Table 3. Genotype distribution of single-nucleotide polymorphisms within *HGF* and c-*met* loci in SSc patients with and those without ESLD*

Gene location	Genotype	SSc cohort 1, ESLD		SSc cohort 2, ESLD	
		Present (n = 17)	Absent (n = 126)	Present (n = 13)	Absent (n = 142)
HGF –1652	CC	2 (12)	47 (37)†	3 (23)	53 (37)‡
	CT	8 (47)	69 (55)	5 (38)	77 (54)
	TT	7 (41)	10 (8)§	5 (38)	12 (8)¶
HGF +44222	CC	10 (59)	97 (77)		
	CT	7 (41)	28 (22)		
	TT	0 (0)	1 (1)		
HGF +63555	GG	8 (47)	48 (38)		
	GT	9 (53)	78 (62)		
	TT	0 (0)	0 (0)		
c-*met* –980	TT	15 (88)	98 (78)		
	TA	2 (12)	28 (22)		
	AA	0 (0)	0 (0)		

* In the analysis of systemic sclerosis (SSc) patient cohort 1, a total of 16 patients with a disease duration of <5 years who had not developed end-stage lung disease (ESLD) were excluded. Values are the number (%).
† Overall corrected $P = 0.003$ versus cohort 1 patients with ESLD.
‡ Overall corrected $P = 0.01$ versus cohort 2 patients with ESLD.
§ Pairwise comparison showed a significant difference between patients with and those without the TT genotype ($P = 0.0004$, odds ratio 8.1 [95% confidence interval 2.5–26.0]).
¶ Pairwise comparison showed a significant difference between patients with and those without the TT genotype ($P = 0.004$, odds ratio 6.7 [95% confidence interval 1.9–23.9]).

snRNP antibody between cohorts 1 and 2, but the frequencies of organ involvement were similar in the two cohorts.

***HGF* and c-*met* SNPs in SSc patients.** The genotype distribution of the 3 *HGF* SNPs and the 1 c-*met* SNP in the SSc patients and healthy controls conformed to Hardy-Weinberg equilibrium. There was no difference in the distribution of any of the SNPs between the SSc patients in cohort 1 and the healthy controls (data available upon request from the authors). No significant association was observed when comparing the SNP distribution between healthy controls and patients with dcSSc or lcSSc, nor was there any difference in the *HGF* and c-*met* SNP distributions when the SSc patients were grouped according to the presence or absence of involvement of each organ system or each SSc-related ANA.

Association between *HGF* SNP and outcomes of ILD. We next investigated the potential association of SNPs in the *HGF* and c-*met* loci with long-term outcomes of ILD in SSc patients in cohort 1. In this analysis, 16 patients with disease duration of <5 years who had not developed ESLD were excluded. Of the remaining 143 SSc patients, 17 (12%) developed ESLD during the course of the disease. When SSc patients were categorized according to the presence or absence of ESLD, the genotype distribution of *HGF* −1652 was found to be significantly different between the two groups (overall $P_{corr} = 0.003$) (Table 3). In pairwise comparisons, *HGF* −1652 TT was significantly more frequent in patients who developed ESLD than in those who did not (41% versus 8%; $P = 0.0004$, OR = 8.1 [95% CI 2.5–26.0]). However, the frequency of *HGF* −1652 TT was similar in SSc patients with and those without ILD (12% versus 10%). The disease duration was similar in CC, CT, and TT carriers (mean ± SD 208 ± 104 months, 193 ± 110 months, and 205 ± 102 months, respectively).

To confirm the association between the *HGF* −1652 TT genotype and ESLD in SSc patients, 155 SSc patients from an independent cohort (cohort 2) were used in a replication study. All patients in cohort 2 had disease duration of >5 years, and 13 of them (8%) developed ESLD. The distribution of *HGF* −1652 CC, CT, and TT genotypes in cohort 2 was nearly identical to that in cohort 1 (36%, 53%, and 11%, respectively). The genotype distribution of *HGF* −1652 was, again, significantly different between the patients with ESLD and those without (overall $P_{corr} = 0.01$) (Table 3). In pairwise comparisons, *HGF* −1652 TT was significantly more frequent in patients who developed ESLD than in those who did not (38% versus 8%; $P = 0.004$, OR = 6.7 [95% CI 1.9–23.9]). There was no difference in disease duration between CC, CT, and TT carriers (mean ± SD 111 ± 50, 121 ± 66, and 109 ± 41 months, respectively). When the two cohorts were combined, the OR for

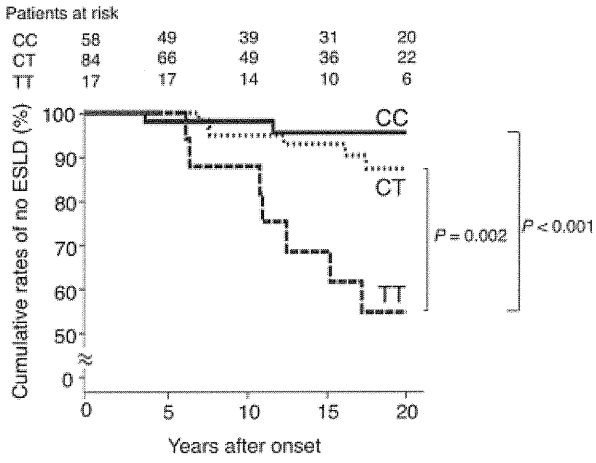

Figure 1. Cumulative rates of survival without end-stage lung disease (ESLD) from the time of systemic sclerosis onset in 159 patients in cohort 1, by *HGF* –1652 genotype. Outcomes were compared using the log rank test. ESLD was defined as the presence of at least 1 of the following 3 features: <50% forced vital capacity, required oxygen supplementation in the absence of pulmonary arterial hypertension, or death due to interstitial lung disease–related causes.

development of ESLD in TT carriers was 7.5 (95% CI 3.2–17.4).

We further evaluated whether the *HGF* −1652 SNP could predict the development of ESLD in the 159 SSc patients in cohort 1. The cumulative rates free of ESLD were compared among the 58 patients with the *HGF* −1652 CC genotype, the 84 with the CT genotype, and the 17 with the TT genotype (Figure 1). The cumulative probability of no ESLD in *HGF* −1652 TT carriers was significantly worse than that in CT or CC carriers ($P = 0.002$ and $P < 0.001$, respectively). There was no significant difference in the cumulative rate between CC carriers and CT carriers.

Influence of the *HGF* −1652 SNP on transcription activity. Since the *HGF* −1652 SNP is located in the promoter region, this SNP may influence transcription activity. To test this hypothesis, we prepared 5 DNA constructs encoding −1756 to −1 (−1652C), −1756 to −1 (−1652T), −1313 to −1, −831 to −1, and −460 to −1, and subjected them to a Dual-Luciferase Reporter Assay system (Figure 2A). The transcription activity of the −1756 to −1 construct carrying the −1652 T allele was significantly lower than the activity of the same region construct carrying the −1652 C allele (mean ± SD 0.16 ± 0.02 versus 0.65 ± 0.03; $P < 0.0001$). The transcription activity of the T allele construct was even lower than the activity of the basic transcriptional activation domain −460 to −1 (0.77 ± 0.05; $P < 0.0001$).

We further assessed interactions between protein and DNA at the *HGF* −1652 polymorphic site, using fibroblast nuclear extracts and EMSA (Figure 2B). Nuclear proteins bound strongly to the T allele probe, but not to the C allele probe. This binding was specific, since an excess amount of the unlabeled probe completely abolished the formation of the complex. This finding indicates that the nuclear factor that bound to the region covering *HGF* −1652 C/T may function as a negative regulator of *HGF* gene transcription.

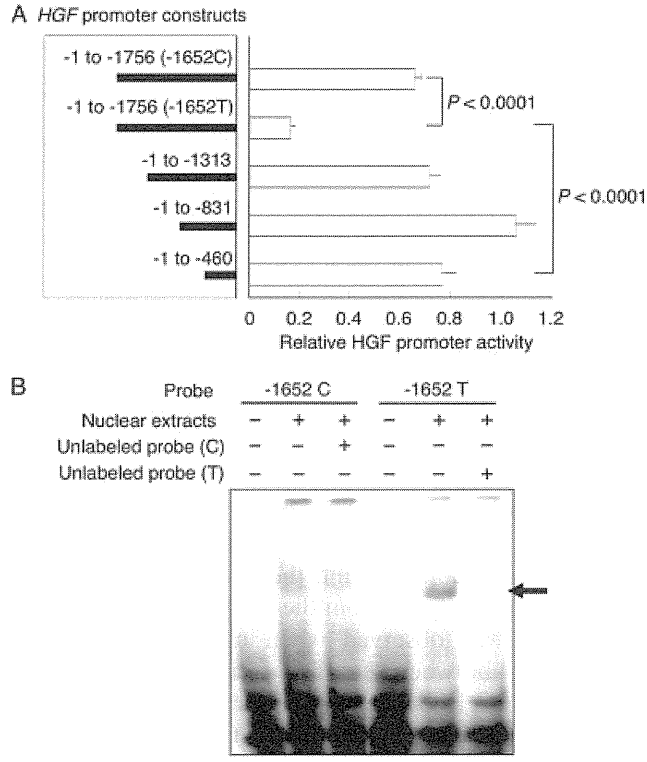

Figure 2. Influence of *HGF* –1652 single-nucleotide polymorphisms (SNPs) on transcription activity. **A,** Transcription activities of reporter constructs harboring *HGF* promoter regions that included nucleotides –460 to –1, –831 to –1, –1313 to –1, –1756 to –1 with a T allele at –1652, and –1756 to –1 with a C allele at –1652. The relative *HGF* promoter activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity. Values are the mean ± SD of pooled data from 4 observations (duplicate cultures) for each of the 5 constructs. **B,** Electrophoretic mobility shift assay with biotin-labeled probes containing the C and T allele at *HGF* –1652. Unlabeled oligonucleotides were used as competitors to demonstrate binding specificity. **Arrow** indicates the band corresponding to the factor that specifically bound to the *HGF* promoter with a T allele at position –1652. Results are representative of 4 independent experiments using nuclear extracts from primary cultures of skin fibroblasts derived from 2 healthy donors.

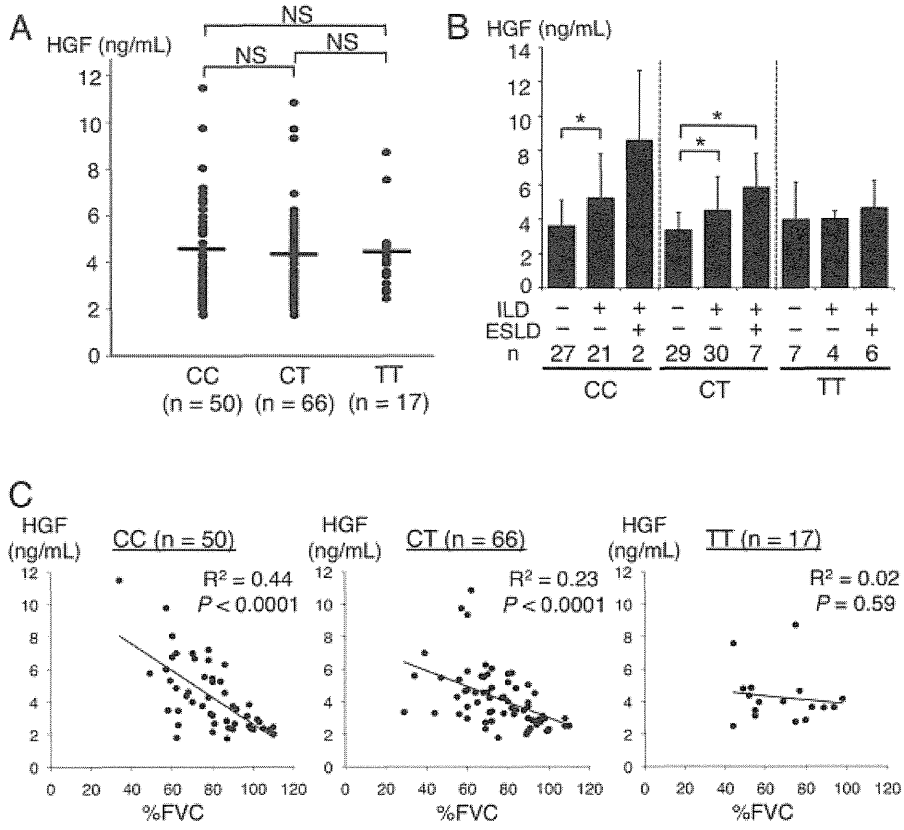

Figure 3. Circulating concentrations of hepatocyte growth factor (HGF) in systemic sclerosis (SSc) patients in cohort 1. **A,** HGF levels in CC, CT, and TT carriers. Each data point represents a single SSc patient; horizontal lines show the mean. NS = not significant ($P > 0.05$). **B,** HGF levels in SSc patients without interstitial lung disease (ILD), those with ILD but without end-stage lung disease (ESLD), and those with ESLD, by carriage of the CC, CT, or TT genotype. * = $P < 0.001$. **C,** Correlation between the serum HGF level and the percentage forced vital capacity (%FVC) in CC, CT, and TT carriers.

Influence of *HGF* −1652 SNP on circulating HGF levels. We measured serum HGF concentrations in 133 SSc patients in cohort 1 and found that there was no difference in the levels between CC, CT, and TT carriers (mean ± SD 4.3 ± 2.1 ng/ml, 4.2 ± 1.8 ng/ml, and 4.2 ± 1.6 ng/ml, respectively) (Figure 3A). We then compared serum HGF levels in the presence or absence of individual organ involvement. ILD was the only organ involvement associated with the HGF level, with a significantly increased level of HGF in patients with ILD than in those without (mean ± SD 4.6 ± 2.0 ng/ml versus 3.8 ± 1.6 ng/ml; $P = 0.008$). We therefore compared the serum HGF levels among patients without ILD, those with ILD and no ESLD, and those with ESLD separately in CC, CT, and TT carriers (Figure 3B). An increased HGF concentration in the presence of ILD or ESLD as compared with the absence of ILD was observed in CC and CT carriers, but not in TT carriers.

We next examined potential correlations between circulating HGF levels and the %FVC in CC, CT, and TT carriers (Figure 3C). A strong negative correlation was observed in CC and CT carriers, suggesting up-regulation of HGF upon progression of ILD. In contrast, TT carriers had a relative inability to increase the level of HGF in the circulation, even in the presence of advanced ILD with low %FVC.

DISCUSSION

In this study, we did not detect any association between the selected SNPs within the *HGF* and c-*met* loci and SSc in the Japanese population. However, our

data, which were obtained from 2 different cohorts of SSc patients, clearly demonstrated that the SNP at *HGF* −1652 was associated with an increased risk of ESLD in SSc patients. Since this SNP did not correlate with the presence or absence of ILD, it may modulate ongoing pathogenic processes in SSc-associated ILD, rather than its susceptibility. Genotyping for *HGF* −1652 SNP in SSc patients may be useful in predicting ILD outcomes and in deciding whether ILD should be actively treated or not.

The functional experiments identified a novel major repressor site in the *HGF* promoter; the presence of a T allele at position −1652 is critical to transcriptional suppression, which would reduce HGF production and impair its antifibrotic effects. SNPs in the promoter region of the gene potentially alter the affinity of DNA–nuclear protein interactions and, in turn, affect transcription efficiency. EMSA findings indicated the presence of a nuclear factor(s) that selectively binds to the *HGF* promoter region, with a T allele at position −1652. Since the T allele displayed lower promoter transcription activity, a nuclear factor(s) bound to the region covering the SNP at *HGF* −1652 might function as a negative transcription regulator.

When potential transcription factors capable of binding to the nucleotide sequence across the polymorphic site were searched using the online prediction site TFBIND (online at http://tfbind.ims.u-tokyo.ac.jp/), several molecules were selected. These included serum response factor, pre-B cell leukemia transcription factor 1, sex-determining region Y, and yin yang 1 (YY-1). Of these, YY-1 is a multifunctional protein that can activate or repress gene expression involved in proliferation, differentiation, and apoptosis, depending on the cell type (21), and is also the factor that preferentially binds to the *HGF* promoter region with a T allele. However, commercially available anti-YY-1 antibodies did not produce a supershift on EMSA (results not shown). Further studies are necessary to identify the nuclear factor(s) that controls the transcription efficiency of the *HGF* gene at this repressor site.

In SSc-associated ILD, extensive extracellular matrix deposits impair the ability of lung epithelial cells to regenerate, resulting in accelerated lung tissue damage and respiratory failure. Numerous studies have shown HGF to be an endogenous antifibrotic factor in the lungs, ameliorating fibrotic lesions and preserving lung function in experimental animal models of pulmonary fibrosis (22–25). HGF exerts its antifibrotic effects on pulmonary function by reducing the production of type I collagen and CTGF, and increasing the production of matrix metalloproteinase 1 in lung fibroblasts (12,26). HGF also assists in regenerating the alveolar structure by promoting alveolar type II cell proliferation (27). Since HGF plays a beneficial role in lung tissue remodeling, the severity and progression of ILD would be influenced by the patient's ability to express this factor.

The association reported here between the functional SNP located on the *HGF* promoter and the SSc patient's risk of developing ESLD may support this theory. In addition, our results suggest that individuals possessing the *HGF* −1652 TT genotype lack the ability to produce HGF in response to ongoing fibrotic processes, such as progression of ILD, and their antifibrotic activity may be impaired by an intrinsically low HGF response. In this regard, data available at the NCBI database (28) show that the *HGF* −1652 TT genotype is more common in African Americans than in Caucasians (32% versus 17%), which may explain why African American SSc patients show a defective HGF response in the circulation and lungs (12) and have more progressive ILD (9–11) as compared to Caucasian patients. Impaired HGF signaling pathways in lung fibroblasts, which have been reported in African American SSc patients (12), also play a role in the ethnic differences in ILD severity.

In summary, our findings suggest that the SNP within the *HGF* promoter region modulates the severity of SSc-associated ILD by controlling *HGF* transcriptional efficiency. The strength of the observed association warrants a larger prospective study of SSc patients of various ethnic backgrounds.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kuwana had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Kuwana.

Acquisition of data. Hoshino, Satoh, Kawaguchi.

Analysis and interpretation of data. Hoshino, Satoh, Kawaguchi, Kuwana.

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Effect of Bosentan on Systemic Sclerosis-associated Interstitial Lung Disease Ineligible for Cyclophosphamide Therapy: A Prospective Open-label Study

YOSHIAKI FURUYA and MASATAKA KUWANA

ABSTRACT. ***Objective.*** To evaluate the clinical benefits of the endothelin receptor antagonist bosentan on interstitial lung disease (ILD) in patients with systemic sclerosis (SSc) who are ineligible for cyclophosphamide (CYC) therapy.

Methods. In this prospective open-label study, 9 patients with SSc and ILD received bosentan for 24 months. The main reasons for avoiding CYC included severely impaired lung function, long disease duration, and relapse after CYC treatment. Pulmonary function tests and Doppler echocardiograms were evaluated every 6 months, and high-resolution computed tomography (HRCT) was performed every 12 months. For an extended survival analysis, 17 historical controls who met the inclusion criteria at referral and had not used any immunosuppressive or antifibrotic agents thereafter were selected from the SSc database.

Results. Two patients did not finish the study; one developed vasculitis requiring high-dose corticosteroids and another died of bacterial pneumonia. The remaining 7 patients tolerated bosentan and completed the study period. There were trends toward mildly reduced forced vital capacity, total lung capacity, and diffusing capacity for carbon monoxide over time. Two patients developed pulmonary hypertension during the 24-month period. HRCT scores for ground-glass opacity, pulmonary fibrosis, and honeycomb cysts gradually increased. In the extended study, there was no difference in cumulative survival rate between the bosentan-treated and historical control groups.

Conclusion. The gradual worsening of pulmonary function and HRCT findings in patients treated with bosentan was consistent with the natural course of SSc-associated ILD. This study does not support the use of bosentan for SSc-associated ILD even when CYC treatment is inadvisable. (First Release Sept 1 2011; J Rheumatol 2011;38:2186–92; doi:10.3899/jrheum.110499)

Key Indexing Terms:
SYSTEMIC SCLEROSIS
INTERSTITIAL LUNG DISEASE
PULMONARY HYPERTENSION
ENDOTHELINS

Interstitial lung disease (ILD) is the leading cause of disease-related morbidity and mortality in patients with systemic sclerosis (SSc)[1]. University of Pittsburgh database records of SSc-related deaths over the past 30 years show that the proportion of patients who died of ILD increased from 6% to 33%, indicating that ILD is the primary cause of SSc-related deaths today[2]. The current treatment for SSc-associated ILD (SSc-ILD) is limited to immunosuppressive agents. For patients with endstage ILD, lung transplantation may offer a viable alternative therapeutic option. A recent randomized placebo-controlled trial (Scleroderma Lung Study; SLS) reported a modest but significant benefit of oral cyclophosphamide (CYC) on lung function and health-related quality of life in SSc patients with active ILD[3], but this small effect was lost at 1 year of followup[4]. Two independent metaanalyses failed to demonstrate any clinically significant improvement in pulmonary function in patients with SSc who were treated with CYC[5,6]. In the Markov decision analytic model for evaluating risk-benefit tradeoffs, 1 year of CYC therapy for SSc-ILD actually resulted in a small loss in the quality-adjusted life-years compared with no CYC[7]. On the other hand, it has been reported that a clinical response to CYC is observed only in a subset of patients with ILD, with the predictors of clinical benefit including a forced vital capacity (FVC) < 70% and moderate pulmonary fibrosis on high-resolution computed tomography (HRCT)[8]. Therefore, the use of CYC should be decided on a patient-by-patient basis by considering the balance between therapeutic efficacy and potential toxicities, such as carcinogenesis and impaired fertility. Alternative reg-

From the Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan.

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Y. Furuya, MD, PhD; M. Kuwana, MD, PhD, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine.

Address correspondence to Dr. M. Kuwana, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
E-mail: kuwanam@z5.keio.jp

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imens for treating SSc-ILD are clearly needed, especially in patients with advanced or endstage ILD, significant risk for toxicity, or relapse after CYC treatment.

Endothelin-1 (ET-1), an endogenous vasoconstrictor, is implicated in the pathophysiology of pulmonary arterial hypertension (PAH) through the constriction and proliferation of vascular smooth muscle cells[9]. Endothelin receptor antagonists (ERA) such as bosentan have been shown to be effective treatments for PAH[10,11]. ET-1 also exerts profibrotic activity by modulating matrix turnover[12] and interacting with transforming growth factor-ß signaling[13]. In addition, ET-1 has been shown to play an important role in ILD pathogenesis, based on the elevated ET-1 levels in the plasma and bronchoalveolar lavage fluids of patients with ILD[14], and reduced collagen deposition in the lung with ERA treatment in a rat model for bleomycin-induced pulmonary fibrosis[15]. Due to its antifibrotic effects, ET-1 blockade would seem to be a logical approach to treating ILD, and randomized, placebo-controlled trials were conducted to investigate the potential efficacy of the dual ERA bosentan on idiopathic pulmonary fibrosis (BUILD-1)[16] and SSc-ILD (BUILD-2)[17]. Disappointingly, both trials failed to show improvement in the 6-min walk distance and in lung function in patients treated with bosentan, although there was a trend in favor of bosentan delaying the time to death or disease progression in patients with biopsy-proven usual interstitial pneumonia[16]. Since the BUILD-2 trial used strict criteria to select SSc patients with active and progressive ILD but without clinically apparent pulmonary hypertension (PH)[17], it is still possible that bosentan is effective for certain subsets of SSc-ILD, such as endstage ILD with severely impaired pulmonary function and complicating PH.

We conducted a 24-month open-label study to evaluate the clinical benefits of bosentan in patients with SSc-ILD ineligible for CYC treatment. The majority of patients enrolled did not satisfy the inclusion criteria of the BUILD-2 trial.

MATERIALS AND METHODS

Study design. This open-label prospective study was conducted at Keio University Hospital, Tokyo, Japan. The protocol was initiated in February 2006, and all the enrolled patients completed the study by February 2010. After assessing a patient's suitability for the study, bosentan was initiated at 62.5 mg twice daily. The dosage was increased to 125 mg twice daily at 4 weeks, and continued for 24 months. Patients were allowed other medications, with the exception of corticosteroids at a dose > 10 mg/day of prednisone or equivalent, or immunosuppressive agents. After completing the 24-month study period, patients were allowed to continue bosentan and were followed to assess the longterm survival and safety profiles. A complete medical history, physical examination, and laboratory analysis were performed for each patient at pretreatment, with more limited evaluations during monthly followup visits. Pulmonary function tests (PFT) and transthoracic echocardiography were done at the 0 (pretreatment), 6, 12, 18, and 24-month visits, and HRCT was performed at 0, 12, and 24 months. Safety profiles, including liver toxicity, were monitored at monthly intervals. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, as reflected in prior approval from the institutional review boards, and each patient gave informed written consent.

Subjects. Patients were considered for inclusion if they satisfied all the following criteria: they (1) were over 18 years of age; (2) met the American College of Rheumatology preliminary classification criteria for SSc[18]; (3) had both exertional dyspnea and ILD as determined by chest radiographs; and (4) were considered ineligible for CYC treatment because of severely impaired lung function [defined as having FVC < 45%, diffusing capacity for carbon monoxide (DLCO) < 30%, oxygen saturation measured by pulse oximetry < 85% during exercise], SSc duration > 7 years after the first non-Raynaud's phenomenon, a relapse after CYC treatment, a recent malignancy, or the patient's refusal[3]. Patients currently treated with corticosteroids > 10 mg/day prednisone or equivalent and/or immunosuppressive agents were excluded. For the extended survival analysis, we selected 17 historical controls from our SSc database, which contains information on 415 patients diagnosed since 1985, based on the following criteria: they (1) met the inclusion and exclusion criteria of this study at time of referral (first visit) to our hospital; and (2) had never been treated with immunosuppressive agents, corticosteroids > 10 mg/day (prednisone), or D-penicillamine since referral to our hospital. Patients with SSc were classified as having diffuse or limited cutaneous disease[19]. Diffuse cutaneous SSc (dcSSc) was considered present if, at any time during the course, skin thickening proximal to the elbows or knees was present, e.g., upper arms, thighs, anterior chest, or abdomen.

Outcome measures. PFT were performed to evaluate FVC, total lung capacity (TLC), and DLCO. Clinically significant worsening and improvement of PFT scores were defined as described[17]. Echocardiography using the Doppler technique was used to evaluate the degree of PH. The tricuspid regurgitation pressure gradient (TRPG) estimated by the tricuspid regurgitation peak velocity was recorded, with tricuspid regurgitation peak velocity > 3.4 m/s or TRPG > 46 mm Hg indicating the presence of PH[20]. The degree of ILD was semiquantitatively assessed by the HRCT scoring system proposed by Goldin, *et al*[21]. Scores (scale 0–24) for ground-glass opacity, pulmonary fibrosis, and honeycomb cysts were recorded individually. The ILD stage, extensive or limited disease, was determined according to the combined evaluation of PFT and HRCT[22]. The modified Rodnan skin thickness score (MRSS; scale 0–51), oral aperture, fingertip-to-palm distance (FTP), Raynaud's condition score (scale 0–10)[23], number of digital ulcers, and the Scleroderma Health Assessment Questionnaire (SHAQ) including the disability index (DI; scale 0–3) and visual analog scale (VAS; scale 0–3)[24,25] were also used to evaluate skin thickening, peripheral vascular disease, and functional status.

Identification of SSc-related antinuclear antibodies (ANA). Serum samples obtained from patients entering the study were analyzed for SSc-related ANA using indirect immunofluorescence and immunoprecipitation assays[26].

Statistical analysis. All continuous values were shown as the mean ± SD, and were compared using the Mann-Whitney U test. The frequencies between 2 groups were tested for statistical significance using the chi-square test or Fisher's 2-tailed exact test, when applicable. Changes in values at different timepoints from the baseline were compared by repeated measures analysis of variance. Cumulative survival rates were calculated according to the Kaplan-Meier method, and comparisons were made using the Cox-Mantel log-rank test.

RESULTS

Patient characteristics at baseline. Nine patients, with the baseline characteristics shown in Table 1, were enrolled. The ratio of men to women was 4:5, and age at entry ranged from 29 to 79 years (55 ± 19). Five were classified as having dcSSc. Evaluation of ANA profiles revealed that all patients with dcSSc were positive for antitopoisomerase I antibodies, and patients with limited cutaneous SSc had either anti-Th/To or anti-U1RNP antibodies. Based on the ILD staging system proposed by Goh, *et al*[22], all except 1 patient had extensive disease, and 4 patients were already receiving supplemental oxygen. Disease duration from the first non-Raynaud's symptom

Table 1. Baseline characteristics of the 9 patients with SSc enrolled.

Patient	Sex/Age at Entry, yrs	SSc Subset	ANA	ILD Stage*	Data at Pretreatment: Disease Duration†, mo	% FVC	% TLC	% DLCO	TRPG, mmHg	MRSS	Reason for Not Using CYC
1	M 29	dcSSc	Topo I	E	25	53	62	44	36	30	Severely impaired lung function
2	F 63	dcSSc	Topo I	E	36	58	57	23	39	20	Relapse after CYC treatment
3	F 65	dcSSc	Topo I	E	293	65	76	30	25	7	Long disease duration
4	M 36	dcSSc	Topo I	E	36	44	53	53	29	14	Severely impaired lung function
5	F 54	dcSSc	Topo I	E	297	34	42	18	53	25	Severely impaired lung function
6	M 62	lcSSc	Th/To	E	167	24	37	16	38	2	Severely impaired lung function
7	F 31	lcSSc	U1RNP	E	72	62	68	22	30	2	Patient refusal
8	M 77	lcSSc	U1RNP	L	10	77	67	40	27	4	Recent malignancy
9	F 79	lcSSc	U1RNP	E	338	107	81	34	30	2	Long disease duration

* E: extensive disease; L: limited disease. † Disease duration from first non-Raynaud's phenomenon. SSc: systemic sclerosis; dcSSc: diffuse cutaneous SSc: lcSSc: limited cutaneous SSc; ANA: antinuclear antibody; TOPO I: topoisomerase I; U1RNP: U1 ribonucleoprotein; FVC: forced vital capacity; TLC: total lung capacity; DLCO: diffusing capacity for carbon monoxide; TRPG: tricuspid regurgitation pressure gradient; MRSS: modified Rodnan skin thickness score; CYC: cyclophosphamide.

ranged from 10 to 338 months (141 ± 134). Patient 5 showed elevated TRPG (53 mmHg), suggesting the coexistence of PH. CYC was not used due to severely impaired lung function in 4 patients, long disease duration in 2, relapse after CYC treatment in 1, the patient's refusal in 1, and recent malignancy in 1.

Safety profiles. During the 24-month study, none of the patients experienced elevated aminotransferase levels (> 3-fold the upper limit of normal) or other adverse events potentially associated with bosentan. Two patients were dropped from the study; 1 because of antineutrophil cytoplasmic antibody-associated vasculitis requiring high-dose corticosteroid therapy at 7 months (Patient 3) and another died of bacterial pneumonia at 17 months (Patient 6). The remaining 7 patients tolerated bosentan and completed the entire study period.

Serial evaluations of outcome measures. Serial measurements of FVC, TLC, DLCO, and TRPG in the 9 patients enrolled are shown in Figure 1. In the 7 patients who completed the 24-month study period, there were trends toward mildly reduced FVC (62.1% ± 24.0% at baseline to 58.7% ± 22.8% at 24 months), %TLC (61.4% ± 12.4% at baseline to 56.0% ± 10.9% at 24 months), and %DLCO (33.4% ± 13.0% at baseline to 27.6% ± 16.4% at 24 months), but only the reduction of TLC was statistically significant ($p = 0.04$). The PFT scores remained stable for all but 1 patient (Patient 9), who experienced clinically significant worsening at 12 and 24 months. None of the patients showed improved PFT scores. TRPG changes were not statistically significant (34.9 ± 9.0 at baseline to 35.4 ± 18.2 at 24 months), but in 2 patients who experienced increased TRPG (Patients 2 and 5), PH was confirmed by right-heart catheterization.

Serial HRCT evaluations revealed stable but slightly increased scores for ground-glass opacity and pulmonary fibrosis (Table 2). The scores for honeycomb cysts gradually increased, and had doubled at 24 months compared with the pretreatment scores ($p = 0.02$). As shown in Table 2, the MRSS and oral aperture were stable, but flexion contractures of the digits as measured by FTP appeared to worsen during the study period. The Raynaud's condition scores tended to increase, but 3 patients experienced a decrease in the number of digital ulcers at 12 and 24 months compared to those observed at pretreatment. There was no treatment effect for the SHAQ-DI or VAS during the study period.

Extended survival analysis. Seven patients continued to take bosentan for an additional 2–24 months after the 24-month study period. None of the patients stopped bosentan due to a potential adverse event during the extension period. Sildenafil was added in 2 patients with PH; one of these died of respiratory failure at 27 months. Therefore, 2 patients (22%) died of ILD-related causes (respiratory tract infection at 17 months and respiratory failure at 27 months) while taking bosentan. The cumulative survival rate in patients treated with bosentan at 2 and 3 years was 86% and 71%, respectively (Figure 2).

We further compared the cumulative survival rate in bosentan-treated patients and historical controls. Table 3 shows baseline characteristics in the 9 bosentan-treated patients and 17 historical controls. There was no statistically significant difference in age at entry, disease duration, or lung function such as FVC and DLCO, but historical controls appeared to be more homogeneous than bosentan-treated patients: historical controls had shorter disease duration, lower %FVC, and higher prevalence of antitopoisomerase I antibody. When an out-

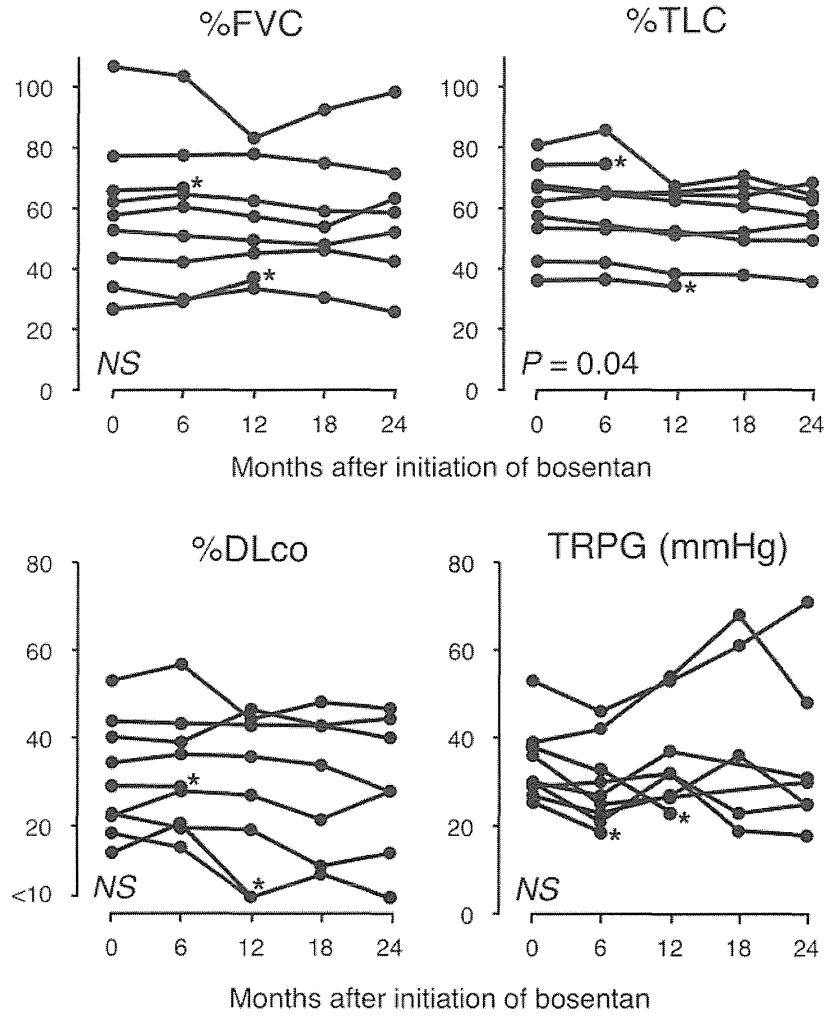

Figure 1. Pulmonary function test results and tricuspid regurgitation pressure gradient (TRPG) changes in 9 patients with SSc-ILD during treatment with bosentan. Statistical analysis was performed for the 7 patients who completed the 24-month study period. *Results from dropout patients. FVC: forced vital capacity; TLC: total lung capacity; DLCO: diffusing capacity for carbon monoxide; NS: not significant.

Table 2. Serial measurements of high resolution computed tomography (HRCT) scores, and measurements of skin thickening, peripheral vascular disease, and functional status in 7 patients who completed the entire study period. Results are shown as the mean ± SD.

Feature	Pretreatment	12 Months	24 Months	p
HRCT scores				
Ground-glass opacity	5.4 ± 4.0	6.1 ± 3.9	5.7 ± 3.5	0.2
Pulmonary fibrosis	8.3 ± 2.4	9.3 ± 2.8	9.1 ± 2.2	0.052
Honeycomb cysts	2.1 ± 3.1	3.0 ± 3.1	4.5 ± 3.7	0.02
Modified Rodnan skin score	13.9 ± 11.6	12.6 ± 9.8	12.7 ± 9.8	0.9
Oral aperture, mm	40.9 ± 13.6	38.9 ± 12.1	43.1 ± 10.8	0.6
FTP, right, mm	1.3 ± 1.3	1.5 ± 2.5	1.7 ± 1.9	0.5
Raynaud's condition score	2.6 ± 2.3	3.5 ± 2.8	3.3 ± 2.5	0.6
No. digital ulcers	0.9 ± 2.3	0.0 ± 0.0	0.1 ± 0.4	0.4
SHAQ-DI	0.7 ± 0.8	0.7 ± 0.9	0.8 ± 0.8	0.4
SHAQ-VAS	1.3 ± 0.5	1.2 ± 0.8	1.4 ± 0.6	0.7

FTP: finger tip-to-palm distance; SHAQ: Scleroderma Health Assessment Questionnaire; DI: disability index; VAS: visual analog scale.

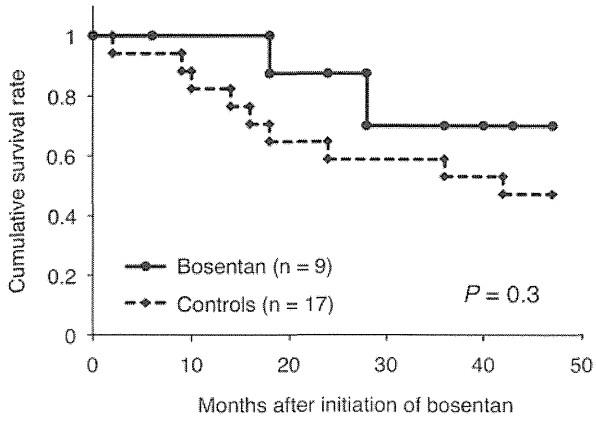

Figure 2. Cumulative survival rates in 9 patients treated with bosentan and 17 historical controls. Comparison between the 2 groups by Cox-Mantel log-rank test.

lier (Patient 9) with very long disease duration (338 months) and preserved %FVC (107%) was excluded from the bosentan-treated groups, disease duration and %FVC were 117 ± 134 and 52.0 ± 17.4, respectively, which were compatible to those in the historical controls. The reasons we did not treat ILD in 17 historical controls included severely impaired lung function in 11, long disease duration in 3, relapse after CYC treatment in 2, and recent malignancy in 1. Nine (53%) of the historical controls died within 48 months, and causes of death were all ILD-related, such as respiratory failure and respiratory tract infection. Although there was a trend toward better cumulative survival rates in the patients treated with bosentan, the difference did not reach statistical significance (p = 0.3). When an outlier (Patient 9) was excluded from the bosentan-treated group, cumulative survival rates at 3 years were almost concordant between bosentan-treated and control groups (67% vs 58%, respectively; p = 0.6).

DISCUSSION

Since the BUILD-2 trial had shown that bosentan does not reduce the frequency of clinically important worsening in SSc patients with active and progressive ILD[17], this prospective open-label study targeted the treatment of CYC-ineligible SSc-ILD. We found that 2-year treatment with bosentan did not have any beneficial effects on lung function or HRCT findings in the target population. Although bosentan was safe and well tolerated, all the outcome variables related to the extent and degree of ILD tended to worsen in spite of bosentan treatment. The relatively slow rate of progression in lung function changes and the HRCT findings in patients treated with bosentan were similar to the natural course of SSc-ILD[27]. Therefore, our data together with the BUILD-2 trial results do not support the use of bosentan as therapy for any forms of established ILD in patients with SSc, including the early disease with exertional dyspnea (SSc duration < 3 years), late active disease (SSc duration ≥ 3 years), or advanced or end-stage disease.

The majority of the patients enrolled in our study had extensive disease, above 20% on HRCT, and the predicted 4-year survival rate of this population was < 60%[22]. In addition, severely impaired lung function requiring oxygen supplementation, ILD concomitant with PH, and relapse after CYC treatment, which were observed in the majority of our patients, are associated with poor prognosis[28,29,30]. Our data showed clearly that bosentan did not improve lung function, but it is still possible that bosentan slowed the lung function deterioration in a patient population highly likely to have poor prognosis. Unfortunately, this issue cannot be evaluated in the setting of a 24-month noncomparative study involving a small number of patients. Our life-table analysis suggests that bosentan may prolong survival in comparison with historical controls, but we have to consider that historical controls might have developed a greater amount of lung deterioration in a shorter period of time in comparison with bosentan-treated patients because of shorter disease duration and lower %FVC. Moreover, historical controls included patients referred to our hospital before 2000, and this may contribute to poor survival

Table 3. Baseline characteristics of bosentan-treated patients and historical controls.

Characteristic	Bosentan-treated Patients, n = 9	Controls, n = 17	p
Years at study entry	2006–2007	1988–2006	
Age at study entry, yrs*	55.1 ± 19.0	54.3 ± 11.8	0.9
Women, %	56	71	0.7
Disease duration, mo*	141 ± 134	109 ± 52	0.4
Diffuse cutaneous SSc, %	56	88	0.3
Extensive disease (ILD staging), %	89	100	0.7
% FVC*	58.2 ± 24.4	50.8 ± 6.3	0.3
% DLCO	31.2 ± 12.5	31.1 ± 6.5	1.0
Oxygen supplementation, %	44	41	0.8
Pulmonary hypertension, %	11	18	0.9
Antitopoisomerase I antibody, %	56	82	0.3
Anti-U1RNP antibody, %	33	12	0.4

* Results are mean ± SD.

rates in historical controls because of outdated equipment and supportive therapies. Larger randomized controlled trials are needed to draw a final conclusion on whether bosentan treatment has some degree of benefit in patients with advanced or endstage ILD.

The potential benefit of ERA to suppress or prevent PH is particularly relevant in patients with SSc-ILD, because these agents have been confirmed to benefit SSc patients with PAH[11]. In this regard, it has been reported that in spite of ERA use, patients with ILD-associated PH have a 5-fold greater risk of death than those with PAH[29], disfavoring the efficacy of ERA in patients with PH owing to ILD. On the other hand, in the subanalysis of the BUILD-2 study assessing bosentan's preventive effect on PH in patients with SSc-ILD, PH developed in 5 patients (5.8%) in the placebo group, but in only one (1.3%) in the bosentan group during a 1-year study period, although this difference did not reach statistical significance[17]. However, in our study, 2 patients (22%) developed PH while being treated with bosentan. Thus, bosentan's efficacy for treating ILD-related PH is apparently inferior to its efficacy for treating PAH.

ET-1 is involved in many pathologic processes, including vasoconstriction, cell proliferation, and promotion of inflammation and fibrosis, and thus blocking ET-1 signaling may result in pleiotropic effects beyond vasodilation. These effects are expected to be beneficial in various aspects of SSc pathogenesis, making ERA attractive potential disease-modifying agents for SSc[31]. Two randomized, prospective, placebo-controlled studies (RAPIDS-1 and -2) have demonstrated that fewer digital ulcers develop in patients treated with bosentan than in those receiving a placebo, although ulcers did not appear to heal better with bosentan treatment[32,33]. Recent open-label studies have shown that bosentan is potentially effective for reducing skin thickening in patients with SSc[34,35]. In contrast, we did not observe any improvement in outcome measures associated with Raynaud's phenomenon, skin thickening, or functional status during bosentan treatment. These negative findings were also reported in the BUILD-2 trial[17]. The lack of therapeutic responses to bosentan may argue against a critical role for ET-1 in SSc pathogenesis, but it is also possible that because of the established and advanced stages of disease in our patient population any effective timing of treatment (in terms of the reversibility of the pathogenic process) might already have passed.

Treatment with bosentan did not improve or stabilize lung function in SSc patients with advanced ILD. Currently, there is no evidence to recommend the use of endothelin receptor antagonists for treating SSc-ILD, even when the disease is ineligible for CYC treatment.

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Prevention of excessive collagen accumulation by human intravenous immunoglobulin treatment in a murine model of bleomycin-induced scleroderma

M. Kajii,* C. Suzuki,* J. Kashihara,* F. Kobayashi,* Y. Kubo,* H. Miyamoto,* T. Yuuki,* T. Yamamoto† and T. Nakae*

**Osaka Research Laboratory, Benesis Corporation, Osaka, and †Department of Dermatology, Fukushima Medical University, Fukushima, Japan*

Summary

Systemic sclerosis (SSc) is an autoimmune disease characterized by fibrotic changes in skin and other organs involving excessive collagen deposition. Here we investigated the effect of intravenous immunoglobulin (IVIG) on fibrosis in a murine model of bleomycin (BLM)-induced scleroderma. Scleroderma was induced in C3H/He J mice by subcutaneous BLM injections daily for 35 days. The collagen content in skin samples from the BLM-injected group (6·30 ± 0·11 mg/g tissue) was significantly higher than the PBS group (5·80 ± 0·10 mg/g tissue), and corresponded with dermal thickening at the injection site. In contrast, mice treated with IVIG for 5 consecutive days after initiating BLM injection showed lesser collagen content significantly (IVIG group, 5·61 ± 0·09 mg/g tissue; BLM vs. IVIG). In order to investigate the cellular and protein characteristics in the early stage of the model, the skin samples were obtained 7 days after the onset of experiment. Macrophage infiltration to the dermis, monocyte chemoattractant protein (MCP-1)-positive cells, and increased TGF-β1 mRNA expression were also observed in the BLM group. IVIG inhibited these early fibrogenic changes; MCP-1 expression was significantly lesser for the IVIG group (1·52 ± 0·19 pg/mg tissue) than for the BLM group (2·49 ± 0·26 pg/mg tissue). In contrast, TGF-β1 mRNA expression was significantly inhibited by IVIG. These results suggest that IVIG treatment may inhibit macrophage recruitment to fibrotic sites by down regulating MCP-1 and TGF-β production, and thus could be a potential drug for managing fibrotic disorders such as SSc.

Keywords: bleomycin-induced fibrosis, immunomodulatory action, intravenous immunoglobulin

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Correspondence: M. Kajii, Osaka Research Laboratory, Benesis Corporation, Osaka 532-8505, Japan.
E-mail: kajii.masahiko@md.mt-pharma.co.jp

Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterized by severe alterations in the microvasculature, prominent inflammatory and immunological changes, and excessive accumulation of extracellular matrix in the skin and internal organs. Despite research efforts, no standard protocol for treating SSc has been established to date. Controlled trials have demonstrated that some immunosuppressive therapies interrupt the immune-mediated portion of its pathogenic cycle, but such findings have been rather inconsistent.

For over 30 years, human intravenous immunoglobulin (IVIG) has been an important treatment option for a number of clinical indications, such as primary immunodeficiency disease, autoimmune disease, and acute inflammatory conditions [1]. IVIG has been also reported to reduce skin stiffness in SSc patients in several open trials [2,3]. Although the precise mechanisms of IVIG are unclear, animal models are crucial for investigating SSc pathogenesis and its various therapeutic approaches. We previously reported several immunomodulatory activities in IVIG using various experimental models of autoimmunity [4–6]. IVIG is classically manufactured from pooled plasma of healthy donors, and contains various antibodies specific to bacteria and toxins. However, this may not be the only mechanism for managing autoimmune diseases. Since pathology of SSc is complicated, IVIG might show some immunological effects on activated fibroblast to produce excessive collagen.

The therapeutic effects of IVIG most likely reflect the functions of natural antibodies in maintaining immune homeostasis in healthy individuals [7]. In this study, we

confirm that infiltration of macrophage in the dermis is suppressed by IVIG.

Animal models that exhibit all aspects of SSc are currently unavailable, but a few experimental systems replicating its pathogenic aspects have been reported by us and others [8–10]. In addition, we previously established a murine SSc model by injecting animals with bleomycin (BLM) daily for 35 days. Histological examination on skin samples of BLM-treated mice revealed thickened dermal areas with collagen bundles, and deposition of homogeneous material with cellular infiltrates mimicking human scleroderma at injection sites thus confirming SSc onset.

We investigated the influence of IVIG administration on inducing dermal sclerosis using the BLM-based murine model, and identified that IVIG attenuated excessive collagen accumulation by inhibiting monocyte chemoattractant protein (MCP-1) and transforming growth factor beta (TGF-β) in this model.

Materials and methods

Mice

Specific, pathogen-free, female C3H/HeJ mice (age: 6 weeks, weight 17–22 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were kept in our animal facility with free access to food and water.

IVIG

Human immunoglobulin preparation (Venoglobulin-IH™, Benesis Corporation, Osaka, Japan) was administrated intravenously at 400 mg/kg/day into tail veins of the IVIG group mice 5 consecutive days after initiating BLM treatment. The same volume of saline was administered to the BLM group. Amount of the drug used are correspond to that used in clinical.

BLM-induced murine fibrosis model

The BLM-induced murine fibrosis model was performed using a previously described method [11]. Briefly, 100 μL of 600 μg/ml BLM (Nippon Kayaku Co., Japan) in PBS was injected subcutaneously into the shaved back of mice using 27-gauge needles daily for 35 days.

Collagen content in the skin

Punched skin samples (diameter: 14 mm) were obtained from shaved backs of mice 35 days after treatment. Each sample was treated with 0·5 mol/l acetic acid containing pepsin (0·3 mg/10 mg tissue) at 4°C overnight. Then the collagen content in skin was determined using the Sircol™ Soluble Collagen Assay kit (Biocolor Ltd, Northern Ireland).

Quantification of MCP-1

Punched skin samples (diameter: 8 mm) were obtained from shaved backs of mice 7 days after BLM treatment. Each sample homogenized in Dulbecco's modified Eagle's medium, centrifuged for 5 min at 200***g***, and the supernatants were collected. Immunoreactive MCP-1 levels were determined using a commercial ELISA kit (Biosciences, San Diego, CA) following the manufacturer's instructions.

Histological examination

Skin samples were obtained from shaved backs of mice 7 days after BLM treatment. Skin sections were routinely stained with hematoxylin and eosin. In addition, collagen production was identified by Masson's trichrome stain. The dermal thickness of BLM- (BLM and IVIG groups) and PBS- (PBS group) injected skin were calculated using an image processor Win Roof (Mitani Corp., Tokyo, Japan). To avoid bias, the histologist was single blinded to the treatment groups of mice.

Immunohistochemical examination

For immunohistochemical experiments, skin samples were cut into halves to divide the BLM-injected region equally, fixed in 10% formalin solution, embedded in paraffin, and sections were stained. Rabbit anti-Iba-1 polyclonal antibody (Wako, Tokyo Japan) with final concentration 500 ng/ml was used for staining macrophages after 7 days of initiating BLM treatment. Iba-1 mainly infiltrate macrophage-like and fibroblastic cells in BLM-treated skin. Primary antibodies against MCP-1 (Novus Biologicals, Littleton, CO) and collagen I (abcam, cambridge, MA) were used according to manufacturers guidelines. Samples were subsequently incubated with a secondary antibody labeled with peroxidase (Nichirei Bioscience Inc., Tokyo, Japan). The skin sections were developed with diaminobenzidine solution as chromogen, counterstained with hematoxylin, dehydrated, cleared, and mounted.

RNA isolation and RT-PCR

Total RNA was isolated from skin samples of the model mice (7 days after initiating BLM treatment) using the Ribo Pure™ extraction kit (Ambion Inc., Austin, TX). All samples were treated with RNA Stabilization Reagent (RNAlater, Qiagen, Valencia, CA) at 37°C overnight and stored at −80°C until use. Total RNA was reverse-transcribed into cDNA according to the manufacturer's protocol for the Reverse Transcription System (Promega, Madison, WI). TGF-β1 mRNA expression was analyzed by quantitative RT-PCR according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Sequence-specific primers and

probes were designed using pre-developed TaqMan® assay reagents (Applied Biosystems). RT-PCR (1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 60 s) was performed using a real-time PCR System 7500 (Applied Biosystems). GAPDH was used to normalize mRNA. To compare TGF-β1 mRNA and housekeeping GAPDH mRNA expression, the relative expression of PCR products was determined using the ΔΔCt method [12]. Fold induction is equal to 2 – [ΔΔCt], where Ct = threshold cycle, and ΔΔCt = [Ct gene interest (unknown) – Ct GAPDH (unknown)] – [Ct gene interest (calibrator) – Ct GAPDH (calibrator)]. One control was chosen as the calibrator. Each was examined in duplicate and the mean Ct was used in the equation.

Statistical analysis

All data are expressed as means ± SEM Statistical significance was determined using Student's *t*-test. *P*-values < 0·05 were considered significant.

Results

IVIG reduced collagen production in BLM-treatment skin

Representative histological skin sections from mice used in the BLM-induced mouse fibrosis model are shown in Fig. 1a. Mice treated with BLM for 35 days exhibited a thicker dermal layer compared to those treated with PBS. However, mice administered IVIG (400 mg/kg/day) for 5 consecutive days after initiating BLM treatment did not exhibit these fibrotic changes. Thickness of dermal layer of each mouse was calculated using an image processor and subsequent statistical analysis (Fig. 1b). The BLM group had significantly increased skin thickness (335·3 ± 14·9 μm) compared to PBS group (159·4 ± 4·9 μm; PBS vs. BLM, $P < 0·01$). Furthermore, IVIG treatment drastically ameliorated the dermal thickening effects of BLM-injected mice (241·1 ± 10·5 μm, BLM vs. IVIG, $P < 0·01$).

Thickening in the dermal layer was corresponded to accumulation of collagen by Masson' s trichrome stain and immunohistochemistry of type I collagen(Fig. 2a). We determined the collagen content in skin samples from mice treated with BLM for 35 days (Fig. 2b), and found that they had significantly higher collagen content (6·30 ± 0·11 mg/g tissue) than the PBS group (5·80 ± 0·10 mg/g tissue, PBS vs. BLM, $P < 0·01$). Supplementary we confirmed that the similar results were obtained when the extent of fibrosis was determined with the contents of hydroxyproline, another index of fibrosis, in a different set of experiment. The content of the amino acid was significantly suppressed in the IVIG group (715 ± 11·8 μg/g tissue, $n = 10$) in the comparison with BLM group (775 ± 15·6 μg/g tissue, $n = 10$). These results show that collagen production was induced by BLM. In another

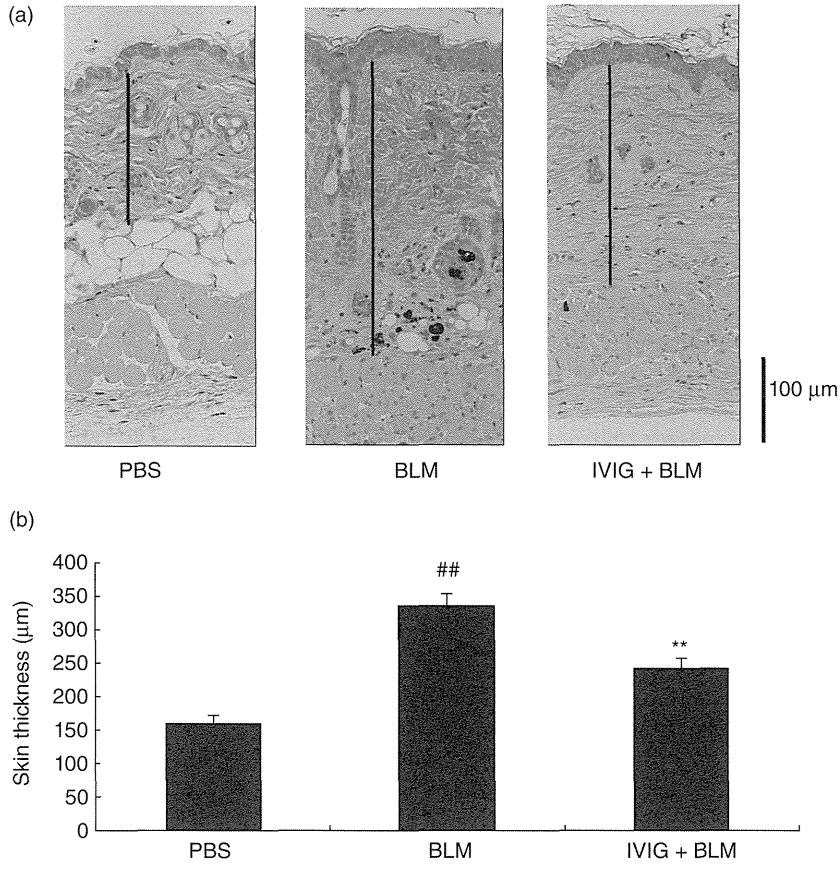

Fig. 1. Effect of IVIG on BLM-induced fibrosis. The murine fibrosis model was induced by subcutaneously injecting mice with BLM for 35 days. Some mice were injected with PBS on the same schedule as negative control. IVIG (400 mg/kg/day) was administered intravenously for 5 consecutive days, and skin samples were taken 35 days after initiating BLM for pathological study. (a) Representative images of each group are shown, with bars and arrows representing the lengths of the dermis and dermal layer, respectively. (b) Thickness of the dermal layer was calculated using an image processor. Data are expressed as the mean ± SEM ($n = 9$–10). $^{\#\#}P < 0·01$ (vs. PBS-treated mice, Student's-test), $^{**}P < 0·01$ (vs. BLM-treated mice, Student's *t*-test).

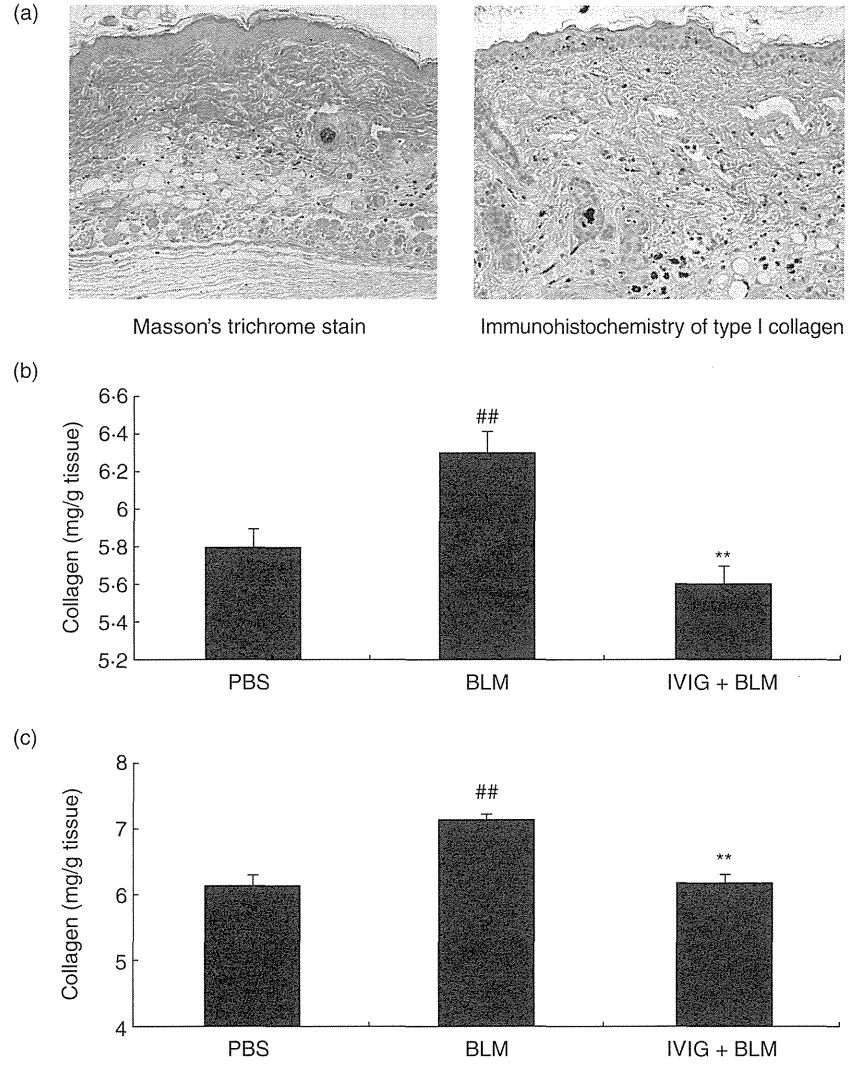

Fig. 2. Collagen content and the effect of IVIG treatment at different time. (a)Collagen in the dermis was observed Masson's trichrome stains and immunohistochemistry of type I collagen in the BLM-treated mice. (b, c) IVIG was administered to mice intravenously (400 mg/kg/day for 5 consecutive days) either immediately (b) or 28 days (c) after initiating BLM treatment. In both experiments, skin samples were obtained 35 days after initiating BLM treatment to determine collagen content (Sircol™ Soluble Collagen Assay kit). Data are expressed as the mean ± SEM ($n = 12$). $^{\#\#}P < 0{\cdot}01$ (vs. PBS-treated mice, Student's *t*-test), $^{**}P < 0{\cdot}01$ (vs. BLM-treated mice, Student's *t*-test).

group of mice, IVIG was given intravenously immediately after initiating BLM treatment. Their results revealed dermal collagen content significantly less than IVIG treatment (5·61 ± 0·09 mg/g tissue; BLM vs. IVIG, $P < 0{\cdot}01$). In another post-onset experiment, IVIG was given intravenously 28 days after initiating BLM treatment. In addition, IVIG treatment suppressed collagen content in the dermis to a similar extent as the previous experiment (Fig. 2c). We discuss the effect of earlier treatment of IVIG later in this study.

IVIG suppressed fibrogenic cytokine and chemokine in the skin

To examine the influence of TGF-β1 on IVIG mechanisms in early SSc, we obtained a skin sample 7 days after initiating the experiment. We subsequently determined mRNA expression levels. We found that TGF-β1 mRNA levels were upregulated in the BLM group compared to the PBS group, and were subsequently suppressed in the IVIG group (BLM vs. IVIG, $P < 0{\cdot}05$) (Fig. 3).

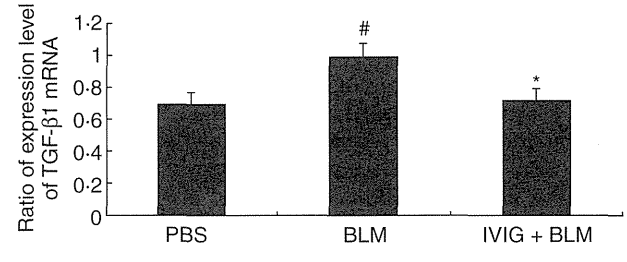

Fig. 3. Expression of TGF-β1 mRNA in lesional skin of mice. Mice were locally treated with either PBS or BLM for 5 days and total mRNA was isolated from skin samples. IVIG was administered intravenously (400 mg/kg/day for 5 consecutive days) immediately after initiating BLM treatment. Skin samples were obtained after 7 days to determine TGF-β1 mRNA expression. Representative data are shown from 3 independent experiments. Relative amounts are expressed as arbitrary units after TGF-β1 m RNA normalized with GAPDH. Data are expressed as means ± SEM ($n = 8$). $^{\#}P < 0{\cdot}05$ (vs. PBS-treated mice, Student's *t*-test), $^{*}P < 0{\cdot}05$ (vs. BLM-treated mice, Student's *t*-test).

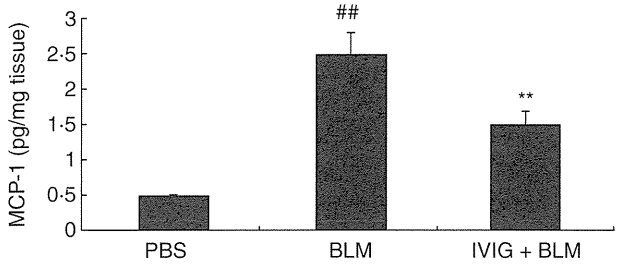

Fig. 4. Immunoreactive MCP-1 levels determined by ELISA. Mice were locally treated with either PBS or BLM for 5 days, and IVIG was administered intravenously (400 mg/kg/day for 5 consecutive days) immediately after initiating BLM treatment. Skin samples were homogenized, centrifuged, and supernatants measured by ELISA. Data are expressed as means ± SEM ($n = 12$). $^{\#\#}P < 0{\cdot}01$ (vs. PBS-treated, Student's *t*-test), $^{**}P < 0{\cdot}01$ (vs. BLM-treated mice, Student's *t*-test).

We examined MCP-1 expression in this experimental *in vivo* model, and found that MCP-1 levels were significantly increased in the BLM group (2·49 ± 0·26 pg/mg tissue) compared to the PBS group (0·46 ± 0·04 pg/mg tissue) as seen for TGF-β1 mRNA expression. IVIG treatment lowered these levels (1·52 ± 0·19 pg/mg tissue; BLM vs. IVIG, $P < 0{\cdot}01$) (Fig. 4).

In a preliminary study, we also measured concentrations of several inflammatory cytokines and chemokines (IL-2, IL-6, KC, MIP-1α, RANTES, GM-CSF, and TNFα) with BD FACSarray Bioanalyzer System and mRNA expression levels of IL-6 and IL-13, all of which might be involved in the IVIG mechanism in this model. Although some of these levels were increased in this model, IVIG treatment did not induce statistically significant changes (data not shown).

Immunohistochemical observation of macrophages and MCP-1

Immunohistochemistry was used to observe cellular infiltration in the dermal layer during early stages of BLM-induction. Staining with Iba-1 (anti-mouse macrophage antibody) revealed that most infiltrated cells were macrophages. Representative images of Iba-1 positive cells are shown in Fig. 5a and c. We also calculated the area of Iba-1 positive cells for each group, which revealed that IVIG treatment suppressed infiltration of macrophages (significantly increased by BLM treatment) in the dermis (Fig. 6). Furthermore, MCP-1 was mainly found in BLM-treated skin samples (Fig. 5b and d). These results suggest this chemokine may be expressed mainly in the infiltrated macrophages because most MCP-1-positive areas colocalized with Iba-1 staining. However, some MCP-1 staining was also shown in fibroblasts. MCP-1-positive area was less for the IVIG group than the BLM group (Fig. 5d).

Discussion

In this study, we used BLM-induced murine fibrotic models to investigate the efficacy and mechanisms of IVIG in treating SSc. In our first experiments, we observed dermal thickening and excess collagen production with infiltration of macrophages in the lesional skin of BLM-treated mice. We also examined how IVIG would affect the lesional skin if administered on the first day of BLM treatment. IVIG was found to almost completely suppress the increase in dermal thickness, as well as collagen content induced by BLM treatment. The concentration of IVIG and the time schedule used in this study was implemented to match typical clinical

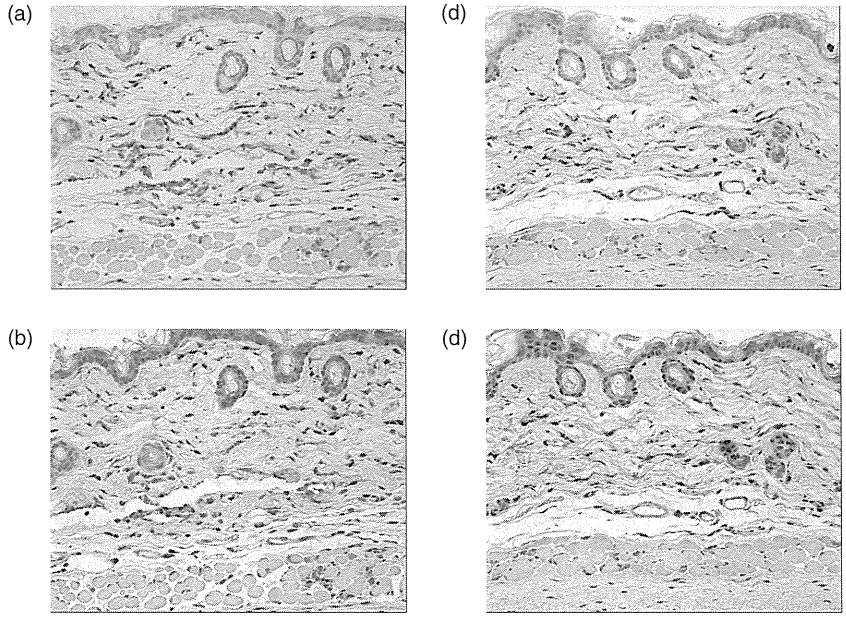

Fig. 5. Immunohistochemical localization of MCP-1. Seven days after the first BLM treatment, immunohistochemistry was performed to identify cells that were positive for Iba-1 (a, c) and MCP-1 (b, d). IVIG was administered intravenously (400 mg/kg for 5 consecutive days) immediately after initiating BLM treatment (c, d). Positive staining for macrophages and MCP-1 were primarily found in BLM-treated skin.

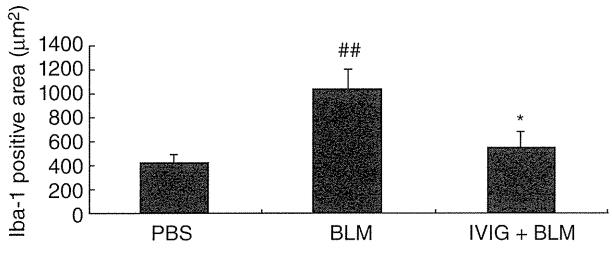

Fig. 6. Immunohistochemical localization of macrophages. Seven days after initiating BLM treatment, immunoreactive cells for Iba-1 were correlated to mainly infiltrating macrophage-like cells in BLM-treated skin. IVIG was administered intravenously (400 mg/kg/day for 5 consecutive days) immediately after initiating BLM treatment. Data are expressed as means ± SEM ($n = 12$). $^{\#\#}P < 0{\cdot}01$ (vs. PBS-treated, Student's *t*-test), $^{*}P < 0{\cdot}05$ (vs. BLM-treated mice, Student's *t*-test).

regimens. We then examined the effect of IVIG on various cytokines and chemokines, which may induce the first step toward fibrosis. Increase in expression of fibrosis-related cytokines, chemokines, and enzymes prior to the increase of collagens and other extracellular matrix proteins in the lesional area of dermis has been investigated [13]. Here we focused on TGF-β1 and MCP-1 because both are crucial factors in the induction of fibrosis. Their increased levels were drastically ameliorated by IVIG. We also examined other cytokines, such as IL-2, IL-6, KC, MIP-1α, RANTES, GM-CSF, and TNFα, but they were largely unaffected (data not shown). In addition, we successfully investigated MCP-1 suppression by IVIG using ELISA and immunohistochemical examination for the first time.

TGF-β is believed to influence the development of tissue fibrosis in SSc patients by directly inducing collagen formation from fibroblasts in the dermal layer [14]. We previously reported that local BLM injections induced collagen production and cellular infiltrates the skin, lung inflammation and fibrosis, and increased production of TGF-β1 *in vivo* [11]. TGF-β has been localized to the sites of mononuclear cell infiltration and fibroblast activation by immunostaining [11]. It is well known that activation of TGF-β cascade is regulated not only by the expression of the molecule but also molecular change from the latent molecule to the active one. Whether this kind of activation is occurred or not in this model and IVIG inhibition is observed at this point is interesting and should be elucidated.

The role of chemokines in BLM-induced scleroderma has been highlighted in literature [15,16]. MCP-1 is a multifunctional inflammatory chemokine belonging to the C-C chemokine superfamily. Upregulation of collagen expression is preceded by monocyte infiltration and increase in TGF-β mRNA expression. In addition, MCP-1 might influence modulation of extracellular matrix deposition by stimulating interstitial collagenase production in human fibroblasts [17]. Furthermore, fibrosis is not caused by BLM in MCP-1-deficient mice [18],. Other studies have shown that this chemokine is also important in other scleroderma models. MCP-1 is upregulated in growth factor-injected mice undergoing simultaneous treatment with bFGF and CTGF increased skin fibrosis [19]. By contrast, these treatments in MCP-1-deficient mice decreased collagen content in the skin suggesting the influence of MCP-1 in recruiting inflammatory cells [19]. These results suggest the MCP-1 may be a key determinant in the development of skin fibrosis induced by BLM [18]. Administration of an anti-MCP-1 neutralizing antibody reduced dermal sclerosis and decreased the skin collagen content usually seen in the pathogenesis of BLM-induced scleroderma. MCP-1 might contribute to the induction of dermal sclerosis through the infiltration of macrophages [16] or through the direct activation of the fibroblasts. This critical role played by the chemokine in SSc pathogenesis has also been demonstrated.

In this study, we mainly focused on effects of IVIG treatment on early fibrotic changes. However, we also showed that the drug significantly reduced the dermal thickness seen in BLM-treated mice, even when administered at the later stage of the experiment (Fig. 2b). This indicates that other mechanisms may exist for the action of IVIG in this study, with continued involvement of cytokines and chemokines resulting in scleroderma through an experiment. However, this point should be further elucidated in future studies. In various experimental models of diseases in mice and rats, pharmacological properties of IVIG have been reported. Indeed, immunological responsibility between human IgG and Fc receptor on the murine cells might be limited in this model. On the other hand, IVIG is considered to contain much more types of antibodies derived from several thousands of donors than that from mice bred in specific pathogen free conditions.

In conclusion, IVIG may act by inhibiting the recruitment of macrophages to the sites associated with fibrotic skin disorders. Furthermore, IVIG is thought to downregulate MCP-1 and TGF-β production by macrophages and monocytes, which is likely to activate fibroblasts, ultimately resulting in excessive accumulation of collagen in skin. This indicates that IVIG may become an important therapy for treating SSc patients.

Disclosure

Nothing to disclose.

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Extended report

Elevated serum interleukin-27 levels in patients with systemic sclerosis: association with T cell, B cell and fibroblast activation

Ayumi Yoshizaki,[1] Koichi Yanaba,[1] Yohei Iwata,[1] Kazuhiro Komura,[2] Asako Ogawa,[1] Eiji Muroi,[1] Fumihide Ogawa,[1] Motoi Takenaka,[1] Kazuhiro Shimizu,[1] Minoru Hasegawa,[2] Manabu Fujimoto,[2] Shinichi Sato[1,3]

[1] Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
[2] Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan
[3] Department of Dermatology, The University of Tokyo Graduate School of Medicine, Tokyo, Japan

Correspondence to
Dr Shinichi Sato, Department of Dermatology, The University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan;
satos-der@h.u-tokyo.ac.jp

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ABSTRACT

Objective To determine serum levels of interleukin 27 (IL-27) in patients with systemic sclerosis (SSc) and relate the results to the clinical features of SSc.

Methods Serum levels of IL-27 in 91 patients with SSc and the production of IL-27 by isolated monocytes were examined by ELISA. The expression of IL-27 receptor in the skin fibroblasts, B cells and T cells was quantified by real-time PCR. The effect of IL-27 on immunoglobulin G (IgG) production of B cells, IL-17 production of CD4 T cells and proliferation and collagen synthesis of fibroblasts was also analysed.

Results Serum IL-27 levels were raised in patients with SSc compared with healthy controls and correlated positively with the extent of skin and pulmonary fibrosis and immunological abnormalities. IL-27 levels also correlated positively with serum levels of hyaluronan, recently identified as an endogenous ligand for Toll-like receptors. The retrospective longitudinal analysis showed a tendency for serum IL-27 levels to be attenuated during the follow-up period. IL-27 production by cultured monocytes was increased by hyaluronan stimulation. IL-27 receptor expression was upregulated in the affected skin fibroblasts, B cells and CD4 T cells of patients with SSc. Moreover, IL-27 stimulation increased IgG production of B cells, IL-17 production of CD4 T cells and proliferation and collagen synthesis of fibroblasts in patients with SSc compared with those in healthy controls.

Conclusion These results suggest that IL-27 and its signalling in B cells, T cells and fibroblasts contributes to disease development in patients with SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterised by excessive accumulation of extracellular matrix in the skin and various internal organs.[1] It is accompanied by a number of immunological abnormalities including autoantibody production and elevated levels of several cytokines such as tumour necrosis factor (TNF)α and interleukin (IL)-1β, IL-4, IL-6, IL-10 and IL-17.[2–4] Furthermore, previous studies have shown that serum levels of endogenous ligands for Toll-like receptors (TLR) such as hyaluronan are increased in SSc.[5–8] In our previous study we showed that serum hyaluronan levels are associated with disease severity and immunological abnormalities.[5] Although these abnormalities were associated with skin fibrosis and involvement of several organs, the mechanism and pathogenesis of SSc remain unknown.[9]

IL-27 is a new member of the IL-12 and IL-6 families which consists of an IL-12 p40-related protein and a newly discovered IL-12 p35-related protein.[10] Examination of cDNA libraries has indicated that human IL-27 is highly induced in activated antigen-presenting cells such as monocytes.[11–13] Although there are no studies of the regulation of IL-27 expression, signalling via TLR4 activated by lipopolysaccharide (LPS) is the key inducer of IL-27.[14–16] IL-27 receptor complex comprises IL-27R (also called WSX-1) and glycoprotein 130 (gp130).[10] IL-27 is the only known ligand for IL-27R. IL-27R and gp130 were found to be coexpressed by a large variety of cells including monocytes, T cells, B cells and fibroblasts, indicating that IL-27 may display pleiotropic functions.[17]

Previous studies have shown that the role of IL-27 in the regulation of immune responses is somewhat controversial. Some studies have shown that IL-27 promotes naive T cell proliferation and initiates Th1 immune responses.[11 18] However, other studies have shown that IL-27 suppresses the expansion of effector and memory T cells and inhibits different cytokine secretion, suggesting anti-inflammatory functions of IL-27.[19 20] The dual role of IL-27 in vitro has also been demonstrated on in vivo infectious and autoimmune inflammatory models. IL-27 neutralisation suppressed inflammation in rodent adjuvant arthritis.[21] In contrast, IL-27R knockout mice displayed deterioration of inflammation in autoimmune encephalomyelitis models.[20 22] However, at present, our understanding of the role of IL-27 in SSc is limited. The objective of this study is therefore to assess the role of IL-27 in SSc.

PATIENTS AND METHODS

Serum samples

Serum samples were obtained from 91 Japanese patients with SSc (81 women and 10 men) at the time of diagnosis. All patients fulfilled the criteria proposed by the American College of Rheumatology.[23] 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.[24–26] Patients were grouped according to the classification system proposed by LeRoy *et al*[27]: 46 patients (44 women and 2 men) had limited cutaneous SSc (lSSc) and 45 patients (37 women

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