated BALB/c recipients is a well-established animal model for human sclerodermatous cGVHD (Scl-cGVHD) and SSc, both of which show many clinical similarities with human SSc. Skin thickening and pulmonary fibrosis develop 21 days after bone marrow transplantation and fibrosis also affects the liver, kidneys, gastrointestinal tract, and parotid glands in mice receiving transplants in murine Scl-cGVHD [3]. Although murine Scl-cGVHD is an ideal animal

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http://dx.doi.org/10.1016/j.jdermsci.2014.02.008
0923-1811/

model for investigating SSc autoimmunity [4], vascular involvement is not seen in Scl-cGVHD model.

Spleen tyrosine kinase (Syk) is a member of zeta-chain associated protein kinase 70 (ZAP70)/Syk family of the non-receptor-type protein tyrosine kinase that contains two Src homology 2 domains and a kinase domain. Syk was initially shown to be critical for the signaling of immunoreceptors, such as Fc receptors (FcRs), B-cell receptors (BCRs), and T-cell receptors (TCRs) in association with immunoreceptor tyrosine-based activation motif (ITAMs) in hematopoietic cells [5]. However, subsequently, it has been revealed that Syk has broader roles in a variety of signal transduction pathways including Toll-like receptor, chemokine receptor, and integrin signalings [6–9]. Furthermore, while the expression of ZAP70 is restricted to T cells and natural killer cells, Syk is broadly expressed in most of hematopoietic cells, including B cells, T cells, granulocytes, monocytes, mast cells, and dendritic cells, and other cells such as fibroblasts, endothelial cells. Activated Syk phosphorylates downstream signaling proteins that eventually control effects such as phagocytosis, cytokine production, cell adhesion, migration, proliferation, and differentiation [10].

Syk-mediated pathways have an important role in various diseases. For example, active T cells are caused by abnormal association of Syk in SLE [11]. Consequently, Syk has been considered as therapeutic target in various diseases, including asthma [12], Wiskott–Aldrich syndrome [13], anaphylactic shock [14], lymphoma [15], carcinoma [16], acute GVHD [17], rheumatoid arthritis [18], systemic lupus erythematosus [11], and multiple sclerosis [19]. Clinical trials of R788 (fostamatinib sodium), an oral prodrug that is rapidly converted to a potent inhibitor of Syk (R406), have demonstrated the significant effects on treatment of allergic disease [20], immune thrombocytopenic purpura [21], rheumatoid arthritis [22], and lymphoma [18]. Syk is also likely to have important roles in transmitting signals in a variety of cells and signaling pathways involved in the GVHD process. Syk regulates the mitogen-activated protein (MAP) kinase cascade, especially JNK-regulated genes such as IL-6 and membrane protein, palmitoylated 3 (MPP-3), in synovial fibroblasts [23]. Syk is also involved in differentiation of 3T3-L1 mouse embryonic fibroblasts [24]. Therefore, this study analyzed the effects of oral blockade of Syk (R788) on Scl-cGVHD.

2. Materials and methods

2.1. Mice

B10.D2 (H-2^d) and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were housed in a specific pathogen-free barrier facility. All studies were approved by the institutional review board.

2.2. Bone marrow transplantation

In this study, 8- to 12-week-old male B10.D2 (H-2^d) and female BALB/c (H-2^d) mice were used as donors and recipients, respectively. Bone marrow (BM) was T cell-depleted (TCD) with anti-Thy1.2 microbeads (Miltenyi Biotech, Auburn, CA). BALB/c recipients were irradiated with 800 cGy (MBR-1520R, Hitachi, Tokyo, Japan) and were injected via the tail vein with 10×10^6 TCD-BM and 10×10^6 splenocytes in 0.5 mL of PBS to generate Scl-cGVHD (allogeneic BMT). A control syngeneic group of female BALB/c mice received male BALB/c TCD-BM and splenocytes (syngeneic BMT).

2.3. Reagents

For *in vivo* studies, R788 (fostamatinib – Biorbyt Limited, Cambridge, UK) was administered to allogeneic recipients by daily oral gavage at a dose of 30 mg/kg twice a day from day 14 to day 42 after BMT. Control mice received distilled water only (allogeneic group). *In vitro* studies were performed with R406 (Biorbyt Limited), the active form of R788.

2.4. GVHD skin score

Clinical cGVHD score was previously described [25]: healthy appearance = 0; skin lesions with alopecia equal or less than 1 cm^2 in area = 1; 1–2 cm^2 = 2; 2–5 cm^2 = 3; 5–10 cm^2 = 4; 10–15 cm^2 = 5; 15–20 cm^2 = 6; more than 20 cm^2 = 7. Additionally, animals were assigned 0.4 points for skin disease (lesions or scaling) on tail, and 0.3 points each for lesions on ears and paws. Minimum score = 0, maximum score = 8. Final scores for dead animals were kept in the data set for the remaining time points.

2.5. Histological analysis

The skin and lung were fixed in 10% formalin and embedded in paraffin. Sections (6 μm in thickness) were stained with H&E and Masson's trichrome. Skin histopathology was scored by a dermatopathologist (blinded to experimental groups) on the basis of epidermal interface changes, dermal collagen thickness, mononuclear cell inflammation, subdermal fat loss, and follicular dropout with scores from 0 to 2 for each category (total score, 0–10) [26]. Collagen deposition was quantified on trichrome stained sections as the ratio of blue-stained area to total stained area using Adobe Photoshop CS4 analysis tools.

2.6. Immunohistochemical (IHC) staining of the skin

Skin sections (6-μm thickness) were applied to slides. Before immunostaining the slides were heated (37 °C) overnight in a drying oven and then deparaffinized in xylene, hydrated through graded alcohols, and washed in distilled water. Antigen retrieval was performed via heat treatment (10 min, in 10 mmol/L sodium citrate buffer at 95 °C). The slides were allowed to cool for 20 min, then rinsed in distilled water and placed into a container of wash buffer (Tris buffered saline). Endogenous peroxidase activity was blocked by incubating the slides for 5 min in 3% hydrogen peroxide. After rinsing in wash buffer, sections were incubated for 1 h at room temperature with phosphor-Syk (Tyr525) antibody (1:100 dilution, NBP1-51392, Novus Biologicals, Littleton, CO) in wash buffer. Slides were rinsed in wash buffer and incubated for 30 min with peroxidase-labeled donkey anti-rabbit IgG antibody (BD Biosciences, San Jose, CA), then avidin–biotin–peroxidase complexes (Vectastain ABC method; Vector Laboratories, Burlingame, CA). Sections were developed with 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide, and then counterstained with methyl green. IHC stains were evaluated for the presence of positively staining cells between in the dermis. The following semiquantitative scale, based on percentage of positively cells, was used: – (no staining), + (<25% staining), ++ (25–50% staining), +++ (50–75% staining), and ++++ (75–100% staining). Stained cells were counted under a high-power microscopic field (original magnification, 400×) on a light microscope. Each section was examined and scored independently by two investigators in a blinded manner (Y.H. and M.H.). The mean score was used for analysis.

2.7. Flow cytometry

The following mAbs were used: FITC-, PE-, PE-Cy5-, PE-Cy7-, PerCP-Cy5.5-, APC-, APC-PECy7-, Pacific Blue-conjugated mAbs to mouse Thy1.2 (30-H12), CD4 (RM4-5), CD11b (M1-70), CD19 (1D3), CD1d (1B1), CD5 (30-H12), CD62L (MEL-14) mAbs (BioLegend, San Diego, CA), CXCR4 (2B11) mAb (eBioscience, San

Jose, CA), CD44 (IM7), and LIVE/DEAD Fixable Aqua Dead cell (Invitrogen, Grand Island, NY). Splenic single-cell suspensions were stained for 20 min for multi-color immunofluorescence analysis at 4 °C using mAbs at predetermined optimal concentrations. Stained samples were analyzed on a FACSCanto II (BD Biosciences). Data were analyzed using FlowJo (Tree Star, Ashland, OR) software.

2.8. Intracellular cytokine staining

B cells and monocytes/macrophages were stimulated for 5 h at 37 °C with LPS (10 μg/mL, Sigma–Aldrich), PMA (50 ng/mL; Sigma–Aldrich), ionomycin (500 ng/mL; Sigma–Aldrich), and brefeldin A (3 μM; BioLegend) for detection of cytokine production. T cells were stimulated for 4 h at 37 °C with PMA (50 ng/mL), ionomycin (1 μg/mL), and brefeldin A (3 μM; BioLegend). After cell-surface staining, the cells were washed, fixed, and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences), followed by staining with anti-IL-10 (JES5-16E3) or anti-IL-6 (MP5-20F3) mAbs (BioLegend), or anti-TNF-α (MP6-XT22), anti-IFN-γ (XMG1.2), anti-IL-17A (TC11-18H10.1) mAbs (BioLegend) or anti-IL-13 (eBio13A) mAb (eBioscience).

Splenic single cell suspension was cultured without stimulants for 30 min in analysis of Syk-phosphorylation of T cells or B cells and CD11b$^+$ cells. After incubation, cells were immediately fixed with Phosflow Lyse/Fix buffer (BD Biosciences) and then permeabilized with Phosflow Perm buffer II (BD Biosciences). After surface staining with Ly9.1 and either Thy1.2 or CD19 and CD11b, cells were stained with anti PE-conjugated phospho-Syk (Tyr525/526) (C87C1) mAb (Cell Signaling, Frankfurt a.M., Germany) or rabbit PE-conjugated mAb IgG isotype control (Cell Signaling).

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from frozen skin specimens using RNeasy spin columns (Qiagen, Hilden, Germany) and digested with DNase I (Qiagen) to remove chromosomal DNA. Total RNA was reverse-transcribed to a cDNA using a reverse transcription system with random hexamers (Promega, Southampton, UK). Cytokine mRNA was analyzed using real-time RT-PCR quantification (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed on an ABI Prism 7000 sequence detector (Applied Biosystems). GAPDH was used to normalize the mRNA. The relative expression of real-time RT-PCR products was determined according to the $\Delta\Delta C_t$ method to compare target gene and GAPDH mRNA expression.

2.10. Cell proliferation assay

Splenic CD4$^+$ T cells and CD11b$^+$ cells were isolated from allogeneic BMT mice 14 days after BMT, using corresponding MACS magnetic microbeads (Miltenyi Biotech). Isolated CD4$^+$ T cells and CD11b$^+$ cells were labeled with 5 μM CFSE (Invitrogen) at 37 °C for 15 min. For CD4$^+$ T cell proliferation: 4×10^5 CFSE-labeled CD4$^+$ T cells were co-cultured with various concentrations of R406 in the presence of plate-bound 1 μg/mL anti-CD3 and 1 μg/mL anti-CD28 for 4 days in 96-well plates. For CD11b$^+$ cell proliferation: 4×10^5 CFSE-labeled CD11b$^+$ T cells were co-cultured with various concentrations of R406 in the presence of recombinant mouse M-CSF (25 ng/mL) for 8 days in 96-well plates. A half of culture media was exchanged every two days. The proliferation rate of cells was measured by CFSE dilution in flow cytometric analysis.

2.11. BrdU incorporation assay

Scl-cGVHD mice were given twice of 1 mg BrdU in 100 μl of PBS by i.p. injection at 30 and 90 min before analysis. Spleen was harvested from Scl-cGVHD mouse. Splenocytes were stained for anti-CD4 Ab, anti-CD11b Ab and stained with BrdU Flow kit (BD Biosciences) according to manufacturer's instructions. Stained samples were analyzed on FACSCanto II.

2.12. Statistics

All data are shown as mean ± standard error of the mean (SEM). The significance of differences between sample means was determined with Student's t test.

3. Results

3.1. Syk phosphorylation is augmented in T cells, B cells, and CD11b$^+$ cells after allogeneic BMT

Syk is expressed in various hematopoietic cells (monocyte/marcrophages, mast cells, lymphocytes, platelets and erythrocytes) [27]. To investigate whether phosphorylation of Syk is increased

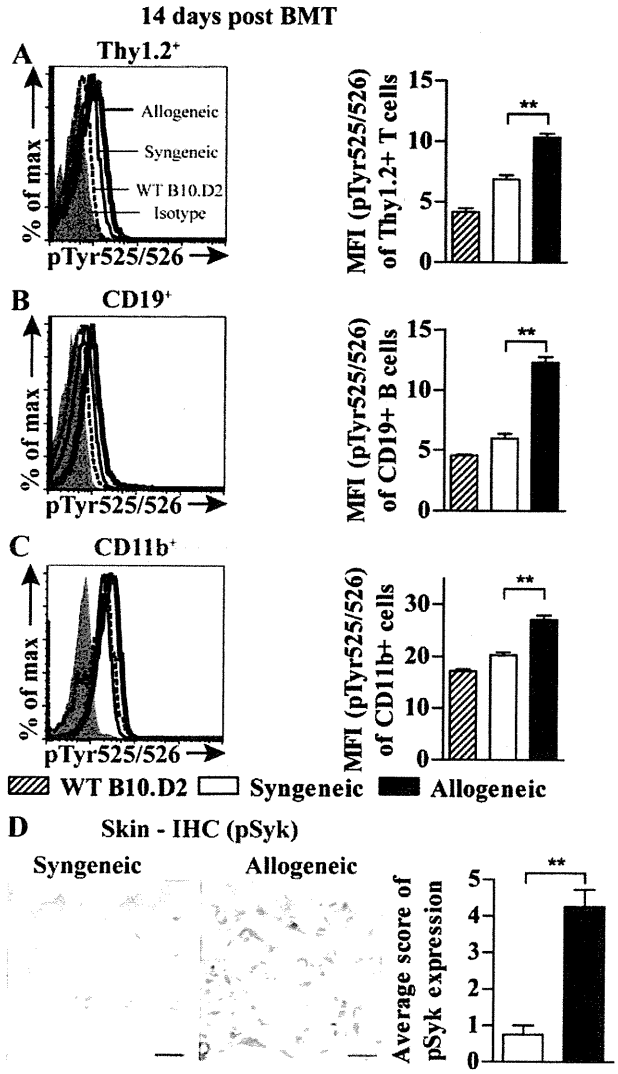

Fig. 1. Phosphorylation of Syk is augmented in splenic T, B, and CD11b$^+$ cells 14 days after allogeneic BMT. Splenic single cell suspension was stained with anti-phospho-Syk (Tyr525/526) antibody after surface staining with Thy1.2 or CD19 and CD11b, cells. The intensity of pSyk in T cells (A), B cells (B), or CD11b$^+$ cells (C) was analyzed in WT B10.D2 (unfilled thin dash line overlay), syngeneic (unfilled thin solid line overlay), and allogeneic (unfilled thick solid line overlay) compared with isotype staining (filled gray overlay) by flow cytometric analysis ($n = 4$ mice per group; $^{**}p < 0.01$). (D) pSyk (Tyr525) in the skin sections from groups of syngeneic and allogeneic mice were investigated at day 14 after BMT ($n = 4$–6 mice per group; scale bar = 20 μm; $^{**}p < 0.01$).

after allogeneic transplantation, phosphorylation of Syk in splenocytes was measured by flow cytometric analysis 14 days after allogeneic BMT (Fig. 1). When compared with syngeneic BMT, constitutive phosphorylation of Syk was significantly higher in Thy1.2$^+$ T cells, CD19$^+$ B cells, and CD11b$^+$ monocyte/macrophages 14 days after BMT ($p = 0.001$, $p = 0.0004$, and $p = 0.012$, respectively, Fig. 1a–c). The hyperphosphorylation of Syk was also observed in the skin infiltrates 14 days after allogeneic BMT when compared with syngeneic BMT ($p < 0.01$, Fig. 1d). Therefore, Syk was activated in T cells, B cells, and monocyte/macrophages after allogeneic BMT.

3.2. The administration of R788 attenuates Scl-cGVHD

R788 were orally administrated twice a day to allogeneic BMT recipients from day 14 to day 42 after BMT. When compared with water-treated group, R788 treatment significantly improved weight loss ($p < 0.05$, Fig. 2a) and skin scores ($p < 0.05$, Fig. 2b). Although early treatment with R788 starting from day 0 after BMT might be more effective for Scl-cGVHD, it is hard to exclude the possibility that the treatment around day 0 after BMT may inhibit the engraftment of BMT. Therefore, R788 treatment was started after engraftment of BMT.

These results were also verified by real-time quantitative PCR analysis, and histopathology. The mRNA expression of collagen type 1 α1, collagen type 1 α2, and especially fibronectin 1 were significantly decreased in R788-treated group ($p < 0.01$, $p < 0.01$, and $p < 0.001$, respectively, Fig. 2c). Histopathologic scores and fibrosis area in the skin and lung were significantly lower in R788-treated group than in the water-treated group ($p < 0.05$, Fig. 2d and e). Collectively, the administration of Syk inhibitor attenuates Scl-cGVHD.

3.3. Elevated CXCR4 expression after allogeneic BMT is reduced by R788 treatment

Chemokine stromal-derived factor 1 (SDF-1/CXCL12) and CXCR4 have been shown to play a crucial role in migration and development of hematopoietic stem cell transplantation [28]. Syk is required for CXCL12/CXCR4-induced cell polarization that occurs in concert with cell adhesion mediated by β-1 integrin [29]. There were higher expressions of CXCR4 on T cells, B cells, and CD11b$^+$ cells after allogeneic BMT (18.60 ± 2.91%, 19.11 ± 2.17%, and 42.90 ± 4.17%) when compared with syngeneic group (2.61 ± 0.27%, 2.28 ± 0.50%, and 12.97 ± 0.64% with $p < 0.01$, $p < 0.005$, and $p < 0.005$, respectively, data not shown). Elevated expressions of CXCR4 on T cells, B cells, and CD11b$^+$ cells after allogeneic BMT were downregulated by R788-treatment (7.99 ± 1.02%, 5.47 ± 0.56%, and 27.25 ± 3.72% with $p = 0.0138$, $p = 0.0009$, and $p = 0.0311$, respectively, Fig. 3a). Accordingly, R788 treatment affects the migration of immune cells in Scl-cGVHD.

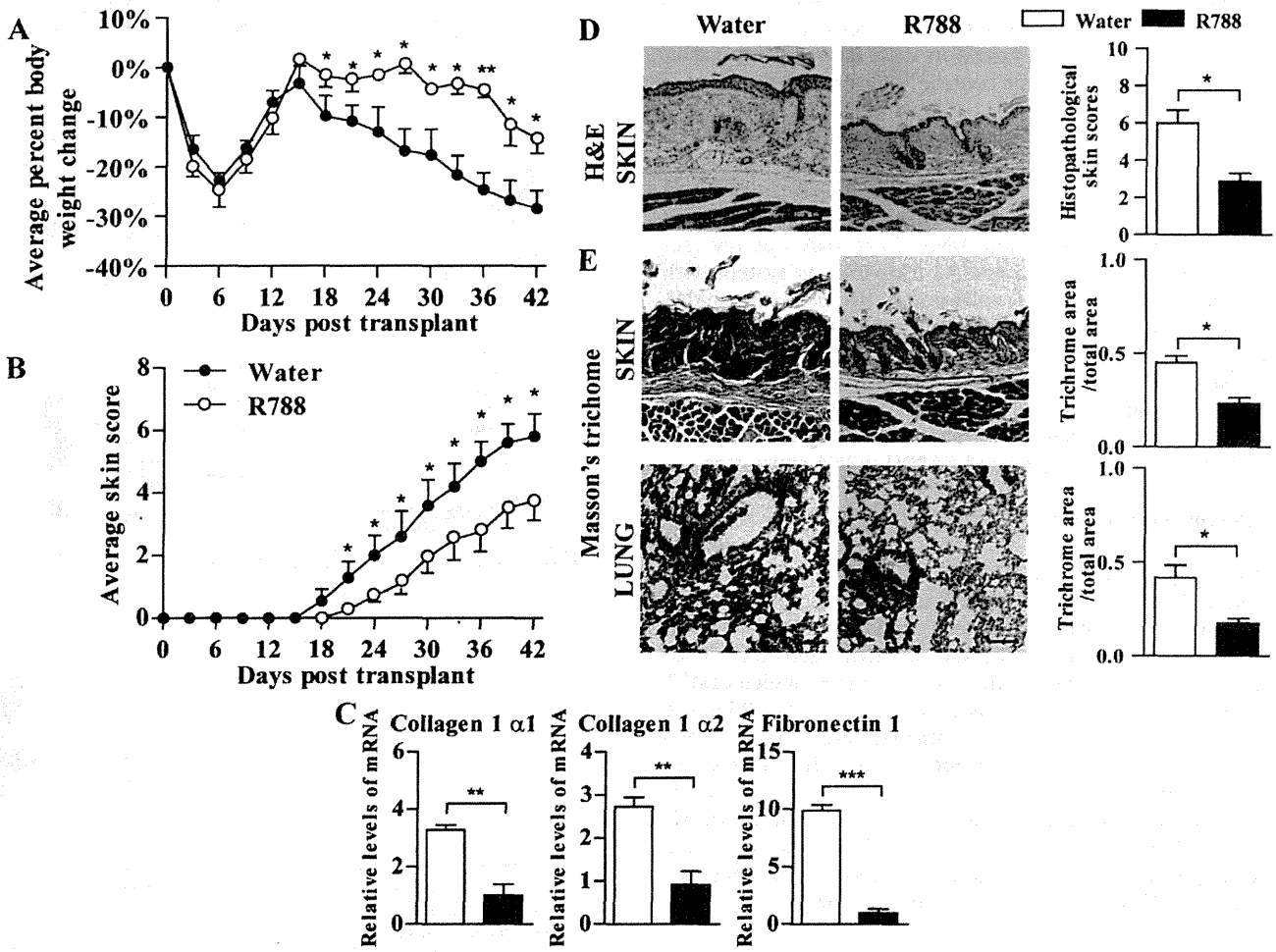

Fig. 2. Oral treatment of R788 attenuates Scl-cGVHD severity and fibrosis. Recipients were given sterile water (●), or were orally administered R788 (30 mg/kg/day, bid) from day 14 to day 42 (R788-treatment group – ○). (A) Average body weight changes, and (B) skin scores were monitored every 3 days ($n = 4$–6 per group; $^*p < 0.05$, $^{**}p < 0.01$ for ○ versus ●). (C) mRNA expression of Collagen 1 α1, Collagen 1 α2, and Fibronectin 1 in the skin of water- and R788-treated groups were measured by real-time quantitative PCR analysis at day 35 after BMT. Skin slides were scored as described in Section 2. (D) Histopathological scores, and (E) ratio of trichome area/total area in the skin and lung were analyzed 42 days after BMT ($n = 4$–6 mice per group; $^*p < 0.05$).

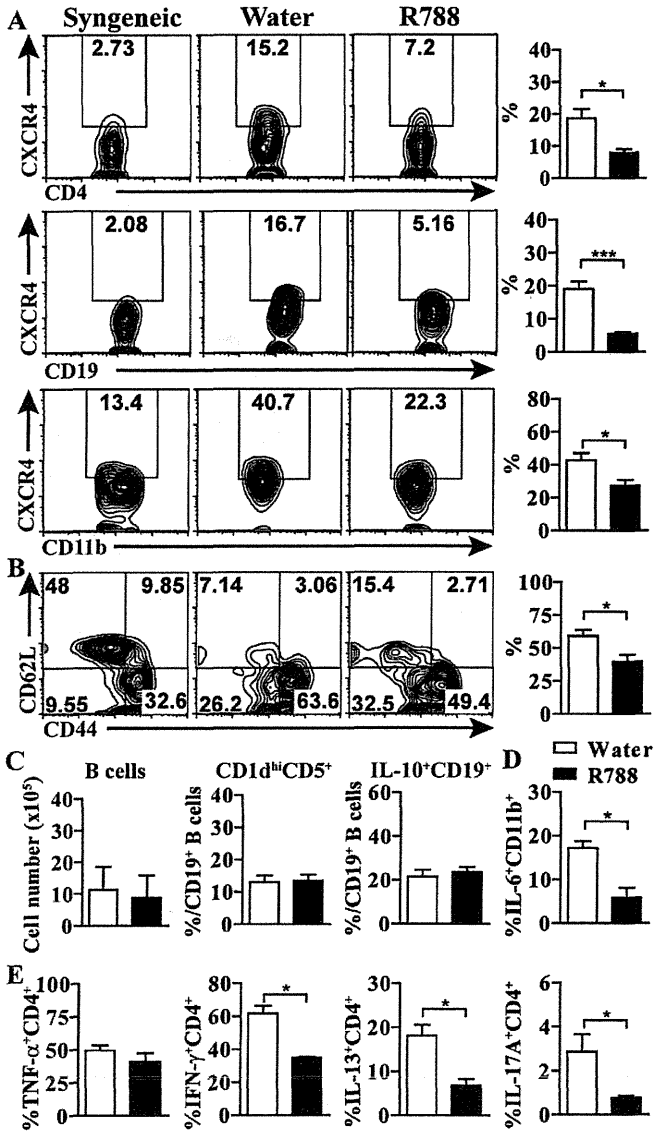

Fig. 3. R788 treatment reduces CXCR4 expression, memory CD4 T cells, and cytokine production of $CD11b^+$ monocytes and $CD4^+$ T cells. Spleen samples from sygeneic, water, and R788-treated groups were harvested 42 days after BMT. Representative results and bar graphs (left) of (A) CXCR4 expression of $CD4^+$ T cells, $CD19^+$ B cells, and $CD11b^+$ cells, (B) memory $CD4^+$ T cells ($CD44^{hi}CD62L^+CD4^+$ T cells) from gate of $CD4^+$ T cells, (C) number and percentages of B cells, $CD1d^{hi}CD5^+$, and regulatory B cells (IL-10-producing $CD19^+$ B cells), (D) IL-6-producing $CD11b^+$ cells, (E) TNF-α-, IFN-γ-, IL-13-, and IL-17A-producing $CD4^+$ T cells were analyzed by flow cytometry ($n = 4$–6 mice per group, $^*p < 0.05$).

3.4. R788 treatment suppresses the expansion of memory $CD4^+$ T cells

A previous study demonstrated that increased memory T cells ($CD44^{hi}CD62L^-$ T cells) from alloantigen-primed donors are responsible for the induction of a chronic form of GVHD, whereas naïve T cells induce acute GVHD [30]. Syk expression is specifically elevated in antigen-specific memory T cells compared with naïve T cells [31]. The frequency of memory $CD4^+$ T cells ($CD44^{hi}CD62L^-CD4^+$) in total $CD4^+$ T cells was increased 14 days after allogeneic BMT compared with about syngeneic mice (85–90% versus 30%; data not shown). In R788-treated group, R788 treatment reduced $CD44^{hi}CD62L^-CD4^+$ T cells when compared with water-treated group ($61.06 \pm 5.34\%$ versus $83.55 \pm 4.30\%$, $p < 0.05$, Fig. 3b). Thus, Syk blockade suppresses the expansion of memory $CD4^+$ T cells.

3.5. R788 treatment do not change the number of B cells, $CD1d^{hi}CD5^+$ B cells, and IL-10-producing regulatory B cells

To determine whether the blockade of Syk regulates B cell reconstitution, B cells and their subsets were analyzed in R788-treated group. The number of $CD19^+$ B cells was not different in R788-treated group when compared with water-treated group (Fig. 3c). Regulatory B cells are considered to suppress cGVHD. IL-10-producing regulatory B cells (B10 cells) are predominantly present in splenic $CD1d^{hi}CD5^+$ B cells. Therefore, we examined frequencies of $CD1d^{hi}CD5^+$ B cells and B10 cells in Scl-cGVHD after R788 treatment. There were no significant differences in frequencies of $CD1d^{hi}CD5^+$ and B10 cells between the two groups 42 days after BMT (Fig. 3c). Therefore, the blockade of Syk after 14 days of BMT did not have an apparent influence on development of B cells and regulatory B cells.

3.6. R788 treatment reduces IL-6-producing $CD11b^+$ monocytes and cytokine-producing $CD4^+$ T cells

IL-6 has been reported to play an important role in the pathogenesis of Scl-cGVHD [25]. To assess the effect of Syk blockade on IL-6 production by $CD11b^+$ cells, splenocytes harvested 42 days after BMT from each group were stimulated with LPS, PMA, ionomycin, and brefeldin A for 5 h. The percentage of IL-6-producing $CD11b^+$ cells was significantly lower in R788-treated group than in water-treated group ($5.81 \pm 2.28\%$ versus $17.23 \pm 1.47\%$, respectively, $p < 0.01$, Fig. 3d). Scl-cGVHD exhibits a mixed Th1/Th2-like cytokine profile with a Th1-like predominance during the early stage and Th2-like profile at the later stage [32]. To determine the effect of Syk blockade on cytokine production by $CD4^+$ T cells, splenocytes were stimulated with PMA, ionomycin and brefeldin A for 4 h. When compared with water-treated group, frequencies of IFN-γ-, IL-13-, and IL-17A-producing $CD4^+$ T cells were significantly decreased in R788-treated groups ($p < 0.05$, Fig. 3e), while there was no difference in TNF-α producing from $CD4^+$ T cells. Therefore, increased productions of inflammatory cytokines were downregulated by the blockade of Syk in Scl-cGVHD.

3.7. Proliferations of GVHD-derived $CD11b^+$ monocyte and $CD4^+$ T cells are blocked by Syk inhibition

It is hypothesized that monocyte activation by host-reactive T cells is an initiating event in scleroderma and Scl-cGVHD. To investigate whether the blockade of Syk affects the development of $CD4^+$ T cells and monocyte/macrophages, GVHD-derived $CD4^+$ T cells were exposed to anti-CD3/CD28, while GVHD-derived monocytes were exposed to M-CSF in presence of R406 *in vitro*. Blockade of Syk inhibited proliferation of $CD4^+$ T cells at 125 nM of R406 ($p < 0.001$, Fig. 4a), and $CD11b^+$ cells at 250 nM of R406 ($p < 0.01$, Fig. 4b) when compared with stimulation of $CD4^+$ T cells or $CD11b^+$ cells alone. In addition, it was also investigated the effect of Syk inhibitor on proliferation of $CD4^+$ T cells and $CD11b^+$ cells *in vivo* using BrdU labeling. The proliferation of $CD11b^+$ cells, but not $CD4^+$ T cells, were significantly decreased in R788-treated group ($p < 0.05$, Fig. 4d). Collectively, these results indicated that the blockade of Syk inhibited the proliferation of $CD4^+$ T cells and monocyte/macrophages in Scl-cGVHD.

3.8. R788 treatment reduces the expressions of chemokines/cytokines in the skin

Up-regulation of cutaneous chemokines/cytokines has been reported in Scl-cGVHD [33]. Expression of mRNA of chemokines including CCL2 (MCP-1), CCL3 (MIP-1α), but not CCL5 (RANTES) and CXCL12, were downregulated in R788-treated group when

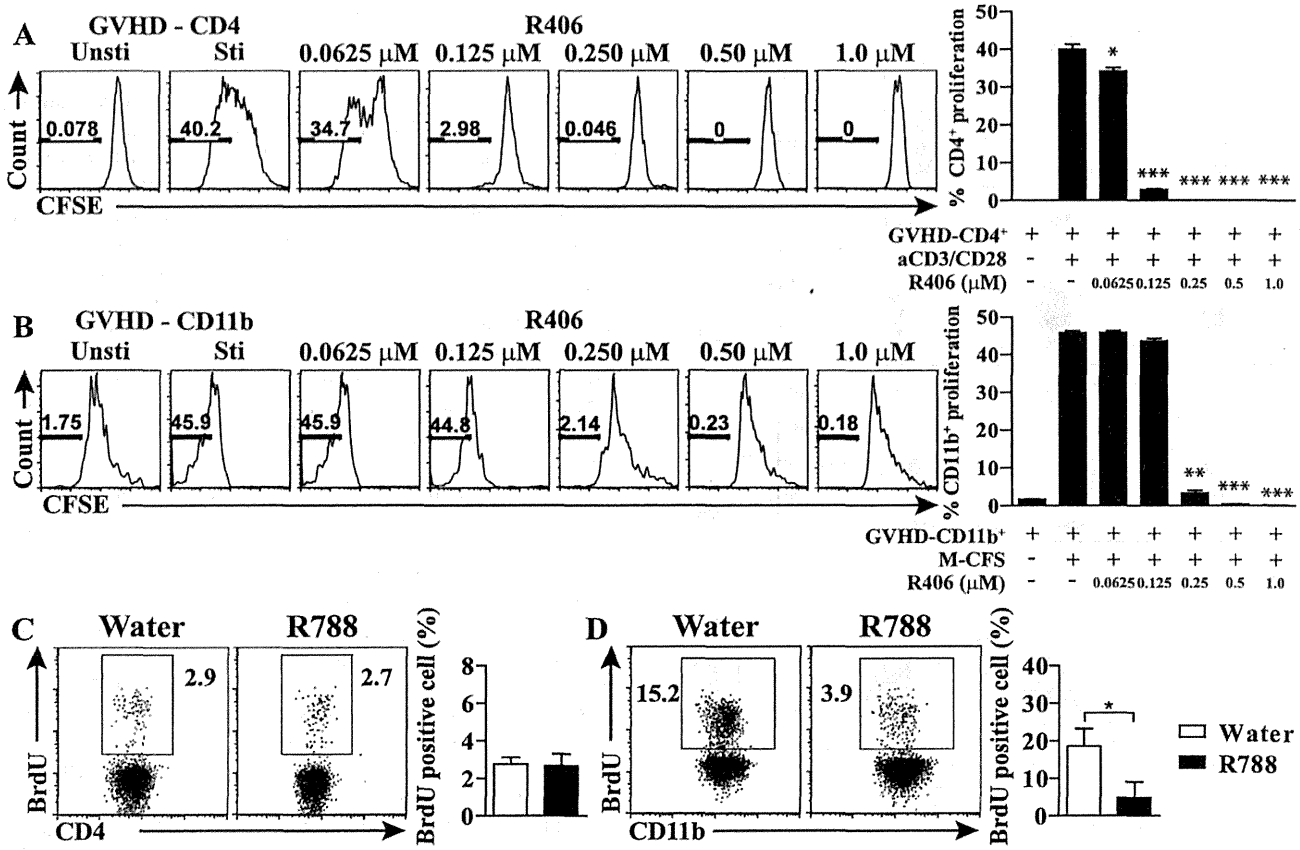

Fig. 4. Syk inhibitor (R406) blocks proliferation of GVHD-derived $CD4^+$ T cells and $CD11b^+$ cells. $CD4^+$ T cells and $CD11b^+$ cells were purified from GVHD spleens 14 days after BMT by MACS microbead, then labeled with CFSE. (A) CFSE-$CD4^+$ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies in presence of various concentrations of R406 for 4 days. (B) CFSE-$CD11b^+$ cells were exposed to M-CSF in presence of various concentrations of R406 for 8 days. Proliferation was measured by CFSE dilution (the experiment was performed in triplicate, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison with stimulated $CD4^+$ T cells or $CD11b^+$ cells alone, respectively). The proliferating $CD4^+$ T cells (C) and $CD11b^+$ cells (D) in the spleen of water- and R788-treated groups were measured by BrdU uptake at day 28 after BMT ($n = 4$–6 mice per group; $^*p < 0.05$).

compared with water-treated group 42 days after BMT (1/5-fold, and 1/6-fold for MCP-1 and MIP-1α with $p = 0.0035$ and $p = 0.0002$, respectively, Fig. 5a). TNF-α mRNA expression was not significantly different between the two groups (Fig. 5b). By contrast, mRNA expressions of IFN-γ, IL-6, IL-13, IL-17A, and TGF-β1 were markedly reduced in R788-treated group when compared with water-treated group 42 days after BMT ($p < 0.05$, Fig. 5b).

4. Discussion

This study demonstrated that Syk inhibitor significantly reduced immune-mediated fibrosis in murine cGVHD model. Syk was activated in T cells, B cells, and monocyte/macrophages in Scl-cGVHD (Fig. 1). Blockade of Syk significantly ameliorated the severity and fibrosis of Scl-cGVHD (Fig. 2a and b). The current study suggested that blockade of Syk inhibited the activation and migration of $CD4^+$ T cells in Scl-cGVHD. Furthermore, elevated frequency of memory $CD4^+$ T cells was inhibited by R788 administration (Fig. 3b). *In vitro*, R406 inhibited the proliferations of Scl-cGVHD-derived $CD4^+$ T cells and $CD11b^+$ cells (Fig. 4). Elevated expression of CXCR4 in immune cells during cGVHD reaction was downregulated by Syk inhibitor. Production of IL-6 by $CD11b^+$ cells and IFN-γ, IL-13, and IL-17A by $CD4^+$ T cells were impaired by the blockade of Syk (Fig. 3d and e). Furthermore, mRNA expressions of IFN-γ, IL-6, IL-13, IL-17A, and TGF-β1 as well as CCL2 (MCP-1) and CCL3 (MIP-1α) were markedly reduced in R788-treated group (Fig. 5). These results suggest that Syk inhibitor may be a promising therapy in cGVHD and SSc.

Syk is involved in the activation of B cells and T cells via FcR-, BCR- and TCR-mediated signal transduction [34]. In addition to ZAP70, it has been shown that Syk undergoes tyrosine phosphorylation following TCR stimulation and serves as a key molecule during TCR-induced activation in T cells [35]. Syk is indicated to mediate T cell proliferation in acute GVHD [17]. In Scl-cGVHD, the elevated frequency of memory $CD4^+$ T cells was decreased by the oral administration of R788 (Fig. 3b). Memory T cells have critical roles in Scl-cGVHD. Memory T cells from allogeneic-primed donors induce a chronic form of GVHD, in contrast to the acute GVHD induced by naïve T cells [36]. Our results are consistent with studies reporting that the blockade of Syk inhibited the expansion of memory $CD4^+$ T cells in murine lupus and that it did not affect acute GVHD [11,17]. While Syk has been considered to have major roles in B cell signaling, the results in this study indicated that the development and differentiation of B cells were not significantly altered by Syk inhibitor (Fig. 3c). Nonetheless, Syk activation in B cells may also promote the expansion of memory $CD4^+$ T cells in Scl-cGVHD, since B cells are required for the generation of memory $CD4^+$ T cells [37].

The migration and recruitment of activated immune cells to a target tissue is a multistep process involving the sequential activation of various chemokine receptors on immune cells and the vascular endothelium as well as expression of a vast array of chemokines/cytokines [38]. It was reported that the inhibition of lymphocyte migration attenuated Scl-cGVHD [39]. The higher expressions of CXCR4 on T cells, B cells, and monocytes were observed after allogeneic BMT (Fig. 3a). Elevated CXCR4 expression

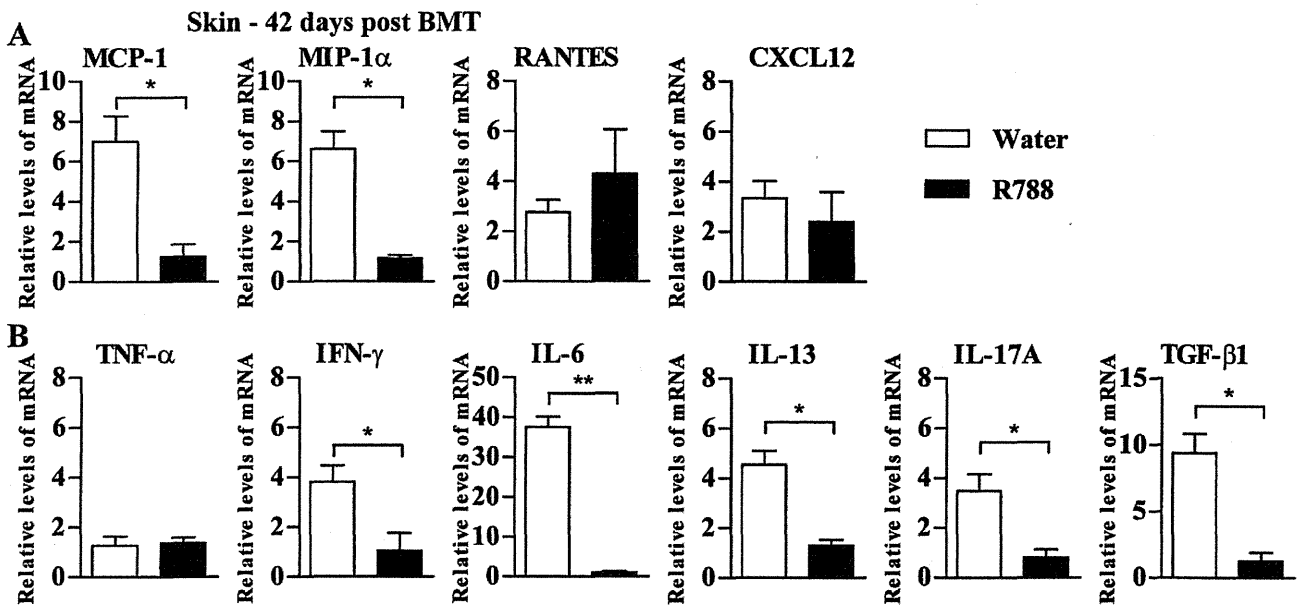

Fig. 5. R788 reduces skin expression of chemokine/cytokines. Skin mRNA expressions of (A) chemokines: MCP-1, MIP-1α, RANTES, and CXCL12, and (B) cytokines: TNF-α, IFN-γ, IL-6, IL-13, IL-17A, and TGF-β1 were measured by real-time quantitative PCR analysis 42 days after BMT ($n = 4$–6 mice per group; $^*p < 0.05$).

in immune cells was down-regulated by Syk inhibitor consequentially resulting in decreased infiltration of immune cells into tissues. Our study suggests blockade of Syk not only inhibited the activation and expansion of $CD4^+$ T cells but also migration of T cells in Scl-cGVHD. The CXCL12/CXCR4 axis regulates haematopoietic stem and progenitor cell trafficking [40]. CXCL12 is highly present in ischemic tissue. CXCR4, the receptor of CXCL12, regulates specific steps in new vessel formation [41]. CXCL12 and CXCR4 are upregulated in the skin of both diffuse and limited cutaneous forms of SSc [42]. Thus, the CXCL12/CXCR4 axis also plays important roles in the vasculopathy of SSc pathogenesis [43]. A previous study demonstrated that a Syk-independent signaling of RANTES production by macrophages through the FcγR, while MCP-1 and IL-6 were produced in a Syk-dependent manner by macrophages [44]. High expressions of MCP-1, MIP-1a, and IL-6 in cGVHD skin were indicated in current study (Fig. 5). MCP-1 is known to be a strong chemoattractant for monocytes/macrophages. Production of MCP-1 and IL-6 by macrophages in this context was largely dependent on Syk and may result in further accumulation of pathogenic macrophages into the inflamed sites.

Monocyte activation by host-reactive T cells is considered to be an initiating event of fibrotic changes in Scl-cGVHD [45]. Skin-infiltrating monocytes produce TGF-β1, resulting in collagen upregulation and leading to skin fibrosis [33]. Phosphorylated Syk induces monocyte activation with consequent synthesis and release of massive amount of inflammatory modulators responsible for inflammatory reaction. Production of IL-6 by $CD11b^+$ cells was impaired by the blockade of Syk (Fig. 3d and e). Both serum IL-6 and sIL-6R levels were significantly higher in patients with SSc. IL-6 has roles in vascular damage and activate fibroblast to produce collagen [46]. In addition, IL-6 plays important roles in development of Scl-cGVHD. Early blockade of IL-6 signaling by anti-IL-6 receptor antibody attenuates Scl-cGVHD severity and fibrosis by promoting T reg differentiation that resulting in suppressing activated immune cells [25]. Production of IFN-γ, IL-13, and IL-17A by $CD4^+$ T cells were also impaired by the blockade of Syk (Fig. 3d and e). Recent reports shed light on the critical role of IL-17A in the pathogenesis of scleroderma [47–49]. Especially, STAT3 signaling in Th17 cell is important for development of Scl-cGVHD [49]. In addition, these T cell-derived cytokines, especially, Th2 and Th17 cytokines, may directly stimulate fibroblasts, or indirectly stimulate monocyte/macrophages to produce TGF-β. Productions of collagen 1 α1, collagen 1 α2, and fibronectin 1 were reduced by Syk inhibitor (Fig. 2c). This is likely to result from decreased immune responses, since Syk inhibitor did not directly affect collagen production from fibroblast in the current study (data not shown). However, Src inhibitor attenuated the activation and production of extracellular matrix (ECM) component in human fibroblasts [50]. Syk activation has been demonstrated to occur as the result of a Src kinase-initiated activation loop phosphorylation in NIH 3T3 cells [51]. Thus, the effect of Syk inhibitor on fibroblast should be carefully investigated in the future.

As a conclusion, we have demonstrated that treatment with R788 effectively reduces Scl-cGVHD severity and fibrosis. Blockade of Syk suppressed the immune response by reducing migration factor of immune cells and antigen-specific memory $CD4^+$ T cells. We also showed that proliferation and activation of $CD4^+$ T cells and $CD11b^+$ cells were inhibited by blockade of Syk signaling. The current study suggested that Syk inhibitor is a potential candidate for use in treating patients with Scl-cGVHD and scleroderma in humans.

Acknowledgments

We thank Ms M. Matsubara and Y. Yamada for technical assistance. This work was supported by grants-in-aid from the Ministry of Education, Science, and Culture of Japan and from the Ministry of Health, Labour and Welfare of Japan.

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膠原病の病型・予後判定に有用な新しい特異的自己抗体検査*

濱口　儒人*1・竹原　和彦*1

要　約　全身性強皮症や皮膚筋炎では複数の疾患特異的自己抗体が検出され，それぞれの自己抗体は特有の臨床像と結びついている．全身性強皮症でみられる自己抗体として，抗セントロメア抗体，抗トポイソメラーゼI抗体，抗RNAポリメラーゼ抗体，抗Th/To抗体，抗U3RNP抗体，抗Ku抗体，抗hUBF抗体，抗セントリオール抗体などがある．一方，皮膚筋炎では，近年，筋症状は乏しいものの急速進行型間質性肺炎を合併する抗MDA5(抗CADM-140)抗体，悪性腫瘍と小児皮膚筋炎と相関する抗TIF1抗体が同定された．さらに，これまでは報告数が少なく臨床症状が明らかでなかった自己抗体(抗NXP-2抗体や抗SAE抗体)についても，その臨床的特徴が明らかになりつつある．しかし，これらの自己抗体の同定には手技の煩雑な免疫沈降法を要するものが多く，ELISA法が利用できる自己抗体は限られる．簡便に測定できる測定法の開発が望まれる．

キーワード　自己抗体，全身性強皮症，皮膚筋炎，免疫沈降法，ELISA

濱口儒人，他：臨皮 68(5増)：58-61，2014

はじめに

膠原病をはじめとする自己免疫疾患ではさまざまな自己抗体が検出される．なかでも，全身性強皮症(systemic sclerosis：SSc)と皮膚筋炎(dermatomyositis：DM)では複数の疾患特異的自己抗体の存在が知られている．本稿では，SScとDMでみられる自己抗体について，最近その臨床的特徴が報告された自己抗体を含めて概説する．

検査・診断の実際

SScやDMに特異的な自己抗体を同定する方法として，二重免疫拡散法(double immunodiffusion assay：DID法)，ELISA法，免疫沈降法などがある．しかし，ELISA法で同定できる自己抗体は限られ，多くは手技が煩雑である免疫沈降法を要する．簡便に自己抗体を同定する方法として，ドイツのEUROIMMUN社が開発した

* Systemic sclerosis- or myositis-specific autoantibodies that are useful for subgrouping patients and predicting prognosis

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〔略語〕CADM：clinically amyopathic dermotomyositis, DID：double immunodiffusion assay, DM：dermatomyositis, SSc：Systemic sclerosis, TIF1：transcriptional intermediary factor 1

表1　全身性強皮症における自己抗体の検出方法と臨床的特徴

自己抗体	蛍光抗体間接法	検出方法	臨床症状
抗セントロメア抗体	Discrete-speckled	IIF, ELISA, WB	lcSSc, 指尖潰瘍・壊疽, 皮膚石灰沈, 逆流性食道炎, 肺動脈圧性肺高血圧症
抗トポイソメラーゼI抗体	Ho and N	DID, ELISA, WB, IP	dcSSc, 指尖潰瘍・壊疽, 肺線維症, 心病変, 腎クリーゼ
抗RNAポリメラーゼ抗体	Sp and/or N	ELISA, IP	dcSSc, 腎クリーゼ
抗Th/To抗体	N	IP	lcSSc, 肺線維症, 肺動脈性肺高血圧症
抗U3RNP抗体	N	IP	dcSSc, 肺線維症, 肺動脈性肺高血圧, 心病変, 筋炎, 下部消化管病変？
抗U1RNP抗体	Sp	DID, ELISA, WB, IP	lcSSc, 肺動脈性肺高血圧, 関節炎, 重複症候群
抗hUBF(NOR90)抗体	N	IP	lcSSc, 指尖潰瘍？
抗PM-Scl抗体	N	DID, IP	lcSSc, SSc-myotisis重複症候群
抗Ku抗体	Sp	DID, IP	lcSSc, SSc-myotisis重複症候群
抗RuvBL1/2抗体	Sp	IP	dcSSc, SSc-myotisis重複症候群
抗セントリオール抗体	セントリオール型	IIF	lcSSc, 指尖潰瘍？　肺動脈性肺高血圧症？

Ho: homogenous, N: nucleolar, Sp: speckled, IIF: indirect immunofluorescence staining pattern on HEp-2 cells, ELISA: enzyme-linked imunosorbent assay, WB: Western blotting, DID: double immunodiffusion, IP: immunoprecipitation, lcSSc: limited cutaneous systemic sclerosis, dcSSc: diffuse cutaneous SSc.

EUROLine® がある．このキットは基本的にはELISA法の変法であり，膠原病で検出される自己抗体を短時間に複数同時に測定できるという利点がある．しかし，本邦では保険適応がないこと，陽性判定基準が確立していないことなどが課題として挙げられる．

疾患特異的自己抗体

1. SScで検出される自己抗体

1) 抗セントロメア抗体

抗セントロメア抗体は，限局型の皮膚硬化，皮膚潰瘍，逆流性食道炎，肺動脈圧性肺高血圧症と相関するが，間質性肺炎の合併は稀である(**表1**)．抗セントロメア抗体陽性SSc患者の予後は他の群と比べ良好である．

2) 抗トポイソメラーゼI抗体(抗Topo I抗体)

抗トポイソメラーゼI(Topo I)抗体はSScの約40%に検出される．抗Topo I抗体はびまん型皮膚硬化，指尖潰瘍，間質性肺炎を高率に合併し，腎クリーゼのリスクも高い(**表1**)．間質性肺炎に対する有効な治療法がないため，抗Topo I抗体は予後不良因子である．

3) 抗RNAポリメラーゼ抗体(抗RNAP抗体)

RNAポリメラーゼ(RNAP)はRNAPI, RNAPII, RNAPIIIの3つのサブユニットから構成される．抗RNAP抗体のELISAはRNAPIIIを抗原としている．抗RNAP抗体は，びまん型皮膚硬化，腎クリーゼと相関しているが，間質性肺炎，指尖潰瘍の合併は少ない(**表1**)．腎クリーゼはACE阻害薬で治療可能となったが，依然として重篤な合併症であり，毎日の血圧測定を患者に徹底させる患者指導がきわめて重要である．

4) 抗U3RNP抗体

抗U3RNP抗体は核小体型を示す．抗U3RNP抗体はびまん型皮膚硬化，指尖潰瘍，間質性肺炎，腎クリーゼと相関しているが，臨床症状の頻度と重症度には人種差がみられ，日本人では欧米の報告に比べ内臓病変は軽症である[1,2](**表1**)．

5) 抗Th/To抗体

抗Th/To抗体は抗U3RNP抗体と同様，核小体型を示す．抗Th/To抗体はSScの2～5%に検出され，限局型の皮膚硬化，間質性肺炎，肺動脈性肺高血圧症と相関する(**表1**)．欧米では抗Th/To抗体は予後不良因子であるが，日本人での重症度は欧米の症例に比べ軽症である[1,2]．

6) 抗hUBF(NOR90)抗体

抗hUBF抗体(以前は抗NOR90抗体と呼ばれていた)は，SSc以外でもRaynaud病や関節リウ

表2　皮膚筋炎・多発性筋炎における自己抗体の検出方法と臨床的特徴

自己抗体	蛍光抗体間接法	検出方法	臨床症状
抗アミノアシル tRNA 合成酵素抗体			
抗 Jo-1 抗体	Cyto	DID, ELISA, WB, IP	PM＞DM, 間質性肺炎, 関節炎
抗 EJ 抗体	Cyto	IP	DM＞PM, 間質性肺炎
抗 PL-7 抗体	Cyto	IP	DM＞PM, 間質性肺炎, Raynaud 症状
抗 PL-12 抗体	Cyto	IP	CADM, 間質性肺炎, Raynaud 症状
抗 OJ 抗体	Cyto	IP	間質性肺炎, 筋炎？
抗 KS 抗体	Cyto	IP	間質性肺炎, Raynaud 症状
抗 Ha 抗体	Cyto	IP	
抗 Zo 抗体	Cyto	IP	
抗 Mi-2 抗体	Sp	IP	典型的な皮膚症状と筋炎, 間質性肺炎・悪性腫瘍は少ない
抗 TIF1 抗体	Sp	WB, IP	悪性腫瘍合併 DM, 小児 DM
抗 MDA5(CADM-140)抗体	Cyto	WB, IP	CADM, 急速進行型間質性肺炎, 皮膚潰瘍
抗 NXP-2(MJ)抗体	Sp	IP	悪性腫瘍合併 DM, 小児 DM, 石灰沈着
抗 SAE 抗体	Sp	IP	CADM? 間質性肺炎, 嚥下障害
抗 SRP 抗体	Cyto	IP	治療抵抗性筋炎

Cyto : cytoplasmic, Sp : speckled, DID : double immunodiffusion, ELISA : Enzyme-linked imunosorbent assay, WB : Western blotting, Ho : homogenous, IP : immunoprecipitation, PM : polymyositis, DM : dermatomyositis, CADM : clinically amyopathic DM.

マチ, SLE などでも検出される. 抗 hUBF 抗体は限局型の皮膚硬化, 軽症型の内臓病変, 良好な予後と相関していることが報告されている(表1).

7) 抗 Ku 抗体

抗 Ku 抗体は筋炎を合併するオーバーラップ例が多く, 半数程度の症例で肺線維症を合併する(表1). 皮膚硬化はステロイドに反応して予後は良好である.

8) 抗 RuvBL1/L2 抗体

最近われわれは, DNA 修復や転写などに関与する核内蛋白である RuvBL1 と RuvBL2 の複合体に対する自己抗体(抗 RuvBL1/2 抗体)が SSc に特異的に検出されることを報告した[3]. 抗 RuvBL1/2 抗体は SSc の 1.9% に検出され, 高齢の男性例が多く, びまん型皮膚硬化と筋炎を合併する頻度が高かった(表1).

9) 抗セントリオール抗体

抗セントリオール抗体は分裂期の紡錘体の両極にある中心体が2つのドット状に染色される. 最近われわれは抗セントリオール抗体陽性の5例について検討し, 全例が SSc でそのうち4例が限局型の皮膚硬化, 全例で難治性の指尖潰瘍, 4例で肺動脈性肺高血圧症を合併していたことを報告した[4](表1).

2. DM で検出される自己抗体

1) 抗 ARS 抗体

これまでに, 抗 Jo-1 抗体, 抗 EJ 抗体, 抗 PL-7 抗体, 抗 PL-12 抗体, 抗 OJ 抗体, 抗 KS 抗体, 抗 Ha 抗体, 抗 Zo 抗体の8種類が報告されている. これらの抗体陽性例は, Raynaud 症状, 間質性肺炎, 関節痛, 発熱, メカニクスハンドなどの共通した臨床症状を有することが知られており, 抗 ARS 抗体症候群と呼ばれる[5](表2). しかし, それぞれの抗 ARS 抗体ごとに臨床症状に差異があることも報告されている[6].

2) 抗 Mi-2 抗体

抗 Mi-2 抗体は蛍光抗体間接法で高力価の Speckeled 型を呈する. 抗 Mi-2 抗体陽性例では定型的な皮疹と筋症状を有するが, 間質性肺炎と悪性腫瘍を合併することは稀である[7](表2). 生命予後は良好であるが, 筋症状はしばしば再燃する.

3) 抗 TIF1 抗体

Kaji らが報告した抗 155/140 抗体と Targoff らが報告した抗 p155 抗体はともに transcriptional

intermediary factor 1(TIF1)を抗原とする同一の自己抗体であり，抗TIF1抗体と呼ばれる．抗TIF1抗体はDMの約25～25%で陽性となる．抗TIF1抗体は成人では50～75%で悪性腫瘍を合併するが，間質性肺炎とは相関しない(表2)．一方，抗TIF1抗体は小児DMの23～29%で陽性になることが報告されている．小児例では悪性腫瘍，間質性肺炎とも合併しない[8]．

4) 抗MDA5抗体(抗CADM140抗体)

抗MDA5抗体はmelanoma differentiation-associated gene 5を抗原とする自己抗体で，抗CADM140抗体と同一である．抗MDA5抗体陽性例は筋症状に乏しく〔amyopathic DMあるいはhypomyopathic DM：両者をまとめてclinically amyopathic DM(CADM)と呼ぶ〕，急速進行型間質性肺炎を高率に合併する[9](表2)．急速進行型間質性肺炎の予後はきわめて悪く，早期からステロイドと免疫抑制薬を併用した治療が望まれる．

5) 抗NXP-2(MJ)抗体

抗NXP-2抗体はかつて抗MJ抗体と呼称されていた．抗NXP-2抗体は小児DMの代表的自己抗体で，石灰沈着を高率に合併する．一方，われわれは抗NXP-2抗体が成人DMの1.6%，多発性筋炎の1%に検出され，半数の患者で悪性腫瘍を合併していたことを報告した[10](表2)．

6) 抗SAE抗体

抗SAE抗体はsmall ubiquitin-like modifier activating enzymeを抗原とする筋炎特異的抗体の1つである．日本人を対象とした検討では，抗SAE抗体はDMの1.5%(433例中7例)に検出され，5例で間質性肺炎を合併していた(表2)．間質性肺炎の活動性は大部分が軽症～中等症だった．7例中悪性腫瘍合併例は1例，小児DMは1例だった[11]．

おわりに

これまで述べてきたように，SScとDMでは個別の疾患特異的自己抗体と臨床像は密接に相関している．しかし，その同定には煩雑な手技を要する免疫沈降法が必要な抗体も多く，ELISA法などの簡便な方法の開発が望まれる．

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VI 関節リウマチの検査・診断

鑑別診断が必要な疾患

全身性強皮症

Systemic sclerosis

長谷川 稔

Key words：線維化，末梢循環障害，自己抗体

はじめに

全身性強皮症は，手指のこわばり，関節拘縮，関節痛などを伴うことがあり，時に関節リウマチとの鑑別が難しい場合がある．また，関節リウマチとの合併も珍しくはない．

本稿では，強皮症の症状，診断，治療などについて概説する．

1 概　念

強皮症は，皮膚や内臓臓器の線維化と末梢循環障害を主体とする膠原病である．ほとんどの症例には，自己抗体が検出される．

その病態はいまだ明らかになっていないが，遺伝的な素因に加えて，何らかの外的要因によって血管内皮細胞や血小板が活性化され，炎症性サイトカイン，ケモカイン，接着分子などが産生される．これらによって組織内にT細胞やマクロファージなどの炎症性細胞が浸潤し，それらが産生するtransforming growth factor(TGF)-β，interleukin(IL)-4，IL-6，IL-13，IL-17Aなどのサイトカインが線維芽細胞からの細胞外基質タンパクの産生を誘導する．また，いったん活性化した線維芽細胞は，TGF-βやconnective tissue growth factor(CTGF)を自己分泌することによって持続的に細胞外基質タンパクが産生され，持続的な線維化が形成されるものと思われる(**図1**)[1]．

2 症　状

ほとんどの強皮症の症例は，レイノー現象で発症する．この時点で，強皮症と関連した自己抗体が末梢血中に検出されたり，爪かく部の出血点などが認められる場合は強皮症の初発症状と考えられ，その後に皮膚硬化などの強皮症の症状が明らかになってくる．皮膚硬化は，手指などの末端から始まり，進行する症例では体幹などの近位側に拡大する．皮膚硬化が肘を超えて上腕や体幹などの近位側に及ぶ場合には，diffuse cutaneous systemic sclerosis(dcSSc)と分類され，肘より遠位にとどまる症例はlimited cutaneous SSc(lcSSc)と分類される．

内臓病変で最も頻度が高いのは，逆流性食道炎である．また，日本人で最も予後を左右する内臓病変は，間質性肺炎である．大半の症例は軽度にとどまるが，進行する症例では呼吸不全に至る．肺動脈性肺高血圧症は，欧米に比べて頻度が少ないものの，発症すると予後不良である．腎クリーゼも我が国では頻度が低く，早期に発見して治療を行えば，予後良好である．ほ

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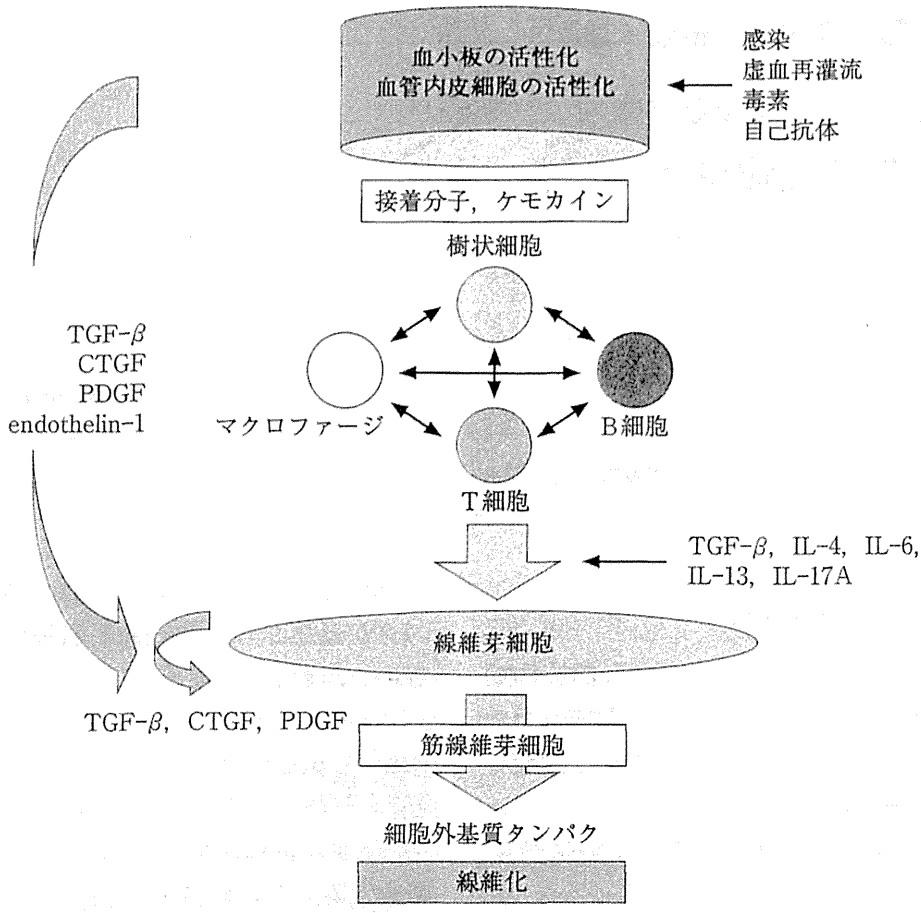

図1 想定される強皮症の病態

TGF: transforming growth factor, CTGF: connective tissue growth factor, PDGF: platelet-derived growth factor, IL: interleukin.

かには，心筋の線維化などによる不整脈，偽イレウスや腸管気腫症などの腸管病変が予後を左右することもある．

3 強皮症にみられる自己抗体

強皮症患者の9割以上の症例では，抗核抗体が陽性である．このうち，30–40％くらいの症例で抗セントロメア抗体が陽性である．本抗体陽性例では，ほとんどが皮膚硬化の軽いlcSScに分類される．逆流性食道炎は本抗体陽性例でも比較的高率にみられるが，間質性肺炎，心筋病変，腎クリーゼはまれである．我が国では，肺動脈性肺高血圧症も非常に少ない．このように線維化と関連した病変は軽症のことが多いが，肺動脈性肺高血圧症や末梢循環障害などの血管病変の程度は症例によって様々である．

また，30–40％程度の症例では，抗トポイソメラーゼI抗体が陽性である．この抗体が陽性の症例の7割くらいは皮膚硬化の範囲の広いdcSScに進展する．逆流性食道炎，間質性肺炎，心筋病変，腎クリーゼなどの内臓病変も抗セントロメア抗体陽性例よりも通常高率で重症である．特に本抗体陽性例では間質性肺炎が重症のことが多く，そのような症例では予後不良である．また，末梢循環障害が強く，難治性，再発性の指尖潰瘍をきたす症例も少なくない．

抗RNAポリメラーゼ抗体陽性例は，我が国では強皮症の5–10％にすぎないが，ほとんどの症例は皮膚硬化の強いdcSScで，腎クリーゼの危険性が高いことから，強皮症の診断目的に抗体検査が保険収載されている．内臓病変も伴いやすいが，間質性肺炎がそれほど重症でないことが多いため，抗トポイソメラーゼI抗体陽

表1 厚生労働省 全身性強皮症診断基準

(1) 大基準
手指あるいは足趾を越える皮膚硬化※1
(2) 小基準
①手指あるいは足趾に限局する皮膚硬化
②手指尖端の陥凹性瘢痕，あるいは指腹の萎縮※2
③両側性肺基底部の線維症
④抗トポイソメラーゼI(Scl-70)抗体または抗セントロメア抗体陽性
(3) 除外基準
①※1 限局性強皮症(いわゆるモルフィア)を除外する
②※2 手指の循環障害によるもので，外傷などによるものを除く
(4) 診断の判定
大基準を満たすものを強皮症と診断する．
大基準を満たさない場合は，小基準の①かつ②-④のうち1項目以上を満たすものを強皮症と判断する．

性例よりも予後は良好である．しかし，10-20％前後の症例では，前述の3つの抗体がいずれも陰性で，免疫沈降法などで検索することによりわかる抗体(抗U3RNP抗体，抗Th/To抗体など)をもつ症例や，既知の抗体が検出されない，あるいは抗核抗体自体がみられない症例も存在する．

4 診　　断

表1に厚生労働省研究班の強皮症の診断基準を示す．これによれば，強皮症では必ず末端の指から皮膚硬化が出現して近位に広がるため，手指や足趾を超える，すなわち手背や足背よりも近位に皮膚硬化がみられる場合は，それだけで強皮症と診断できる．皮膚硬化が手指や足趾に限局している場合，手指尖端の陥凹性瘢痕または指腹の萎縮，CTによる間質性肺炎の所見，あるいは強皮症に特異的な自己抗体(抗セントロメア抗体または抗トポイソメラーゼI抗体)が見つかれば強皮症と診断できる．皮膚硬化の有無は，両手の親指で皮膚をつまみあげるようにした際に，健常な人の同部位よりも皮膚が厚ぼったい(浮腫による一時的なものでなく)，あるいはつまみ上げにくいということから判断する．ただし，いずれの場合も，限局性強皮症(モルフィア)，POEMS症候群，Werner症候群，慢性移植片対宿主病(GVHD)などの他の疾患による皮膚硬化を除外する必要がある．また，強皮症と皮膚筋炎の症例では，爪かく部の出血点や毛細血管ループの拡張が高率に認められる．なお，毛細血管顕微鏡を用いて爪かく部の毛細血管を観察することで，肉眼でみるよりも血管の変化が鋭敏に観察でき，特徴的な所見が早期診断の参考になる(図2)[2]．

5 治　　療

強皮症の治療法は，いまだ十分に確立されていない[3]．皮膚硬化に対しては，放置すると関節拘縮を残すと考えられる症例に対して，ステロイドの少量(プレドニゾロン20mg/日未満)投与や免疫抑制薬(シクロホスファミド，シクロスポリン，またはタクロリムス)の併用が行われている．我が国の強皮症の最大の死因である間質性肺炎に対しては，少量のステロイドとシクロホスファミドの併用療法が最も行われている．しかし，シクロホスファミド無効例に対して，あるいはシクロホスファミド治療終了後の後療法として，アザチオプリン，シクロスポリン，タクロリムスなどが使用されることがある．

6 リウマチとの関連

強皮症の症例が，同じ膠原病に属する関節リ

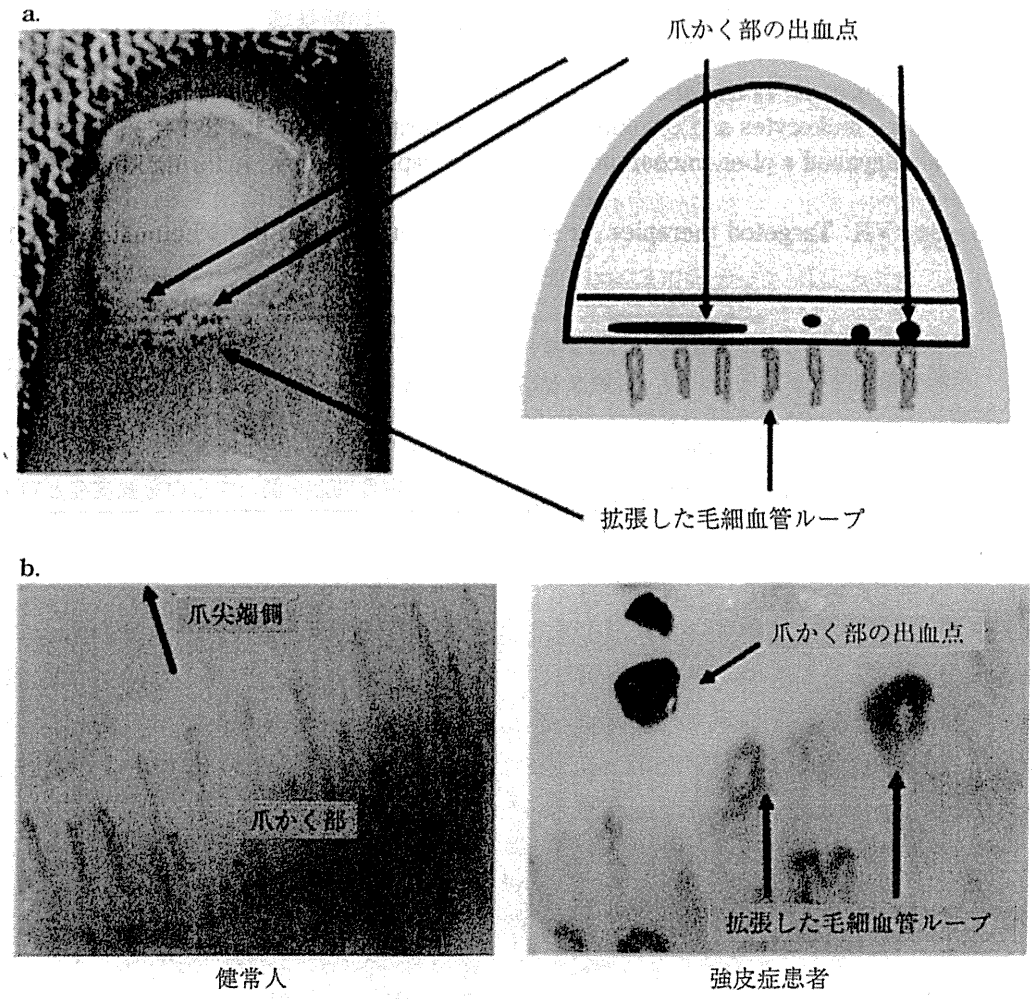

図2-a 強皮症にみられる爪かく部出血点と毛細血管ループの拡張の臨床写真とイラスト

-b 健常人と強皮症患者の爪かく部における毛細血管顕微鏡所見

ウマチを合併する，あるいは関節リウマチの症例が強皮症を合併することは珍しくない．また，強皮症の半数近くでは経過中に関節病変がみられることがある．通常は全身性の関節痛や関節のこわばりなどとして認められるが，まれには関節に炎症をきたす症例があり，関節リウマチなどの他の膠原病の合併なのか診断に迷う症例がある．部位としては，手指関節，手関節，足関節に多い傾向がみられる．リウマチ因子陽性はしばしばみられるが，特に関節変形や骨びらんをきたす症例で陽性率が高いとする報告もある．X線でみられる所見としては，関節近傍の脱灰，関節腔の狭小化，骨びらんなどである．皮膚硬化の強い強皮症では，関節の拘縮をきたすことが少なくないが，関節に炎症などを伴う場合には，更に拘縮が進むので注意が必要である．

おわりに

明らかな皮膚硬化がみられる症例では，強皮症の診断は容易である．しかし，早期や軽症で皮膚硬化がはっきりしない症例では，レイノー現象がみられ，更に強皮症に特異的な自己抗体が検出されるか，爪かく部に出血点がみられる場合には，強皮症の可能性が非常に高い．関節症状を有する強皮症においては，強皮症と関節リウマチの鑑別を慎重に行っていく必要がある．

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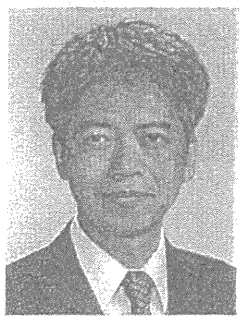
皮膚臨床 56(10)；1455～1463，2014

臨床講義



皮膚科医による膠原病診療
—強皮症と皮膚筋炎について—

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① はじめに

膠原病は，結合組織と血管を病変の主座とし，自己抗体産生を伴う多臓器性の慢性難治性疾患である。これには，関節リウマチ，全身性エリテマトーデス（systemic lupus erythematosus，以下SLE），全身性強皮症（systemic scleroderma，以下SSc），皮膚筋炎・多発性筋炎，結節性多発動脈炎，Sjögren症候群，混合性結合組織病などが含まれる。

米国の大規模な疫学調査[1]からは，SLEを発症した症例はまず抗核抗体が陽性となるが，その時点（発症の平均2.3年前）ではSLEに特異的な抗体は検出されない。しかし，その後，SLEに特異的な抗Sm抗体が出現すると，間もなく（平均0.47年で）症状が出現するとのデータが得られている。すなわち，自己抗体は必ず関連した症状に先行して認められ，そういう意味で早期診断を含めた診断に大変有用である。

本稿では，誌面の関係上，膠原病のなかでもSScと皮膚筋炎にしぼって解説したい。これらの疾患では，何らかの皮膚症状が初期からみられることが多く，最初に皮膚科を受診されることが少なくない。この際に，皮膚症状をよく観察し，自己抗体に関して十分理解していれば，皮膚科医がもっとも早期にそして正確に診断を下せる可能性が高い。

SScと皮膚筋炎の皮膚症状，自己抗体をどのようにしてとらえ，皮膚科医がどのように診療していけばよいかを概説する。

② 全身性強皮症（SSc）

2013年に，米国リウマチ会議（ACR）と欧州リウマチ会議（EULAR）は合同で，SScの新しい分類基準案を発表した（**表1**）[2]。8つのカテゴリーの点数の合計によるが，そのうちの6つは皮膚の所見である。指だけでなく手背にも皮膚の肥厚や硬化が及ぶ場合，他のSSc様の症状をきたす疾患が除外できればSScと分類される。指の腫脹または硬化がない症例はその時点で除外されるが，いずれかがある症例では，他の項目の有無が重要となる。このなかには，内臓病変として肺動脈性肺高血圧症および/または間質性肺炎という項目がひとつだけ含まれており，強皮症関連自己抗体（抗トポイソメラーゼI抗体，抗セントロメア抗体，抗RNAポリ

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別刷請求先　長谷川稔：福井大学医学部感覚運動医学講座皮膚科学（〒910-1193 福井県吉田郡永平寺町松岡下合月23-3）
Key words　強皮症，皮膚筋炎，自己抗体，末梢循環障害，ダーモスコピー

表1　全身性強皮症分類基準2013（ACR/EULAR）

MCP関節より近位の皮膚の肥厚や硬化（9点）
指の皮膚肥厚：腫れぼったい指（2点），PIPからMCPまでの指の硬化（4点）
指尖の病変：指尖部の潰瘍（2点），指尖部陥凹性瘢痕（3点）
毛細血管拡張（2点）
爪かく部の毛細血管異常（2点）
肺動脈性肺高血圧症（2点）および/または間質性肺炎（2点）
Raynaud現象（3点）
強皮症関連自己抗体（2点）：抗セントロメア抗体，抗トポイソメラーゼI抗体，抗RNAポリメラーゼIII抗体のいずれか

合計9点以上で，指の腫脹か硬化があり，強皮症様の症状を呈する他の疾患を除外できる場合に全身性強皮症と分類する。各カテゴリーの中では，高いほうのポイントを使用する。

（文献2）より引用，改変）

メラーゼIII抗体）の有無もひとつの項目として含まれている。注目すべきは，皮膚の血管病変に関する所見が4項目も含まれていることである。SScの診断において，皮膚の血管病変が軽症例や早期例の診断に有用であることを反映している。

SScでは，血管内皮障害とその修復機転の異常によって血管病変，引き続いて線維化が生じると考えられている。実際に，寒冷刺激で指が一時的に蒼白になるRaynaud現象を初発症状とする。その後，やはり末梢循環障害がもっとも強い手指などの末端から皮膚硬化が出現するが，そのまま肘や膝より遠位に皮膚硬化の範囲がとどまる症例群（limited cutaneous SSc，以下lcSSc，**図1-a**）と，皮膚硬化が上腕，大腿，体幹など近位にも拡大する症例群（diffuse cutaneous SSc，以下dcSSc，**図1-b**）に分類される。

皮膚硬化の有無は両手の親指で皮膚を挟み込んだ場合に，皮膚が厚い（浮腫性硬化ないし軽度の硬化）あるいは皮膚が硬くて持ち上がらない（強い硬化）などから判断する。皮膚硬化の重症度の指標としては，modified Rodnan total skin thickness score（MRSS）が使用され，51点満点で点数が高いほど重症である[3]。皮膚硬化の判断が臨床的に難しい場合は皮膚生検を施行し，膠原線維の等質膨化がみられるかどうかを確認する。lcSScでは，長年にわたって皮膚硬化が進行しないことが多いが，dcSScでは発症5年以内の早期に皮膚硬化が急速に進行し，その後は緩徐に軽快する傾向がある。

その他の皮膚症状としては，末梢循環障害によるものとして，指尖部の虫喰い状の陥凹性瘢痕（**図2-a**）があり，SScにかなり特徴的な所見である。さらに末梢循環障害が強い場合には，指尖潰瘍や壊疽を呈してくる（**図2-b**）。また，顔面や手などの末端に，毛細血管拡張がしばしばみられるのも，SScにかなり特異的な所見である（**図3-a，b**）。他には皮内や皮下の石灰沈着がみられることも珍しくない。

皮膚硬化に対する治療としては，放置すると手指関節などに屈曲拘縮を残しうる症例に限って，プレドニン® 20 mg/日以下のステロイド内服治療を開始する。そして，関節の拘縮が残らないようにゆっくりと漸減して5〜10 mg/日程度で維持することが多い。Raynaud現象や指尖潰瘍・壊疽に対しては，保温がもっとも大事なのはいうまでもない。保険収載されている薬はないが，プロスタグランディン誘導体の内服や注射が行われることが多い。SScにみられる指尖潰瘍や壊疽に対して，指を切断することは原