Table 1 Clinical features of ECDS patients

Case	Sex	Age	Disease duration	Clinical manifestations							Location	Progression	Central nervous system involvement		
				Erythema	Hyper-pigmentation	Hypo-pigmentation	Atrophy	Sclerosis	Depression	Hair loss			Clinical symptoms	Neuroimaging abnormalities	Abnormal EEG
1	F	59	6 months	+	–	–	–	–	+	+	Forehead	+	–	–	N.A.
2	M	23	2 months	+	–	–	+	–	–	+	Parietal	+	–	N.A.	N.A.
3	F	34	2.5 years	+	–	–	–	–	–	+	Parietal	+	–	–	N.A.
4	F	32	7 years	–	+	–	–	+	+	–	Forehead	+	Head ache	–	N.A.
5	F	38	6 years	–	–	+	–	+	+	+	Temple	+	Head ache	–	–
6	F	53	7 years	–	+	–	+	+	+	+	Parietal	+	–	Intracranial calcification	+
7	F	16	2 months	+	–	–	+	–	–	–	Forehead	+	–	–	–
8	F	46	8 years	–	+	–	–	–	–	+	Forehead	+	–	–	+
9	F	11	1 year	–	–	+	–	–	–	–	Forehead	+	–	–	N.A.
10	F	42	7 years	–	–	+	+	+	+	–	Parietal	–	–	–	–
11	F	21	1.5 years	–	+	–	+	+	+	–	Forehead	+	–	–	–
12	M	14	2 months	+	–	–	–	–	–	–	Forehead	+	–	–	–
13	M	9	1 year	–	+	–	+	–	–	–	Forehead	+	–	–	–
14	F	10	6 months	+	–	–	–	–	–	–	Forehead	+	–	N.A.	N.A.
15	F	17	1 years	–	+	–	+	+	+	+	Forehead	+	–	Intracranial calcification	+
16	F	15	8 years	–	–	+	+	+	+	+	Forehead	+	–	–	N.A.

M, male; F, female; +, present; –, absent; N.A., not assessed; EEG, electroencephalogram.

Table 2 Result of histological evaluation of ECDS patients

Case	Early or late	Epidermal atrophy changes	Spongiosis	Vacuolar changes	Satellite cell necrosis	Basal pigmentation	Melanin incontinence	Perivascular infiltrate	Perineural infiltrate	Periappendageal infiltrate	Vacuolar changes within hair follicles	Dermal fibrosis
1	E	–	+	+	–	++	+	+++	+	++	++	+
2	E	–	+++	++	–	++	–	++	+	++	+++	+++
3	E	–	+	++	–	+	+	++	+	++	–	++
4	L	–	++	++	–	+++	++	+	–	–	+	++
5	L	+	+	+	–	++	++	+	–	–	–	++
6	L	+	+	++	–	+	–	+	–	+	–	+++
7	E	–	+	+	–	–	–	++	+	–	–	++
8	L	+	+	+	–	+++	++	+	–	–	+	+
9	E	+	+	++	+	–	–	+	++	++	++	+++
10	L	+	+	+	–	–	–	+	–	–	–	++
11	E	+	+	+	–	++	+	++	++	++	++	++
12	E	–	++	+	–	++	+	+++	++	+++	+++	++
13	E	–	+	++	–	+++	+++	++	+	+	+++	++
14	E	–	+	++	+	+++	+++	+++	+++	+++	+++	++
15	E	–	+	+	–	+++	+	+	–	++	+++	+++
16	L	+	+	+	–	–	+	+	+	+	–	++

E, early; L, late; –, none; +, mild; ++, moderate; +++, severe.

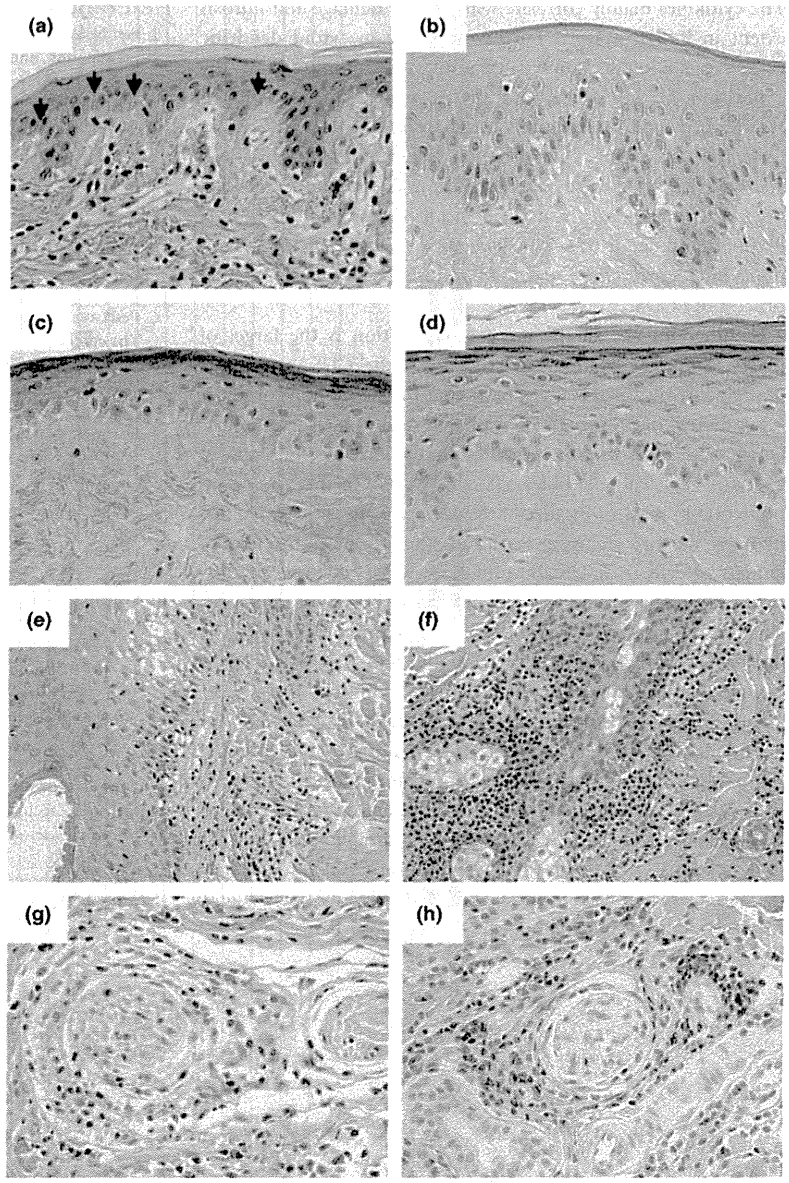

Figure 1 Histopathological features of ECDS (a) Moderate vacuolar changes with necrotic keratinocytes (shown with arrows) and melanin incontinence were seen in early ECDS (Case 14). (b) Moderate vacuolar changes with melanin incontinence were seen in late ECDS (Case 4). (c, d) Moderate vacuolar changes were seen in early ECDS (c, Case 9) and late ECDS (d, Case 6). (e, f) Severe vacuolar changes were seen within the epithelium of hair follicles accompanied with perifollicular lymphocytic infiltrate in early ECDS (e, Case 2; f, Case 14). (g, h) Moderate to severe perineural lymphocytic infiltrate with scattered plasma cells was observed in early ECDS (g, Case 9; h, Case 14). (haematoxylin-eosin stain, original magnification × 400).

atrophy was less frequently seen, in early ECDS patients than in late ECDS patients. Importantly, the intensity of interface dermatitis in epidermis was comparable between early and late ECDS lesions. Although the only characteristic histological finding in early stage localized scleroderma reported so far is perivascular lymphocytic infiltrate,[1–3] the present observations suggest that vacuolar changes in epidermis is a common histological feature in lesional skin of ECDS patients and vacuolar changes in follicular epithelium and periappendageal infiltrate serve as a histological marker of early and active ECDS lesions in addition to perivascular infiltrate.

Although the pathogenesis of localized scleroderma still remains unknown, the present observation that interface dermatitis is a common histological feature in ECDS provides us a new clue to further speculate the developmental process of this disorder. A wealth of evidence has suggested that a number of interface dermatitis skin disorders share a common inflammatory signalling pathway involving the actions of plasmacytoid dendritic cell-derived interferon-α,[7,8] which induces the polarization towards Th1 and Th17 immune responses.[9] Being still controversial, it is generally accepted that Th1 and Th17 cytokines are elevated during the early stage of localized scleroderma, whereas

Th2 cytokines mainly correlate with disease damage and fibrosis extent in its late stage.[10] Furthermore, plasmacytoid dendritic cells are abundant among inflammatory infiltrate and the expression of myxovirus protein A, which is produced in response to type I interferons, are increased around inflammatory cells, including epidermis, in lesional skin of localized scleroderma.[11] Taken together, these present and previous data suggest that the local tissue production of type I interferons by plasmacytoid dendritic cells, which skew Th1 and Th17 polarization, induces interface dermatitis during the developmental process of ECDS.

Another interest of the present observation is the target of injury in interface dermatitis associated with ECDS. An important clue to answer this question is that the skin lesions of linear scleroderma including ECDS generally follow Blaschko's lines which represent the pathways of embryonic cell development of the skin, especially of epidermal cells.[12,13] Given that the developmental process of localized scleroderma is possibly explained by tissue damage and resultant fibrosis due to the elimination of epidermal cells with somatic mutation, which theoretically distribute along Blaschko's lines, by immune surveillance, it is plausible that interface dermatitis is a common histological characteristic in ECDS. Supporting this idea, the episodes of trauma and vaccination, which activate immune surveillance, are often seen as a trigger of the onset in ECDS patients.[14,15]

In summary, this is the first study reporting that interface dermatitis is a common histological feature in ECDS and perivascular and/or periappendageal infiltrate and vacuolar changes in follicular epithelium serve as a histological marker of early and active ECDS lesions. These results are useful to determine the activity of skin lesions in ECDS and provide us a new clue to further understand the pathogenesis of this disorder.

References

1 Fett N, Werth VP. Update on morphea: part I. Epidemiology, clinical presentation, and pathogenesis. *J Am Acad Dermatol* 2011; **64**: 217–228.
2 McNiff JM, Glusac EJ, Lazova RZ, Carroll CB. Morphea limited to the superficial reticular dermis: an underrecognized histologic phenomenon. *Am J Dermatopathol* 1999; **21**: 315–319.
3 Falanga V. Localized scleroderma. *Med Clin North Am* 1989; **73**: 1143–1156.
4 Peterson LS, Nelson AM, Su WP. Classification of morphea (localized scleroderma). *Mayo Clin Proc* 1995; **70**: 1068–1076.
5 Succaria F, Kurban M, Kibbi AG, Abbas O. Clinicopathological study of 81 cases of localized and systemic scleroderma. *J Eur Acad Dermatol Venereol* 2013; **27**: e191–e196.
6 Peterson LS, Nelson AM, Su WP, Mason T, O'Fallon WM, Gabriel SE. The epidemiology of morphea (localized scleroderma) in Olmsted County 1960–1993. *J Rheumatol* 1997; **24**: 73–80.
7 Wenzel J, Tüting T. An IFN-associated cytotoxic cellular immune response against viral, self-, or tumor antigens is a common pathogenetic feature in "interface dermatitis". *J Invest Dermatol* 2008; **128**: 2392–2402.
8 Sontheimer RD. Lichenoid tissue reaction/interface dermatitis: clinical and histological perspectives. *J Invest Dermatol* 2009; **129**: 1088–1099.
9 Santini SM, Lapenta C, Donati S, Spadaro F, Belardelli F, Ferrantini M. Interferon-α-conditioned human monocytes combine a Th1-orienting attitude with the induction of autologous Th17 responses: role of IL-23 and IL-12. *PLoS ONE* 2011; **6**: e17364.
10 Kurzinski K, Torok KS. Cytokine profiles in localized scleroderma and relationship to clinical features. *Cytokine* 2011; **55**: 157–164.
11 Ghoreishi M. Vera Kellet C, Dutz JP. Type 1 IFN-induced protein MxA and plasmacytoid dendritic cells in lesions of morphea. *Exp Dermatol* 2012; **21**: 417–419.
12 Bolognia JL, Orlow SJ, Glick SA. Lines of Blaschko. *J Am Acad Dermatol* 1994; **31**: 157–190.
13 Weibel L, Harper JI. Linear morphoea follows Blaschko's lines. *Br J Dermatol* 2008; **159**: 175–181.
14 Christen-Zaech S, Hakim MD, Afsar FS, Paller AS. Pediatric morphea (localized scleroderma): review of 136 patients. *J Am Acad Dermatol* 2008; **59**: 385–396.
15 Zulian F, Athreya BH, Laxer R *et al.* Juvenile localized scleroderma: clinical and epidemiological features in 750 children. An International Study.. *Rheumatology (Oxford)* 2006; **45**: 614–620.

Japan College of Rheumatology
MODERN RHEUMATOLOGY

http://informahealthcare.com/mor
ISSN 1439-7595 (print), 1439-7609 (online)

Mod Rheumatol, 2014; Early Online: 1–4

DOI: 10.3109/14397595.2014.885377

informa
healthcare

CASE REPORT

Effect of ambrisentan on peripheral circulation in patients with systemic sclerosis

Hayakazu Sumida[1], Yoshihide Asano[1], Masaru Hatano[2], Naohiko Aozasa[1], Tetsuo Toyama[1], Kaname Akamata[1], Miki Miyazaki[1], Takashi Taniguchi[1], Takehiro Takahashi[1], Yohei Ichimura[1], Shinji Noda[1], Yoshihiro Kuwano[1], Koichi Yanaba[1], and Shinichi Sato[1]

[1]*Department of Dermatology, Faculty of Medicine, The University of Tokyo, Tokyo, Japan and* [2]*Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*

Abstract

Systemic sclerosis (SSc) is characterized by disturbed blood circulation. The effect of ambrisentan, an endothelin-A receptor-selective antagonist, on impaired peripheral circulation in SSc remains largely elusive. Here we show SSc patients, whose clinical symptoms such as cyanosis and Raynaud's phenomenon, were ameliorated by the treatment with ambrisentan. Additionally, objective evaluations with thermography showed improvement of hand coldness in steady-state and cold challenge tests. Ambrisentan might have a potential to improve peripheral circulation in SSc.

Keywords

Ambrisentan, Cyanosis, Raynaud's phenomenon, Systemic sclerosis, Thermography

History

Received 17 December 2013
Accepted 16 January 2014
Published online 24 February 2014

Introduction

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by initial vascular injuries and resultant fibrosis of the skin and certain internal organs. In particular, vascular damages link to impaired peripheral circulation including cyanosis of the fingers, Raynaud's phenomenon (RP), and digital ulcers (DUs) [1]. In the pathology of SSc, vascular changes are classified into two subgroups: destructive vasculopathy and proliferative obliterative vasculopathy [2]. Destructive vasculopathy contributes to progressive loss of capillaries and impaired angiogenesis; whereas, proliferative obliterative vasculopathy is characterized by the proliferation of vascular smooth muscle cells and resultant intravascular stenosis in arterioles and arteries [3].

Increased serum levels of endothelin-1 (ET-1) were reported to be well correlated with the extent of vascular damage in SSc [4]. In addition, ET-1 exerts its major vascular effects of vasoconstriction and cell proliferation through ET_A and ET_B receptors on vascular smooth muscle cells [5]. Therefore, endothelin receptor antagonists (ETRAs) have been thought to be effective for SSc-related proliferative obliterative vasculopathy including SSc-associated pulmonary arterial hypertension (SSc-PAH). In addition to prostanoids and phosphodiesterase type 5 (PDE-5) inhibitors, ETRAs have also been shown to improve not only idiopathic PAH but also SSc-PAH [1].

Two randomized controlled trials demonstrated that bosentan, a dual ET_A/ET_B receptor antagonist, reduced the appearance of new DUs in SSc patients [6,7]. Probably, these successes of bosentan might be due to not only its vasodilatory effects but also its reverse remodeling effects, which are not shared by prostanoids or PDE-5 inhibitors. To further complicate matters, endothelial ET_B receptor alone has an opposing role in vasodilation [5]. Therefore, selective ET_A receptor antagonists have been expected to exert beneficial effects especially on vasodilatation.

Ambrisentan, an ET_A selective antagonist, has recently proven effective in SSc-PAH [8]. Given few data on the use of ambrisentan for SSc patients [8,9], it remains largely unknown whether ambrisentan would show efficacy as well as bosentan in the SSc-related impaired peripheral circulation.

Patients and methods

Patients

Among eight patients starting ambrisentan for PAH between October 2011 and March 2013, three SSc patients with severe cyanosis and RP in the distal extremities were selected. PAH was diagnosed using ultrasound cardiography and/or right ventricular catheterization. Patients were treated with ambrisentan 5 mg daily. Concomitant medications were not substantially modified during the follow-up period.

Thermographic examination

Thermographic examination was performed using thermotracer (TH5100, NEC, Japan). In steady-state tests, the finger temperature was measured in an air conditioned room at 25°C and 60% relative humidity. After 15 minutes of habituation, the thermographic images of the dorsal and ventral sides of hands were taken. In cold challenge tests, hands were immersed in water at 10°C for 60 s after the equilibration phase of 25°C for 15 min. Immediately after the hands were taken out of the water, thermographic images were recorded at 5-min intervals over 20 min.

Results

Clinical features and information of all three patients are summarized in Table 1. Efficacy of ambrisentan for impaired peripheral

Correspondence to: Yoshihide Asano, MD, PhD, Department of Dermatology, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655 Japan. Tel: +81 3 5800 8661. Fax: +81 3 3814 1503. E-mail: yasano-tky@umin.ac.jp

Table 1. Clinical features and information of the patients treated with ambrisentan.

Patient	Patient 1	Patient 2	Patient 3
Age (years)/Gender	75/F	61/F	68/F
Disease duration (years)	6	15	3
Type of SSc	Diffuse	Diffuse	Diffuse
Specific antibodies	ssDNA, Ro/SS-A	Topo-I	Topo-I, ssDNA
Cyanosis of the distal extremities	+	+	+
Raynaud's phenomenon	+	+	+
Digital pitting scars and/or ulcers	+	+	+
Efficacy of ambrisentan for impaired peripheral circulation	+	+	+
Concomitant drugs aimed at improving peripheral circulation	Beraprost sodium, Sarpogrelate hydrochloride, Tocopherol nicotinate, Ethyl icosapentate	Beraprost sodium, Sarpogrelate hydrochloride, Limaprost alfadex, Tocopherol nicotinate	Sarpogrelate hydrochloride, Tocopherol nicotinate

F, Female; SSc, systemic sclerosis; ssDNA, anti-single-stranded DNA antibody; Topo-I, anti-topoisomerase I antibody.

circulation was observed in all patients, as described in detail below. Although all patients had interstitial lung disease (ILD), deterioration of respiratory manifestation and ILD parameters were not found during observation periods. Ambrisentan did not affect the serum levels of C-reactive protein, immunoglobulin G, and anti-topoisomerase I antibodies.

Patient 1

A 75-year-old woman with a 6-year history of SSc had suffered from RP with triphasic color changes and small pitting scars on her fingers from 74 years. High levels of antibodies to single-stranded DNA and Ro/SS-A were detected in her laboratory examinations. Within one week after the start of treatment with ambrisentan, her cyanosis of the fingers was promptly improved (Figure 1a, b). To provide objective evaluations, we conducted thermographic examination, since thermography shows promise as a diagnostic tool for cyanosis or RP in the distal extremities [10]. The steady-state tests showed the increase in finger temperature after starting the therapy with ambrisentan (Figure 1c, d). Furthermore, cold challenge tests showed that ambrisentan treatment markedly reduced recovery time of finger temperature (Figure 1e, f).

Patient 2

A 61-year-old woman with a 15-year history of SSc had suffered from severe RP with biphasic color changes and recurrent DUs for 15 years. Antibodies to topoisomerase I was detected in her laboratory examinations. Within two weeks after the treatment with ambrisentan, the degree of cyanosis of the fingers was improved (Figure 1g, h). Moreover, DUs also showed healing tendency. She showed slight and controllable edema on the lower leg, which is known to be a frequent side effect of ambrisentan [11].

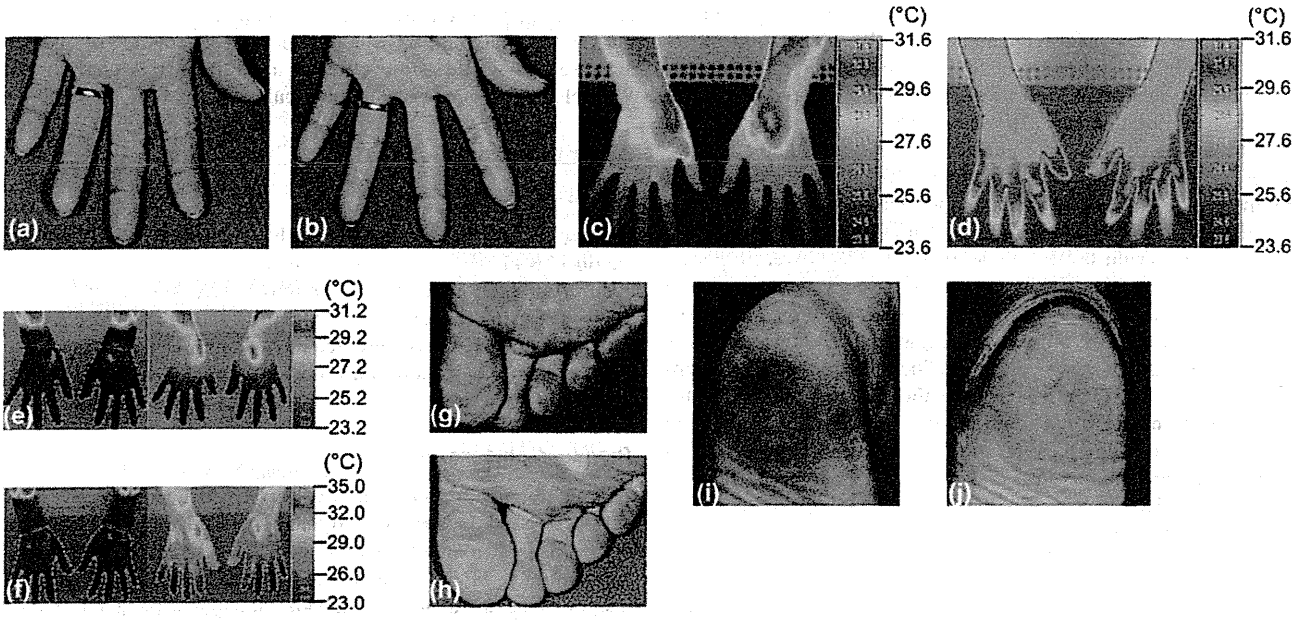

Figure 1. Clinical course of peripheral vascular involvement in SSc patients treated with ambrisentan. (a and b; Patient 1) Raynaud's phenomenon (RP) in the left hands before (a) and after (b) the administration of ambrisentan. (c and d; Patient 1) Thermographic images of the dorsal sides of both hands before (c) and after (d) starting the therapy with ambrisentan at room temperature condition (25°C and 60% relative humidity for 15 min). Median temperature, 27.6°C. Thermal sensitivity (temperature resolution), 1.0°C. (e; Patient 1 before the treatment with ambrisentan) Thermographic images of the dorsal sides of both hands just after the immersion in water at 10°C for 60 s (left image) and after the following 20-min exposure to an room temperature condition (right image). Median temperature, 27.2°C. Thermal sensitivity (temperature resolution), 1.0°C. (f; Patient 1 after the treatment with ambrisentan) Thermographic images as is the case before the treatment. Median temperature, 29.0°C. Thermal sensitivity (temperature resolution), 1.5°C. (g and h; Patient 2) RP in the right toes before (g) and after (h) the administration of ambrisentan. (i and j; Patient 3) RP in the right heels before (i) and after (j) the administration of ambrisentan.

Patient 3

A 68-year-old woman with a 3-year history of SSc had severe cyanosis and RP in the distal extremities. Antibodies to topoisomerase I and single-stranded DNA were detected in her laboratory data. Within two or three weeks after starting treatment with ambrisentan, the degree of cyanosis was obviously improved (Figure 1i, j). Furthermore, the pain of lower legs probably due to the blood flow disturbance was also attenuated.

Discussion

The current study demonstrated that an ET_A-selective antagonist was useful for the improvement of peripheral circulation in SSc patients. Specifically, peripheral vascular involvement manifesting as peripheral cyanosis or RP was attenuated after the administration of ambrisentan. ETRAs have short-term vasodilator and long-term reverse vascular remodeling effects [12]. In PAH animal model, positive vasodilatory effects have been observed in both selective ET_A-receptor and nonselective antagonism [13]. Furthermore, several studies in animal models revealed that both nonselective and ET_A-selective blockade attenuated vascular remodeling [14,15]. Considering these evidences and the fact that peripheral vasculopathy is attributed to constriction and vascular remodeling in SSc patients, the vasodilator and reverse vascular remodeling effects of ETRAs might have a great influence on our observations. In particular, our patients showed the amelioration of cyanosis of the fingers soon after starting the treatment with ambrisentan. Therefore, the short-term vasodilator mechanism must be greatly involved in the improvement of impaired vascular perfusion in our patients. Furthermore, thermographic images from Patient 1 showed improved peripheral vascular perfusion by ambrisentan both in steady-state and under cold challenge tests. Steady-state and cold challenge tests are suitable for the evaluation of cyanosis of the distal extremities without attack and RP with attack, respectively. RP is mainly induced by vasoconstriction; whereas, cyanosis is closely related to vascular remodeling in addition to vasoconstriction. Therefore, the results of this study suggest that ambrisentan has both reverse remodeling and vasodilator effects.

Clinical efficacy of ambrisentan has been reported for the symptoms such as PAH and DUs in SSc [8,9]. In addition to these symptoms, this report suggests the effects of ambrisentan on cyanosis and RP. These all clinical symptoms were considered to be caused by proliferative obliterative vasculopathy. Moreover, reverse vascular remodeling effects of ETRAs are exerted by restoring proliferative vascular changes [5]. Therefore, ETRAs including ambrisentan may achieve those clinical utilities via reverse vascular remodeling effects.

In terms of safety of ambrisentan, systemic vasodilators have been shown to interfere with ventilation/perfusion matching in fibrotic lung disease [16]. Therefore, there is a concern that ETRAs may have similar disadvantageous effects on gas exchange. Recently, a study of patients with PAH and ILD has reported very small incidence of worsening ventilation/perfusion mismatch [17]. Similarly, deterioration of respiratory manifestation and ILD parameters were not found in our all three patients with SSc-related ILD. Meanwhile, a recent double-blind study of ambrisentan for idiopathic pulmonary fibrosis, not SSc-related ILD, showed the possibility that ambrisentan might increase the risk for disease progression [18]. Therefore, it must be still important to keep in mind the possibility of ILD exacerbation in the use of ETRAs for SSc patients. For more discussion about side effects, compared with bosentan, ambrisentan appears to have a better safety profile with regards to hepatic dysfunction and drug–drug interactions [19]. Therefore, ambrisentan is a viable option for SSc patients, who have liver damage resulting from bosentan or other reasons. However, ambrisentan shows a higher rate of other adverse events, such as peripheral edema [11]. It is consistent that one of our patients (Patient 2) showed edema on the legs. Therefore, the use of ambrisentan for SSc patients requires special care for the presence of edema.

We herein reported three SSc patients with severely impaired vascular perfusion, which was attenuated by ambrisentan probably due to its vasodilator and reverse remodeling effect. The current observation strongly supports the possibility that ambrisentan might be useful for the treatment of impaired peripheral circulation leading to RP or DUs in SSc patients.

Acknowledgments

None.

Conflicts of interest

None.

References

1. Asano Y. Future treatments in systemic sclerosis. J Dermatol. 2010;37(1):54–70.
2. Horn A, Distler JH. Vascular alterations upon activation of TGFbeta signaling in fibroblasts–implications for systemic sclerosis. Arthritis Res Ther. 2010;12(3):125.
3. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med. 2009;360(19):1989–2003.
4. Vancheeswaran R, Magoulas T, Efrat G, Wheeler-Jones C, Olsen I, Penny R, Black CM. Circulating endothelin-1 levels in systemic sclerosis subsets–a marker of fibrosis or vascular dysfunction? J Rheumatol. 1994;21(10):1838–44.
5. Spieker LE, Noll G, Ruschitzka FT, Luscher TF. Endothelin receptor antagonists in congestive heart failure: a new therapeutic principle for the future? J Am Coll Cardiol. 2001;37(6):1493–505.
6. Korn JH, Mayes M, Matucci Cerinic M, Rainisio M, Pope J, Hachulla E, et al. Digital ulcers in systemic sclerosis: prevention by treatment with bosentan, an oral endothelin receptor antagonist. Arthritis Rheum. 2004;50(12):3985–93.
7. Matucci-Cerinic M, Denton CP, Furst DE, Mayes MD, Hsu VM, Carpentier P, et al. Bosentan treatment of digital ulcers related to systemic sclerosis: results from the RAPIDS-2 randomised, double-blind, placebo-controlled trial. Ann Rheum Dis. 2011;70(1):32–8.
8. Saggar R, Khanna D, Shapiro S, Furst DE, Maranian P, Clements P, et al. Brief report: effect of ambrisentan treatment on exercise-induced pulmonary hypertension in systemic sclerosis: a prospective single-center, open-label pilot study. Arthritis Rheum. 2012;64(12):4072–7.
9. Parisi S, Peroni CL, Lagana A, Scarati M, Ambrogio F, Bruzzone M, Fusaro E. Efficacy of ambrisentan in the treatment of digital ulcers in patients with systemic sclerosis: a preliminary study. Rheumatology (Oxford). 2013;52(6):1142–4.
10. O'Reilly D, Taylor L, el-Hadidy K, Jayson MI. Measurement of cold challenge responses in primary Raynaud's phenomenon and Raynaud's phenomenon associated with systemic sclerosis. Ann Rheum Dis. 1992;51(11):1193–6.
11. Shapiro S, Pollock DM, Gillies H, Henig N, Allard M, Blair C, et al. Frequency of edema in patients with pulmonary arterial hypertension receiving ambrisentan. Am J Cardiol. 2012;110(9):1373–7.
12. Opitz CF, Ewert R, Kirch W, Pittrow D. Inhibition of endothelin receptors in the treatment of pulmonary arterial hypertension: does selectivity matter? Eur Heart J. 2008;29(16):1936–48.
13. Opitz CF, Ewert R. Dual ET(A)/ET(B) vs. selective ET(A) endothelin receptor antagonism in patients with pulmonary hypertension. Eur J Clin Invest. 2006;36 Suppl 3:1–9.
14. Chen SJ, Chen YF, Meng QC, Durand J, Dicarlo VS, Oparil S. Endothelin-receptor antagonist bosentan prevents and reverses hypoxic pulmonary hypertension in rats. J Appl Physiol. 1995;79(6):2122–31.
15. Tilton RG, Munsch CL, Sherwood SJ, Chen SJ, Chen YF, Wu C, et al. Attenuation of pulmonary vascular hypertension and cardiac hypertrophy with sitaxsentan sodium, an orally active ET(A) receptor antagonist. Pulm Pharmacol Ther. 2000;13(2):87–97.

16. Olschewski H, Ghofrani HA, Walmrath D, Schermuly R, Temmesfeld-Wollbruck B, Grimminger F, Seeger W. Inhaled prostacyclin and iloprost in severe pulmonary hypertension secondary to lung fibrosis. Am J Respir Crit Care Med. 1999;160(2):600–7.
17. Gunther A, Enke B, Markart P, Hammerl P, Morr H, Behr J, et al. Safety and tolerability of bosentan in idiopathic pulmonary fibrosis: an open label study. Eur Respir J. 2007;29(4):713–9.
18. Raghu G, Behr J, Brown KK, Egan JJ, Kawut SM, Flaherty KR, et al. Treatment of idiopathic pulmonary fibrosis with ambrisentan: a parallel, randomized trial. Ann Intern Med. 2013;158(9):641–9.
19. Vizza CD, Fedele F, Pezzuto B, Rubin LJ. Safety and efficacy evaluation of ambrisentan in pulmonary hypertension. Expert Opin Drug Saf. 2012;11(6):1003–11.

International Journal of
Rheumatic Diseases

International Journal of Rheumatic Diseases 2014

ORIGINAL ARTICLE

Circulating galectin-1 concentrations in systemic sclerosis: potential contribution to digital vasculopathy

Koichi YANABA, Yoshihide ASANO, Kaname AKAMATA, Shinji NODA, Naohiko AOZASA, Takashi TANIGUCHI, Takehiro TAKAHASHI, Tetsuo TOYAMA, Yohei ICHIMURA, Hayakazu SUMIDA, Yoshihiro KUWANO, Miki MIYAZAKI and Shinichi SATO

Department of Dermatology, Faculty of Medicine, University of Tokyo, Tokyo, Japan

Abstract

Aim: To determine serum galectin-1 levels and their clinical associations in patients with systemic sclerosis (SSc).

Method: Serum galectin-1 levels were examined by enzyme-linked immunosorbent assay in 66 patients with SSc and 24 healthy individuals.

Results: No significant differences were observed in serum galectin-1 levels between patients with SSc (9.4 ± 5.6 ng/mL), and healthy individuals (8.9 ± 1.3 ng/mL). Among patients with SSc, no significant differences were seen in serum galectin-1 levels between those with diffuse cutaneous SSc (8.8 ± 5.7 ng/mL; $n = 31$) and those with limited cutaneous SSc (10.0 ± 5.4 ng/mL; $n = 35$). Patients with SSc who had increased galectin-1 levels less often had pitting scars/digital ulcers than those with normal galectin-1 levels (17% *vs.* 49%; $P < 0.01$). Consistently, galectin-1 levels were significantly lower in SSc patients with pitting scars/digital ulcers than in those without pitting scars/digital ulcers (6.9 ± 4.8 *vs.* 10.9 ± 5.5 ng/mL; $P < 0.01$).

Conclusion: These results suggest that galectin-1 is a protective factor against the development of digital vasculopathy in SSc. In addition, measurement of serum galectin-1 levels may be useful for risk stratification for the development of digital vasculopathy in the early phase of SSc.

Key words: digital ulcer, ELISA, galectin-1, systemic sclerosis, vasculopathy.

INTRODUCTION

Systemic sclerosis (SSc) is a heterogeneous disorder characterized by microvascular damage and excessive fibrosis in the skin and various internal organs. Although the pathogenesis of SSc remains unknown, microangiopathies, including the activation and damage of endothelial cells, precede the development of fibrosis.[1] Microangiopathies underlie numerous manifestations of SSc, such as digital ulcers, scleroderma renal crisis and pulmonary arterial hypertension.

Galectins are a family of β-galactoside-binding lectins, which are highly conserved throughout animal species, while 15 members of galectins have been identified thus far.[2] Among them, both galectin-1 and galectin-3 are co-expressed in a variety of tissues, including the thymus and lymph nodes,[3] and bind to a number of glycoproteins, including CD29, CD43 and CD45 on T cells,[4] and to extracellular matrix proteins, such as laminin, fibronectin and vitronectin.[3,5] It has been suggested that galectin-1 has an immune regulatory effect. Binding of galectin-1 induces apoptosis of immature thymocytes[4] and activated T cells but not resting T cells.[6,7] The administration of recombinant galectin-1 reduces

Correspondence: Dr Koichi Yanaba, Department of Dermatology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
Email: yanaba@jikei.ac.jp

disease severity in animal models of rheumatoid arthritis,[8] systemic lupus erythematosus,[9] multiple sclerosis,[10] graft versus host disease,[11] type-1 diabetes[12] and inflammatory bowel disease.[13] Galectin-1 deficiency enhances inflammation in a mouse model of multiple sclerosis.[2] Moreover, serum galectin-3 levels are increased in SSc patients with digital ulcers and elevated right ventricular systolic pressure.[14] Thus, we hypothesized that galectin-1 may also play a role in the pathogenesis of SSc. Therefore, we examined the levels of serum galectin-1 in patients with SSc and evaluated the results with respect to clinical features of the disease.

METHODS

Patients

Serum samples were obtained from 66 Japanese patients with SSc (59 women and deven men) with a mean age of 52 years (range 12–77) on their first visit. All patients fulfilled criteria for SSc proposed by the American College of Rheumatology.[15] Patients were classified[16] into diffuse cutaneous SSc (dcSSc; n = 31) and limited cutaneous SSc (lcSSc; n = 35) groups. Overall, 24 patients were positive for anti-topoisomerase I antibodies, 30 for anticentromere antibodies, seven for anti-RNA polymerase I and III antibodies, one for anti-U1RNP (ribonucleoprotein) antibodies and two for anti-U3RNP antibodies. Two patients were negative for all antibodies. The mean disease duration was 5.8 ± 6.0 years (range 0.5–27). Duration was calculated from the time of onset of the first clinical event (other than Raynaud's phenomenon) that was a clear manifestation of SSc. None of the patients had received corticosteroids or other immunosuppressants. A further 24 age- and sex-matched healthy Japanese individuals were included as healthy controls.

Clinical assessment

Complete medical histories, physical examinations and laboratory tests were conducted for all patients at their first visit. Organ system involvement was defined as previously described[17,18]: lung, bibasilar fibrosis on chest radiography and high-resolution computed tomography (CT) scan; esophagus, hypomotility shown by barium radiography; heart, pericarditis, congestive heart failure or arrhythmias requiring treatment; kidney, malignant hypertension and rapidly progressive renal failure with no other explanation; joint, inflammatory polyarthralgias or arthritis; and muscle, proximal muscle weakness and elevated serum creatine kinase. Interstitial lung disease was defined as bibasilar interstitial fibrosis on chest high-resolution CT scan. In addition, a pulmonary function test, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLco), was also evaluated to examine the severity of interstitial lung disease. When DLco and VC were < 75% and < 80%, respectively, of the predicted normal values, they were considered to be abnormal.

Patients with SSc who were smokers or who had respiratory disorders that could have affected DLco or VC were excluded from this study. Pulmonary artery pressure was estimated by Doppler echocardiogram. The modified Rodnan total skin thickness score was measured by summing the skin thickness measurements, and was determined by palpation on a scale of 0–3 in 17 body areas.[19] The protocol was approved by The University of Tokyo and The University of Tokyo Hospital, Tokyo, Japan and informed consent was obtained from all patients.

Measurement of serum galectin-1 levels

Fresh venous blood samples were drawn into pyrogen-free blood collection tubes without additives, immediately immersed in melting ice, and allowed to clot for 1 h before centrifugation. All serum samples were stored at −70°C before use. Serum galectin-1 levels were measured using a specific enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis,

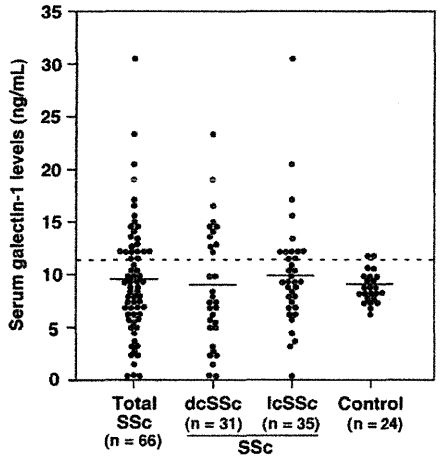

Figure 1 Serum galectin-1 levels in patients with systemic sclerosis (SSc), diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc) and in healthy individuals (controls). Serum galectin-1 levels were determined using a specific enzyme-linked immunosorbent assay. Bars indicate mean values in each group. Dashed line indicates the cut-off value (mean + 2 SD of control values).

MN, USA), according to the manufacturer's protocol. Each sample was tested in duplicate. The detection limit of this assay was 0.022 ng/mL.

Statistical analysis

The Kruskal–Wallis test was used to compare galectin-1 levels, Fisher's exact probability test to compare frequency, and Bonferroni's test for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between continuous variables. A P-value < 0.05 was considered significant. All data are presented as mean ± standard deviation (SD) values.

RESULTS

Serum galectin-1 levels in patients with SSc

The mean serum galectin-1 levels at the first visit were comparable between patients with SSc (9.4 ± 5.6 ng/mL) and healthy individuals (8.9 ± 1.3 ng/mL; Fig. 1). Among SSc subgroups, there was no significant difference in serum galectin-1 levels between patients with dcSSc (8.8 ± 5.7 ng/mL) and lcSSc (10.0 ± 5.4 ng/mL) (Fig. 1). Thus, serum galectin-1 levels were not significantly increased in patients with SSc.

Correlation between clinical characteristics and serum galectin-1 levels

Clinical and laboratory parameters evaluated at the first visit were compared between patients with elevated galectin-1 levels and those with normal galectin-1 levels. Values higher than the mean + 2 SD (11.6 ng/mL) of the healthy control serum samples were considered to be elevated in this study. Elevated galectin-1 levels were observed in 35% (23/66) of all patients with SSc, 35% (11/31) of patients with dcSSc and 34% (12/35) of patients with lcSSc. The prevalence of pitting scars/

Table 1 Clinical and laboratory findings in patients with SSc showing elevated serum galectin-1 levels on the initial visit

	Elevated galectin-1 $n = 23$	Normal galectin-1 $n = 43$	Odds ratio (95% CI)	Relative risk (95% CI)
Age at onset, years (mean ± SD)	46 ± 15	49 ± 19		
Male : female	2 : 21	5 : 38		
Duration, years (mean ± SD)	5.5 ± 6.3	5.9 ± 5.9		
MRSS, points (mean ± SD)	10.2 ± 8.0	11.7 ± 10.6		
Clinical features %				
dcSSc	48	47	0.95 (0.34–2.61)	0.98 (0.60–1.58)
lcSSc	52	53	1.05 (0.38–2.91)	1.04 (0.53–2.00)
Pitting scars/digital ulcers	17*	49	4.53 (1.32–15.6)	2.90 (1.11–7.54)
Contracture of phalanges	39	47	1.35 (0.48–3.79)	1.22 (0.62–2.41)
Diffuse pigmentation	43	40	0.85 (0.30–2.37)	0.90 (0.46–1.75)
Telangiectasia	39	42	1.12 (0.40–3.15)	1.08 (0.55–2.12)
Organ involvement %				
Interstitial lung disease	35	42	1.35 (0.47–3.86)	1.22 (0.60–2.46)
Decreased %VC	22	16	0.70 (0.19–2.52)	0.80 (0.37–1.73)
Decreased %DLco	74	58	0.49 (0.16–1.49)	0.62 (0.28–1.35)
Pulmonary hypertension	13	7	0.50 (0.09–2.71)	0.67 (0.28–1.60)
Esophagus	65	74	1.55 (0.52–4.65)	1.32 (0.67–2.59)
Heart	9	12	1.38 (0.25–7.75)	1.25 (0.37–4.22)
Kidney	4	2	0.52 (0.03–8.79)	0.69 (0.17–2.86)
Joint	17	35	2.55 (0.73–8.86)	1.92 (0.75–4.90)
Muscle	13	21	1.77 (0.43–7.29)	1.48 (0.52–4.19)
Laboratory findings %				
Anti-topoisomerase I antibodies	35	37	1.11 (0.39–3.20)	1.07 (0.53–2.15)
Anticentromere antibodies	52	42	0.66 (0.24–1.83)	0.76 (0.40–1.48)
Increased IgG	26	19	0.65 (0.19–2.17)	0.76 (0.37–1.57)
Elevated ESR	43	44	1.03 (0.37–2.86)	1.02 (0.52–1.98)
Elevated CRP	9	14	1.70 (0.31–9.21)	1.45 (0.42–5.01)

CI, confidence interval; MRSS, modified Rodnan total skin thickness score; dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous SSc; VC, vital capacity; DLco, diffusion capacity for carbon monoxide; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.
Unless otherwise stated, values are expressed as percentages. *$P < 0.01$ versus SSc patients with normal serum galectin-1 levels.

digital ulcers in SSc patients with elevated galectin-1 levels was lower than in those with normal galectin-1 levels (17% *vs.* 49%; $P < 0.01$; Table 1). Consistently, galectin-1 levels were significantly lower in SSc patients with pitting scars/digital ulcers (6.9 ± 4.8 ng/mL) than in those without pitting scars/digital ulcers (10.9 ± 5.5 ng/mL; $P < 0.01$; Fig. 2). Also consistent with these findings, the estimated relative risks of pitting scars/digital ulcers were increased in SSc patients with normal galectin-1 levels (odds ratio, 4.53; relative risk, 2.90). Thus, raised galectin-1 levels were associated with a lower frequency of digital vasculopathy.

DISCUSSION

To our knowledge, this is the first study to evaluate serum galectin-1 levels in patients with SSc. Although mean serum galectin-1 levels were comparable between patients with SSc and healthy individuals (Fig. 1), SSc patients with elevated galectin-1 levels had pitting scars/digital ulcers less often than those with normal galectin-1 levels (Fig. 2, Table 1). Thus, elevated galectin-1 levels may be protective against the development of digital vasculopathy in SSc.

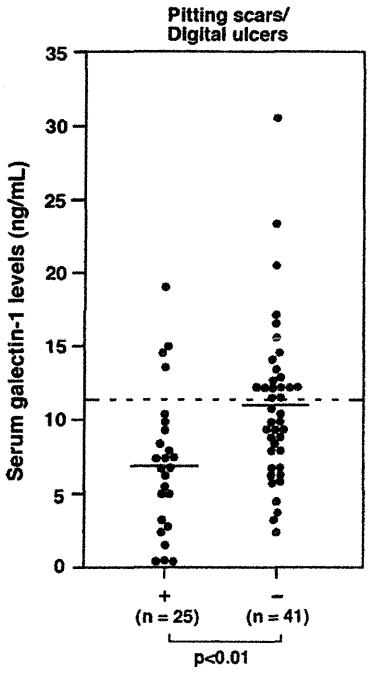

Figure 2 Serum galectin-1 levels in patients with systemic sclerosis with (+) and without (−) pitting scars/digital ulcers. Serum galectin-1 levels were determined using a specific enzyme-linked immunosorbent assay. Bars indicate mean values in each group. Dashed line indicates the cut-off value.

Oxidative stress is thought to be involved in the development of SSc.[20] Raynaud's phenomenon is observed in the majority of SSc patients,[16] and is characterized by vasospasm in response to cold or other stimuli, leading to impaired oxygenation of the distal extremities and the development of digital ulcers.[21] Ischemia–reperfusion injury following Raynaud's phenomenon can generate reactive oxygen species that induce apoptotic cell death, resulting in vascular endothelial damage.[22,23] Galectin-1 plays an important role in angiogenesis by enhancing the proliferation and migration of endothelial cells.[24] Activated endothelial cells increase galectin-1 expression and secrete soluble galectin-1 proteins into the extracellular space,[25,26] whereas galectin-1 also has the ability to protect cells from oxidative stress-induced cell apoptosis.[27] Furthermore, treatment with recombinant galectin-1 was found to be beneficial in an animal model of myocardial ischemia–reperfusion injury.[28] Collectively, these findings suggest that galectin-1 likely has a protective effect in ischemia–reperfusion injury. The results of this study showed that elevated serum galectin-1 levels were associated with a lower frequency of pitting scars/digital ulcers in patients with SSc. Considering that pitting scars/digital ulcers are a manifestation of vasculopathy of the fingers and toes, increased serum galectin-1 may suppress the development of pitting scars/digital ulcers by protecting endothelial cells from apoptosis in SSc.

There are several potential limitations of the present study that should be considered. First, the population was relatively small; a larger study is necessary to confirm our results. Second, our study did not take the clinical features of digital ulcers into account. There could be an association between the severity of digital ulcers and serum galectin-1 levels. Moreover, the severity of digital ulcers in patients with SSc shows seasonal fluctuation. Therefore, it is essential to investigate the longitudinal changes of serum galectin-1 levels in patients with SSc and assess the association with disease activity and severity of digital ulcers in future studies. Third, it is unclear whether the raised galectin-1 levels were specific for SSc patients with digital ulcers. Therefore, it is required to assess serum galectin-1 levels in patients with several other autoimmune diseases and non-autoimmune diseases who have skin ulcers, such as diabetic foot ulcers and venous leg ulcers. Nonetheless, the results of this study suggest that the measurement of serum galectin-1 levels in patients with early SSc may be useful for risk stratification for the development of

digital ulcers, because digital ulcers are refractory to treatment and are the main cause of pain, impaired hand function and disability in SSc. Furthermore, galectin-1 could be a possible treatment for SSc patients with severe digital ulcers.

ACKNOWLEDGEMENT

This work was supported by a grant for Research on Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan.

AUTHOR CONTRIBUTIONS

KY and SS designed research; KY performed research; KY, YA, KA, SN, NA, TT, TT, YI, HS, YK and MM analyzed data; KY wrote the paper.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

REFERENCES

1 Matucci-Cerinic M, Kahaleh B, Wigley FM (2013) Review: evidence that systemic sclerosis is a vascular disease. *Arthritis Rheum* **65**, 1953–62.
2 Toscano MA, Bianco GA, Ilarregui JM *et al.* (2007) Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. *Nat Immunol* **8**, 825–34.
3 Rabinovich GA, Baum LG, Tinari N *et al.* (2002) Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? *Trends Immunol* **23**, 313–20.
4 Stillman BN, Hsu DK, Pang M *et al.* (2006) Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J Immunol* **176**, 778–89.
5 Thijssen VL, Poirier F, Baum LG, Griffioen AW (2007) Galectins in the tumor endothelium: opportunities for combined cancer therapy. *Blood* **110**, 2819–27.
6 Perillo NL, Pace KE, Seilhamer JJ, Baum LG (1995) Apoptosis of T cells mediated by galectin-1. *Nature* **378**, 736–9.
7 Blaser C, Kaufmann M, Muller C *et al.* (1998) β-galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells. *Eur J Immunol* **28**, 2311–9.
8 Rabinovich GA, Daly G, Dreja H *et al.* (1999) Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med* **190**, 385–98.
9 Liu SD, Lee S, La Cava A, Motran CC, Hahn BH, Miceli MC (2011) Galectin-1-induced down-regulation of T lymphocyte activation protects (NZB x NZW) F1 mice from lupus-like disease. *Lupus* **20**, 473–84.
10 Offner H, Celnik B, Bringman TS, Casentini-Borocz D, Nedwin GE, Vandenbark AA (1990) Recombinant human β-galactoside binding lectin suppresses clinical and histological signs of experimental autoimmune encephalomyelitis. *J Neuroimmunol* **28**, 177–84.
11 Baum LG, Blackall DP, Arias-Magallano S *et al.* (2003) Amelioration of graft versus host disease by galectin-1. *Clin Immunol* **109**, 295–307.
12 Perone MJ, Bertera S, Shufesky WJ *et al.* (2009) Suppression of autoimmune diabetes by soluble galectin-1. *J Immunol* **182**, 2641–53.
13 Santucci L, Fiorucci S, Rubinstein N *et al.* (2003) Galectin-1 suppresses experimental colitis in mice. *Gastroenterology* **124**, 1381–94.
14 Taniguchi T, Asano Y, Akamata K *et al.* (2012) Serum levels of galectin-3: possible association with fibrosis, aberrant angiogenesis, and immune activation in patients with systemic sclerosis. *J Rheumatol* **39**, 539–44.
15 Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee (1980) Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* **23**, 581–90.
16 LeRoy EC, Black C, Fleischmajer R *et al.* (1988) Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* **15**, 202–5.
17 Steen VD, Powell DL, Medsger TAJ (1988) Clinical correlations and prognosis based on serum autoantibodies in patients with systemic sclerosis. *Arthritis Rheum* **31**, 196–203.
18 Sato S, Ihn H, Kikuchi K, Takehara K (1994) Antihistone antibodies in systemic sclerosis: association with pulmonary fibrosis. *Arthritis Rheum* **37**, 391–4.
19 Clements PJ, Lachenbruch PA, Seibold JR *et al.* (1993) Skin thickness score in systemic sclerosis: an assessment of interobeservar variability in 3 independent studies. *J Rheumatol* **20**, 1892–6.
20 Murrell DF (1993) A radical proposal for the pathogenesis of scleroderma. *J Am Acad Dermatol* **28**, 78–85.
21 Generini S, Matucci Cerinic M (1999) Raynaud's phenomenon and vascular disease in systemic sclerosis. *Adv Exp Med Biol* **455**, 93–100.
22 Suematsu M, Wakabayashi Y, Ishimura Y (1996) Gaseous monoxides: a new class of microvascular regulator in the liver. *Cardiovasc Res* **32**, 679–86.
23 Butler AR, Flitney FW, Williams DL (1995) NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective. *Trends Pharmacol Sci* **16**, 18–22.
24 Thijssen VL, Postel R, Brandwijk RJ *et al.* (2006) Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. *Proc Natl Acad Sci USA* **103**, 15975–80.
25 Baum LG, Seilhamer JJ, Pang M, Levine WB, Beynon D, Berliner JA (1995) Synthesis of an endogeneous lectin,

galectin-1, by human endothelial cells is up-regulated by endothelial cell activation. *Glycoconj J* **12**, 63–8.
26 He J, Baum LG (2006) Endothelial cell expression of galectin-1 induced by prostate cancer cells inhibits T-cell transendothelial migration. *Lab Invest* **86**, 578–90.
27 Ito K, Scott SA, Cutler S *et al.* (2011) Thiodigalactoside inhibits murine cancers by concurrently blocking effects of galectin-1 on immune dysregulation, angiogenesis and protection against oxidative stress. *Angiogenesis* **14**, 293–307.
28 Seropian IM, Cerliani JP, Toldo S *et al.* (2013) Galectin-1 controls cardiac inflammation and ventricular remodeling during acute myocardial infarction. *Am J Pathol* **182**, 29–40.

THE JOURNAL OF DERMATOLOGY

doi: 10.1111/1346-8138.12619

Journal of Dermatology 2014; **41**: 912–914

CONCISE COMMUNICATION

Late-onset anaphylactic reactions following i.v. cyclophosphamide pulse in a patient with systemic sclerosis and systemic lupus erythematosus overlap syndrome

Takashi TANIGUCHI, Yoshihide ASANO, Zenshiro TAMAKI, Takehiro TAKAHASHI, Yohei ICHIMURA, Tetsuo TOYAMA, Shinji KAGAMI, Hiroshi MITSUI, Shinichi SATO

Department of Dermatology, University of Tokyo Graduate School of Medicine, Tokyo, Japan

ABSTRACT

Late-onset anaphylactic reaction is a rare adverse effect of i.v. cyclophosphamide pulse (IVCY), which is caused by cyclophosphamide metabolites. We herein report a case of scleroderma and lupus overlap syndrome who developed anaphylactic reactions 3 h to 4 days after IVCY. Long time lapses between IVCY and appearance of symptoms are compatible with type I hypersensitivity to cyclophosphamide metabolites, appearing as late-onset anaphylactic reaction. Albeit unusual, this is an important anaphylactic reaction to be aware of following IVCY.

Key words: cyclophosphamide, interstitial lung disease, late-onset anaphylactic reactions, systemic lupus erythematosus, systemic sclerosis.

INTRODUCTION

Cyclophosphamide (CY) is an immunosuppressive and antineoplastic agent widely used for the treatment of systemic rheumatic diseases and malignant neoplasms. Common undesirable toxic effects of CY include bone marrow suppression, infertility and hemorrhagic cystitis. On the other hand, various type I and III hypersensitivity reactions to CY have been reported, including urticaria, angioedema, anaphylactic reactions with cutaneous, respiratory and vascular manifestations, and cutaneous vasculitis.[1–3] Importantly, there also have been reports of late-onset type I hypersensitivity to CY in patients with malignant neoplasms, which occurs several hours to several days after the infusion and is associated with acute allergic reaction to CY metabolites, such as phosphoramide mustard and 4-hydroxycyclophosphamide.[4] Although immediate anaphylactic reactions related to i.v. CY pulse (IVCY) have been reported in systemic lupus erythematosus (SLE) patients,[5,6] there is no report of late-onset type I hypersensitivity in patients with collagen vascular diseases. We herein present a case of late-onset type I hypersensitivity following IVCY in a patient with systemic sclerosis (SSc)/SLE overlap syndrome.

CASE REPORT

A 46-year-old Japanese woman with diffuse cutaneous SSc/SLE overlap syndrome presented to us in December 2004. Laboratory work-up at presentation revealed hypocomplementemia, positive antinuclear antibody of 1:2560 with a speckled pattern, elevated anti-dsDNA antibody, anti-topoisomerase I antibody and anti-U1-RNP antibody. Anti-Sm antibody was not present. Her serological test results showed slightly elevated KL-6 (526 U/mL; normal, <500) and normal surfactant protein-D (SP-D, 106 ng/mL; normal, <110) values. Chest computed tomography (CT) revealed patchy ground-glass opacity in the lower lobes of both lungs, compatible with interstitial lung disease (ILD) related to SSc. Since November 2009, 5 years after first presentation, she progressively developed dyspnea and chest CT showed worsening of her ILD. In Mach 2010, arterial blood gas test and pulmonary function test showed hypoxia with a decreased level of PaO_2 (65.7 mmHg), decreased vital capacity (VC; 1.30 L: 49.7% of the predicted value) and low diffusion capacity of the lung for carbon monoxide (D_{LCO}; 5.26 mL/min per mmHg: 31.2% of the predicted value). In addition, the disease activity of ILD was confirmed based on the increased serum levels of KL-6 and SP-D (1642 U/mL and 136 ng/mL, respectively). Therefore, in April 2010, we decided to start monthly IVCY (a test dose of 350 mg/m^2 at the first month followed by monthly IVCY at a dose of 500 mg/m^2) with moderate dose of prednisolone (30 mg/day) for her SSc-related ILD. During and after the first IVCY, there was no adverse effect related to the therapy. However, 4 days after the second IVCY, she felt feverish and experienced dyspnea and mild wheezing. She then developed generalized macular erythema, pruritus and muscle pain (Fig. 1). Repeated blood cultures were all negative. These symptoms subsided spontaneously in 24 h but recurred after subsequent pulses, with the

Correspondence: Yoshihide Asano, M.D., Ph.D., Department of Dermatology, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: yasano-tky@umin.ac.jp

Received 2 June 2014; accepted 5 August 2014.

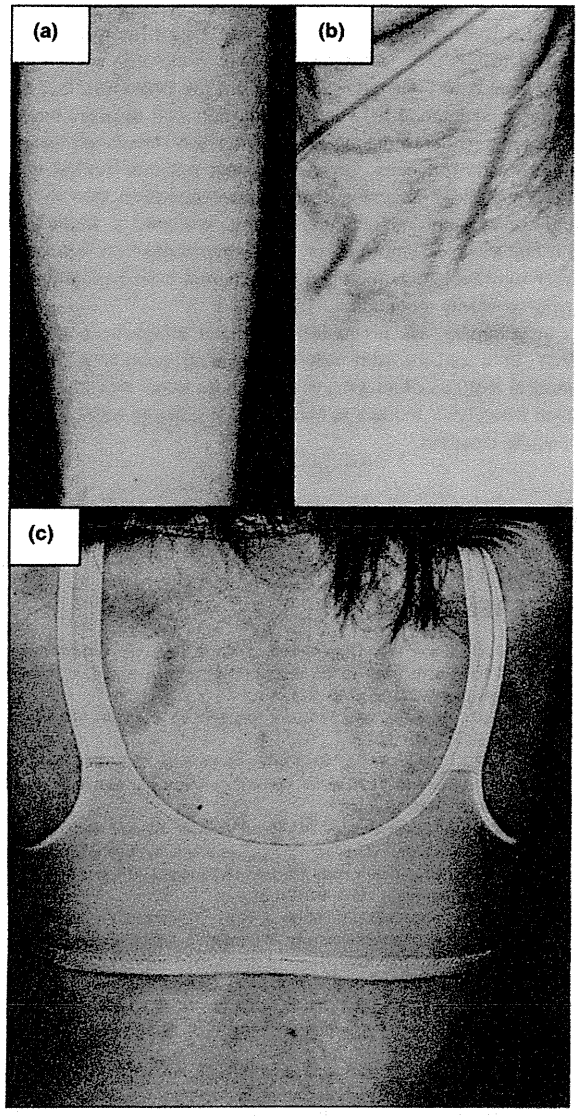

Figure 1. Cutaneous manifestations at the first episode of adverse reactions. Bean-sized faint erythema disseminated on the patient's (a) forearm, (b) neck and (c) trunk.

time lapse between infusion and appearance of symptoms getting shorter and these symptoms getting severer. Three hours after the fifth IVCY, she suddenly deteriorated with marked tachycardia, hypotension, respiratory distress and diffuse maculopapular rush, suggesting that she was in a preshock state (Fig. 2). Her blood systolic pressure decreased by 48 mmHg from baseline to 78 mmHg, and her pulse rate increased by 38 b.p.m. to 145 b.p.m. At that time, arterial blood gas test showed decreased PaO_2 (44.2 mmHg). The patient immediately received diphenhydramine, methylprednisolone (125 mg i.v.) and i.v. fluids. The blood pressure stabilized without vasopressors, and other symptoms gradually resolved within 24 h. These series of events made us notice the relationship between these anaphylactic reactions and IVCY because her symptoms appeared only after IVCY. Despite the extraordinarily long time lapse between CY infusion and appearance of these anaphylactic reactions, these events were consistent with late-onset anaphylactic reactions caused by type I hypersensitivity to CY metabolites. Although prick tests and i.d. skin tests with suspected drug or metabolites were not performed for fear of anaphylactic shock induction, we diagnosed her, based on the characteristic clinical courses, as having late-onset anaphylactic reactions related to IVCY. Although monthly IVCY were effective, confirmed by improvement of dyspnea, VC and D_{LCO} on pulmonary function test (1.75 L and 5.80 mL/min per mmHg representing 66.5% and 34.5% of the predicted values, respectively), chest CT manifestations, and decreased serum KL-6 and SP-D levels after the fifth IVCY (1510 U/mL and 85 ng/mL, respectively), we did not perform further IVCY to prevent the induction of anaphylactic reactions. Even after recovering from the preshock state, serum biomarkers kept similar levels. Her ILD had been well controlled with mizoribine (300 mg/day) as a p.o. maintenance immunosuppressive therapy for 1.5 years until there was an increased frequency of dry cough accompanied by increased biomarkers (1621 U/mL and 107 ng/mL for KL-6 and SP-D, respectively) and deterioration in respiratory function (1.49 L and 5.34 mL/min per mmHg, representing 57.2% and 31.8% of the predicted values for VC and D_{LCO}, respectively) in 2011. After that, her ILD was successfully treated with rituximab.[7]

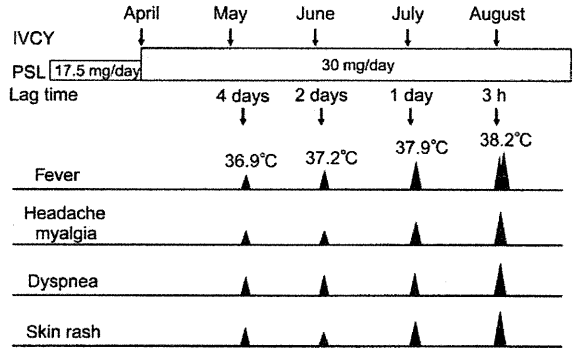

Figure 2. Time course of anaphylactic symptoms during i.v. cyclophosphamide pulse (IVCY) therapy. Degree of anaphylactic reactions became stronger and time lapse between IVCY and appearance of anaphylactic symptoms became shorter as the number of IVCY increased. PSL, prednisolone.

DISCUSSION

Although CY has been reported as a possible causative drug of late-onset anaphylactic reactions, it is clinically difficult to notice the relationship between late-onset anaphylactic reactions and IVCY because there is a long time interval between CY infusion and appearance of symptoms. In our case,

although skin allergy tests were not performed, anaphylactic reactions appeared to be associated with CY infusion because re-administration of CY reproduced these reactions and the degree of reactions got stronger as the number of IVCY increased. Given that mild clinical symptoms related to type I allergic reaction are occasionally similar to manifestations of collagen vascular diseases and easily confused with exacerbations of the underlying diseases, it is important to be aware of late-onset allergic reactions when IVCY is performed for patients with those diseases.

Clinical delay in late-onset anaphylactic reactions is thought to reflect the time to metabolize the drug and then generate metabolite-dependent allergens.[8] Popescu *et al.*[4] demonstrated the positive immediate skin test results of CY metabolites, such as 4-hydroxycyclophosphamide and phosphoramide mustard, but not CY itself, in all of the five patients with late-onset type I hypersensitivity, including four patients with 8–16 h of delay and a patient with 10 days of delay, indicating that immunoglobulin E-mediated allergic reaction to these two metabolites is a major mechanism underlying late-onset anaphylactic reactions. Given that 4-hydroxycyclophosphamide and phosphoramide mustard reach peak levels within 0.5–3 h and 3–6 h after IVCY, respectively,[9] there may be other mechanisms due to underlying autoimmune diseases, such as altered metabolism of CY in the liver, to induce these reactions several days after IVCY. Importantly, although serum levels of aspartate aminotransferase and alanine aminotransferase before IVCY were within normal limits (23 and 15 U/L, respectively), they were slightly reduced before the third IVCY (14 and 9 U/L, respectively) and kept the reduced levels thereafter. These results may support the idea that repeated IVCY therapies ameliorated altered CY metabolism due to the patient's autoimmune disease, leading to earlier appearance of allergic reactions afterwards.

Another problem related to adverse reactions after IVCY is that it is difficult to distinguish late-onset type I hypersensitivity from type III hypersensitivity occurring several hours to a few day after administration of the allergens and anaphylactoid reactions showing a quite similar clinical picture to that of anaphylaxis due to non-allergic hypersensitivity reactions. The former two types of allergic reactions are different from each other in that type III hypersensitivity does not trigger anaphylaxis, while type I hypersensitivity does, though some symptoms such as urticarial rash, macular erythema, muscle pain and arthralgia are common manifestations. Regarding discrimination between anaphylactic and anaphylactoid reactions, escalating intensity of her symptoms suggests that her reactions were of an allergic nature. Although high-grade fever is not a common symptom in anaphylactic reactions, presence of pyrexia does not necessarily deny the possibility that her reactions were due to type I hypersensitivity because pyrexia is not a rare symptom in patients with type I hypersensitivity related to CY.[4,8,10] Taken together, her symptoms seemed to be typical type I allergic reactions from the beginning, but the longer time interval between CY infusion and appearance of symptoms hindered diagnosis and she finally developed severe anaphylactic reactions. Given that most patients treated with IVCY are under an immunosuppressive condition due to CY and concomitant immunosuppressants, we need to know that the first clinical symptoms of type I hypersensitivity following IVCY may be largely modified and different from typical type I hypersensitivity reactions.

In summary, we presented late-onset anaphylaxis due to IVCY in a patient with SSc/SLE overlap syndrome. These unusual features of anaphylaxis should be taken into consideration when IVCY therapy is conducted in patients with collagen vascular diseases.

CONFLICT OF INTEREST: None.

REFERENCES

1 Lakin JD, Cahill RA. Generalized urticaria to cyclophosphamide: type I hypersensitivity to an immunosuppressive agent. *J Allergy Clin Immunol* 1976; **58**: 160–171.
2 Lakin JD. Possible anaphylactic reaction to cyclophosphamide. *JAMA* 1977; **237**: 1827.
3 Green RM, Schapel GJ, Sage RE. Cutaneous vasculitis due to cyclophosphamide therapy for chronic lymphocytic leukemia. *Aust N Z J Med* 1989; **19**: 55–57.
4 Popescu NA, Sheehan MG, Kouides PA *et al.* Allergic reactions to cyclophosphamide: delayed clinical expression associated with positive immediate skin tests to drug metabolites in five patients. *J Allergy Clin Immunol* 1996; **97**: 26–33.
5 Thong BY, Leong KP, Thumboo J, Koh ET, Tang CY. Cyclophosphamide type I hypersensitivity in systemic lupus erythematosus. *Lupus* 2002; **11**: 127–129.
6 García Hernández FJ, Ocaña Medina C, Sánchez Román J, Castellano M, Hevia E. [Hypersensitivity response to cyclophosphamide in a patient with systemic lupus erythematous]. *Farm Hosp* 2005; **29**: 216–217.
7 Sumida H, Asano Y, Tamaki Z *et al.* Successful experience of rituximab therapy for systemic sclerosis-associated interstitial lung disease with concomitant systemic lupus erythematosus. *J Dermatol* 2014; **41**: 418–420.
8 Kim HC, Kesarwala HH, Colvin M, Saidi P. Hypersensitivity reaction to a metabolite of cyclophosphamide. *J Allergy Clin Immunol* 1985; **76**: 591–594.
9 de Jonge ME, Huitema AD, Rodenhuis S, Beijnen JH. Clinical pharmacokinetics of cyclophosphamide. *Clin Pharmacokinet* 2005; **44**: 1135–1164.
10 Knysak DJ, McLean JA, Solomon WR, Fox DA, McCune WJ. Immediate hypersensitivity reaction to cyclophosphamide. *Arthritis Rheum* 1994; **37**: 1101–1104.

ARTHRITIS & RHEUMATOLOGY
Vol. 66, No. 6, June 2014, pp 1636–1647
DOI 10.1002/art.38380

Decreased Interleukin-20 Expression in Scleroderma Skin Contributes to Cutaneous Fibrosis

Hideo Kudo,[1] Masatoshi Jinnin,[1] Yoshihide Asano,[2] Maria Trojanowska,[3] Wakana Nakayama,[1] Kuniko Inoue,[1] Noritoshi Honda,[1] Ikko Kajihara,[1] Katsunari Makino,[1] Satoshi Fukushima,[1] and Hironobu Ihn[1]

Objective. **To clarify the role of interleukin-20 (IL-20) in the regulatory mechanism of extracellular matrix expression and to determine the contribution of IL-20 to the phenotype of systemic sclerosis (SSc).**

Methods. **Protein and messenger RNA (mRNA) levels of collagen, Fli-1, IL-20, and IL-20 receptor (IL-20R) were analyzed using polymerase chain reaction (PCR) array, immunoblotting, immunohistochemical staining, enzyme-linked immunosorbent assay, and real-time PCR.**

Results. **PCR array revealed that IL-20 decreased gene expression of α2(I) collagen (0.03-fold), Smad3 (0.02-fold), and endoglin (0.05-fold) in cultured normal dermal fibroblasts. Fli-1 protein expression was induced by IL-20 (~2-fold). The inhibition of collagen by IL-20, the induction of Fli-1 by IL-20, and the reduction of Smad3 and endoglin by IL-20 were also observed in SSc fibroblasts. Serum IL-20 levels were reduced only slightly in SSc patients but were significantly decreased in patients with scleroderma spectrum disorders (the prodromal stage of SSc) compared with those in normal subjects (111.3 pg/ml versus 180.4 pg/ml; $P < 0.05$). On the other hand, IL-20 mRNA expression in SSc skin was decreased compared with that in normal skin ($P < 0.05$), which may result in the induction of collagen synthesis in SSc dermal fibroblasts. IL-20R was expressed in normal and SSc fibroblasts. Moreover, IL-20 supplementation by injection into the skin reversed skin fibrosis induced by bleomycin in mice (~0.5-fold).**

Conclusion. **IL-20 reduces basal collagen transcription via Fli-1 induction, while down-regulation of Smad3 and endoglin may cancel the effect of transforming growth factor β in SSc fibroblasts. To confirm the therapeutic value of IL-20 and IL-20R, their function and expression in vivo should be further studied.**

Systemic sclerosis (SSc) or scleroderma is one of the rheumatic diseases characterized by tissue fibrosis of the skin and internal organs. In the skin, thickened dermis due to uncontrolled excessive deposition of extracellular matrix (ECM), mainly type I collagen (which consists of α1[I] and α2[I] collagen), is a hallmark of this disease (1,2). The source of the increased ECM is thought to be dermal fibroblasts activated by interactions with endothelial cells, lymphocytes, or macrophages, via various mediators including cytokines and growth factors (3,4). For example, many researchers have suggested that transforming growth factor β1 (TGFβ1) may play a central role in fibroblast activation (5–7). In addition, connective tissue growth factor (7), platelet-derived growth factor (8), insulin-like growth factor, interleukin-1 (IL-1), IL-6, and IL-7 are reported to be involved in the pathogenesis of this disease (9–12). Accordingly, investigating the cytokine network mediating fibroblast activation of SSc is essential to understand the molecular mechanism(s) of this disease.

IL-20 is identified as a member of the IL-10 family (13), which includes IL-10, IL-19, IL-22, IL-24 (melanoma differentiation–associated protein 7), and IL-26 (AK155). Although IL-19, IL-20, and IL-24 have partial homology in their amino acid sequences and share their receptor (14), the main biologic effects of these 3 cytokines seem quite distinct. IL-19 and IL-24 are mainly implicated in immune activity and tumor

[1]Hideo Kudo, MD, Masatoshi Jinnin, MD, PhD, Wakana Nakayama, MD, PhD, Kuniko Inoue, MD, PhD, Noritoshi Honda, MD, PhD, Ikko Kajihara, MD, PhD, Katsunari Makino, MD, PhD, Satoshi Fukushima, MD, PhD, Hironobu Ihn, MD, PhD: Kumamoto University, Kumamoto, Japan; [2]Yoshihide Asano, MD, PhD: University of Tokyo Graduate School of Medicine, Tokyo, Japan; [3]Maria Trojanowska, MD, PhD: Boston University School of Medicine, Boston, Massachusetts.

Address correspondence to Masatoshi Jinnin, MD, PhD, Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan. E-mail: mjin@kumamoto-u.ac.jp.

Submitted for publication May 4, 2013; accepted in revised form January 21, 2014.

apoptosis, respectively (15–17). On the other hand, IL-20 is expressed by multiple cell types, including monocytes and skin keratinocytes, and is implicated in the pathogenesis of autoimmune diseases. IL-20 was reportedly elevated in synovial fluid from patients with rheumatoid arthritis (18). Moreover, IL-20 is thought to be correlated with the etiology of lupus nephritis (19,20). In addition, IL-20 expression and IL-20 receptor (IL-20R) complexes are dramatically up-regulated in psoriatic skin lesions (21). However, there have been no reports that validate the function of IL-20 in the regulation of ECM or the pathogenesis of SSc.

In the present study, we investigated the effect of IL-20 on ECM expression in normal fibroblasts. In addition, we compared the expression pattern of IL-20 in the sera and skin between normal subjects and SSc patients, and we demonstrated the involvement of IL-20 signaling in the abnormal ECM regulation seen in SSc.

PATIENTS AND METHODS

Reagents. Recombinant human IL-19, IL-20, and IL-24 and mouse IL-20 were obtained from R&D Systems.

Patients. Serum samples were obtained from 33 patients with SSc (5 males and 28 females, mean age 58.7 years [range 24–85 years]); 13 had diffuse cutaneous SSc (dcSSc), and 20 had limited cutaneous SSc. All patients fulfilled the classification criteria proposed by the American College of Rheumatology (ACR) (22). We also included in the study 10 patients with systemic lupus erythematosus (SLE), 12 patients with dermatomyositis (DM), and 9 patients with scleroderma spectrum disorders who did not fulfill the ACR classification criteria for SSc but who we thought would meet these criteria in the future (23–25) (further information available from the corresponding author). Control serum samples were also obtained from 15 healthy volunteers. Skin biopsy specimens of lesional skin were obtained from SSc patients. Control samples of routinely discarded skin were obtained from healthy human subjects undergoing skin grafts. Institutional review board approval and written informed consent according to the Declaration of Helsinki were obtained before patients and healthy volunteers were entered into this study.

Diagnosis of scleroderma spectrum disorders by the point system. A total score was evaluated as the sum of 5 factors for the diagnosis of scleroderma spectrum disorders (23–25):

1. Skin sclerosis: swollen fingers = 1 point, sclerodactyly = 3 points, proximal sclerosis = 5 points, diffuse sclerosis = 10 points.
2. Pulmonary changes: pulmonary fibrosis accompanied by forced vital capacity (FVC) ≥80% predicted = 2 points, pulmonary fibrosis accompanied by FVC <80% predicted = 4 points.
3. Antinuclear antibodies (ANAs): anti–topoisomerase I antibody = 5 points, anticentromere or anti–U1 RNP antibody = 3 points, antinucleolar antibody = 2 points, other ANAs = 1 point.
4. Pattern of Raynaud's phenomenon: biphasic or bilateral = 1 point, biphasic and bilateral = 2 points, triphasic = 3 points.
5. Nailfold bleeding: 1 or 2 fingers = 1 point, ≥3 fingers = 2 points.

A total score of 5–8 is consistent with scleroderma spectrum disorders, and a total score of ≥9 is consistent with SSc.

Cell cultures. Human dermal fibroblasts were obtained by skin biopsies of the affected areas (dorsal forearm) of 7 healthy human subjects and dcSSc patients (26). Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. Independently isolated monolayer cultures of fibroblasts obtained from different individuals were maintained at 37°C in 5% CO_2 in air. Cells were serum-starved for 24 hours before all experiments.

Cell lysis and immunoblotting. Cultured fibroblasts were washed twice with cold phosphate buffered saline (PBS) and lysed in Denaturing Cell Extraction Buffer (BioSource International). Aliquots of the cell lysates (normalized for protein concentrations) were separated by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto PVDF filters. The PVDF filters were then incubated with antibodies against type I collagen (SouthernBiotech) or against Ets-1, Fli-1, IL-20R, or β-actin (all from Santa Cruz Biotechnology). The filters were incubated with secondary antibody, and the immunoreactive bands were visualized using an ECL system (Amersham Biosciences).

Immunohistochemistry. Wax-embedded sections (4-μm thickness) were dewaxed in xylene and rehydrated in graded alcohols. For the immunostaining of IL-20 and Smad3, antigens were retrieved by incubation with citrate buffer (pH 6) for 9 minutes with a microwave. Endogenous peroxidase activity was inhibited with a solution of 0.3% H_2O_2 in methyl alcohol, after which sections were blocked with 5% donkey serum for 20 minutes and then reacted with antibodies to IL-20 (Abcam) or Smad3 (Santa Cruz Biotechnology) at 4°C. After excess antibody was washed out with PBS, samples were incubated with horseradish peroxidase (HRP)–labeled anti-mouse antibody (Nichirei Biosciences) at 37°C.

For immunostaining of IL-20R or Fli-1, antigens were retrieved by incubation with citrate buffer (pH 9) for 9 minutes with a microwave. Antibodies to IL-20R (Abcam) and Fli-1 (Santa Cruz Biotechnology) and HRP-labeled anti-rabbit antibody (Nichirei Biosciences) were used.

The reaction was visualized using a diaminobenzidine substrate system (Dojin). Slides were counterstained with Mayer's hematoxylin and examined under a light microscope (BX50; Olympus).

RNA isolation, array analysis, and quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted from culture cells with Isogen (Nippon Gene) or from paraffin-embedded sections with an RNeasy FFPE kit (Qiagen). For the array, first-strand complementary DNA (cDNA) was synthesized from RNA using an RT^2 First Strand Kit (Qiagen). Complementary DNA was mixed with RT^2 SYBR Green/Rox qPCR Master Mix (Qiagen), and the mixture was added to a 96-well Extracellular Matrix and Adhesion Molecules PCR Array (Qiagen). PCR was performed on a Takara Thermal Cycler Dice (TP800) instrument (Takara Bio). The threshold cycle (C_t) for each RNA was extracted using Ther-

mal Cycler Dice Real Time System software, version 2.10B (Takara Bio). The raw C_t was normalized using the values of housekeeping genes. For quantitative real-time PCR, first-strand cDNA was synthesized using a PrimeScript RT reagent Kit (Takara Bio) (27). GAPDH primer was purchased from Qiagen, and other primers were purchased from Takara Bio. Using a Takara Thermal Cycler Dice instrument, DNA was amplified for 40 cycles of denaturation for 5 seconds at 95°C and annealing for 30 seconds at 60°C. The relative expression of each gene of interest and GAPDH was calculated using a standard curve method.

Immunoprecipitation. Phosphorylated Fli-1 was detected as described previously (28). For the detection of acetylated Fli-1, cells were lysed in Pierce IP lysis buffer with the Halt phosphatase inhibitor cocktail (Thermo Scientific Pierce). Cell lysates were precleared with protein G–Sepharose (GE Healthcare) and then were incubated with monoclonal mouse anti–Fli-1 antibody (BD Biosciences) and protein G–Sepharose beads overnight at 4°C. The immunoprecipitated proteins were washed with Pierce buffer. Agarose-bound proteins were extracted by incubation in sample buffer at 95°C. The sample was then assessed by immunoblotting with anti–acetylated lysine antibody (Cell Signaling Technology). The membrane was stripped and reprobed with rabbit polyclonal anti–Fli-1 antibody (Santa Cruz Biotechnology) (29).

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using an EpiQuik ChIP kit (Epigentek) (28). Briefly, after cells were treated with 1% formaldehyde for 10 minutes, the crosslinked chromatin was sonicated. The DNA fragments were immunoprecipitated with IgG isotype control antibody or polyclonal anti–Fli-1 antibody at room temperature. After crosslinking was reversed, the immunoprecipitated chromatin was amplified by PCR of a specific region of the α2(I) collagen genomic locus. The primers used were 5′-CTGGACAGCTCCTGCTTTGAT-3′ (forward) and 5′-CTTTCAAGGGGAAACTCTGACTC-3′ (reverse). The amplified DNA products were run out on an agarose gel containing ethidium bromide.

Plasmid construction. A construct consisting of the full-length human α2(I) collagen gene fragment linked to the chloramphenicol acetyltransferase (CAT) reporter gene and a series of 5′-deletions of the construct were generated as previously described (30).

Transient transfection. For CAT assay, fibroblasts were transfected with promoter constructs employing Lipofectamine 2000 (Invitrogen), as described previously (30). In order to correct minor variations in transfection efficiency, pSV-β-galactosidase vector (Promega) was included in all transfections.

For reverse transfection of small interfering RNAs (siRNAs), siRNAs were mixed with Lipofectamine RNAiMAX (Invitrogen) and then added when the cells were plated, followed by incubation at 37°C in 5% CO_2. Small interfering RNA against Fli-1 and control siRNA were purchased from Dharmacon.

CAT assay. After 48 hours of incubation after the transfection of constructs with CAT reporter, cells were harvested. CAT activity in cell lysates was assayed colorimetrically using a CAT ELISA kit (Roche) (31).

Immunofluorescence microscopy. Fibroblasts were grown in 4-well LAB TEK chambers (Nunc) to subconfluence as described above. Immunofluorescence was performed using antibody to IL-20R as primary antibody and fluorescein isothiocyanate–conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) as secondary antibody. A Zeiss fluorescence microscope was used (6) to visualize fluorescence.

Measurement of serum IL-20 concentrations. Serum IL-20 levels were measured with a specific enzyme-linked immunosorbent assay (ELISA) kit (Human IL-20 Immunoassay; R&D Systems) (27,32). Briefly, antibody to IL-20 was precoated onto microtiter wells. Aliquots of serum were added to each well, followed by peroxidase-conjugated antibodies to IL-20. Color was developed with H_2O_2 and tetramethylbenzidine peroxidase, and absorbance at 450 nm was measured. Wavelength correction was performed by absorbance at 540 nm. The concentration of IL-20 in each sample was determined by interpolation from a standard curve.

Intradermal treatment with bleomycin. Bleomycin (Nippon Kayaku) was dissolved in PBS at a concentration of 1 mg/ml and sterilized by filtration. Bleomycin (300 μg) or PBS was injected intradermally into the shaved backs of 6-week-old BALB/c mice 6 times per week for 1 month, as described previously (33,34). Injection was performed using a 27-gauge needle. The back skin was removed on the day after the final injection. The skin samples were then fixed in 10% formalin solution and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). Dermal thickness was evaluated by 2 investigators (HK, WN) in a blinded manner who measured the distance between the epidermal–dermal junction and the dermal–fat junction in H&E-stained sections under 100× magnification.

Collagen assay. The amount of collagen in the paraffin-embedded skin sections was quantified using a Semi-Quantitative Collagen Assay Kit (Chondrex). After being dewaxed in xylene and rehydrated in graded alcohols, 20-μm thick sections were immersed in staining solution at room temperature for 30 minutes. The staining solution was removed and a bleaching solution was added to measure the absorbance at 540 nm and 605 nm in an ND-1000 spectrophotometer (NanoDrop Technologies). The collagen concentration was calculated based on the following formula: collagen (μg/section) = (optical density [OD] at 540 nm − [OD_{605} × 0.291])/37.8 × 1,000 (35).

Statistical analysis. Data presented as bar graphs are the mean ± SEM of at least 3 independent experiments. The statistical analysis was carried out using the Mann-Whitney U test for the comparison of medians and Fisher's exact probability test for the analysis of frequency. *P* values less than 0.05 were considered significant.

RESULTS

Effect of IL-20 on expression of ECM-related genes in cultured normal dermal fibroblasts. As an initial experiment, to determine the effect of IL-20 on ECM expression, we performed a PCR array of 84 ECM-related genes using RNA obtained from 3 dermal fibroblasts either unstimulated or stimulated with IL-20 for 12 hours. When a 16-fold difference by the $\Delta\Delta C_t$ method was considered meaningful, 3 of the 84 genes were up-regulated and 13 genes were down-regulated in