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ORIGINAL ARTICLE

# Clinical correlation of brachial artery flow-mediated dilation in patients with systemic sclerosis

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**Abstract**

*Objective* To investigate the clinical significance of flow-mediated dilation (FMD) in systemic sclerosis (SSc).

*Methods* Thirty-three SSc patients and 12 healthy controls were studied. Ultrasound assessment of the brachial artery FMD was performed on all subjects. The results were expressed as the percentage of increase in brachial artery diameter following hyperemia.

*Results* Limited cutaneous SSc (lcSSc) patients had significantly lower FMD values than healthy controls ($5.3 \pm 2.7$ versus $7.7 \pm 2.0$ %, $p < 0.05$), while the values in diffuse cutaneous SSc (dcSSc) patients ($6.7 \pm 4.0$ %) were comparable to those in lcSSc patients and healthy controls. Although FMD values did not correlate with any clinical features in dcSSc patients, there was an inverse correlation between FMD values and disease duration in lcSSc patients ($r = -0.64$, $p < 0.05$). Furthermore, lcSSc patients with decreased FMD values showed significantly higher prevalence of digital ulcers and elevated right ventricular systolic pressure than those with normal values (for each; 75 versus 10 %, $p < 0.05$).

*Conclusion* The FMD values represent the severity of vascular damages, which progress along with disease duration and lead to digital ulcers and pulmonary arterial hypertension, in lcSSc patients.

**Keywords** Systemic sclerosis · Flow-mediated dilation · Digital ulcers · Pulmonary arterial hypertension

## Introduction

Systemic sclerosis (SSc) is a multisystem autoimmune disorder characterized by initial vascular injuries and resultant fibrosis of the skin and certain internal organs [1]. Although the pathogenesis of SSc still remains unknown, a wealth of evidence suggests that the functional and morphological changes of blood vessels resulting from aberrant angiogenesis underlie the mechanism of vasculopathy and fibrosis in this disorder [2–4]. Vascular changes associated with SSc are classified into two subgroups, such as destructive vasculopathy and proliferative obliterative vasculopathy [5]. Destructive vasculopathy is characterized by progressive loss of capillaries early in the disease course, while proliferative obliterative vasculopathy is featured by proliferation of vascular cells resulting in intimal hyperplasia, intimal fibrosis, and luminal narrowing of arterioles and arteries, which gradually progresses along with disease duration and eventually becomes clinically evident in the late stage. Both vascular changes occur in almost all of SSc patients with variable degrees of severity.

Flow-mediated dilation (FMD), which represents the vasodilatory response to blood flow-associated shear stress, of the brachial artery is a validated, noninvasive physiological measure widely used as a research tool to quantify endothelial function [6]. Since the increased shear stress stimulates the endothelial production and release of several vasodilators, primarily nitric oxide (NO) [7], the resulting

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augmentation of arterial diameter estimates the endothelium-dependent vasodilatation. Thus, a small percentage of FMD response is interpreted as low NO bioavailability. On the other hand, calcification and fibrosis reduce vascular compliance and subsequently affect NO signaling by limiting vascular stretch [8, 9], indicating that vascular stiffness itself is an important factor determining FMD induced by reactive hyperemia. Importantly, it is already established that the decrease in FMD values is associated with the increased risk of cardiovascular events [10].

The clinical significance of FMD measurement in SSc patients has already been well studied. The first report by Lekakis et al. [11, 12] demonstrated that FMD values are decreased in patients with Raynaud's phenomenon, either primary or secondary to SSc. Following this, there have been several reports regarding the impaired FMD in SSc patients [13–18], but a couple of reports revealed no difference in FMD values between SSc and control subjects [19, 20]. Regarding the clinical association of FMD among SSc patients, Rossi et al. [16] reported that FMD values correlate with pulmonary artery pressure, while Rollando et al. [15] reported that FMD values are already decreased in SSc patients with early nailfold capillary changes and further reduced along with the progression of microvascular damage. Thus, the impairment of FMD in SSc is generally accepted, but the clinical significance of FMD in SSc, which appears to reflect the severity of micro- and macrovascular impairments, still leaves a room for further investigation.

Based on these backgrounds, we herein evaluated the clinical association of FMD in SSc and found that FMD values may serve as a marker of patients at high risk for the development of proliferative obliterative vasculopathy. The pathological process of SSc explaining this observation was also discussed.



## Materials and methods

### Patients

Thirty-three SSc patients and 12 healthy controls (all women) were studied after getting informed consent and institutional approval (University of Tokyo Graduate School of Medicine). Patients were grouped by the LeRoy's classification system [21]: 19 patients with diffuse cutaneous SSc (dcSSc) and 14 patients with limited cutaneous SSc (lcSSc). All patients fulfilled the criteria proposed by the American College of Rheumatology. Ten of the dcSSc patients, but none of the lcSSc, had already been treated with corticosteroids (initial dose; 20–30 mg/day) and/or other immunosuppressants, including intravenous cyclophosphamide pulse, when FMD values were measured. No smokers were included in this study. Characteristics of patients and controls are summarized in Table 1. None of the patients had signs or symptoms of cardiovascular disease. Sinus rhythm, but no other major abnormalities, were found at electrocardiography in all subjects. No major differences were observed between patients with SSc and healthy controls in means of age, height, weight, body mass index, systolic and diastolic blood pressure, or levels of triglycerides, low-density or high-density lipoprotein cholesterol. Anti-centromere antibody and anti-topoisomerase I antibody were positive in 12 (36.4 %) and 17 patients with SSc (51.5 %), respectively.

### The measurement of brachial artery FMD

All assessments were performed in an air-conditioned room at 21–24 °C, after 4 h fasting and following a 30-min rest. Heavy foods, including high-fat foods and caffeine-containing beverages, were prohibited the night before the study. Ultrasound scans were performed using the amplitude and brightness-mode ultrasonography with the use of a linear-array 10-MHz transducer (UNEXEF18G, UNEX). The brachial artery was scanned over a longitudinal section 2–3 cm above the antecubital fossa, based on individual anatomical variability. When an adequate image was obtained, the ultrasound probe was positioned using a mechanical probe stabilizer. After baseline measurements of the brachial artery diameter, the pressure cuff was kept inflated for 5 min at 50 mmHg over systolic blood pressure on the proximal portion of the forearm. No patients experienced significant discomfort during or after cuff occlusion. Post-ischemic artery diameter recording was started after rapid deflation of the blood pressure cuff and the longitudinal image of the artery was recorded continuously for up to 2 min. All diameters were taken in the end-diastolic phase, which was defined as the beginning of the R wave in the electrocardiogram. The FMD was expressed as a percentage of increase in arterial diameter from baseline to the post-occlusive period.

### Clinical assessments

Disease onset was defined as the first clinical event of SSc other than Raynaud's phenomenon. Disease duration was defined as the interval between the onset and the time of FMD measurement. The clinical and laboratory data were obtained when FMD values were determined. Skin score was measured using the modified Rodnan total skin thickness score (MRSS) [22]. Interstitial lung disease (ILD) was defined as bibasilar interstitial fibrosis on chest radiographs, and, in patients with no abnormalities on chest radiographs, early ILD was defined as alveolitis on high-resolution

**Table 1** Clinical characteristics of SSc patients and healthy controls

| | Total SSc | dcSSc | lcSSc | Controls |
|---|---|---|---|---|
| Subjects (*n*) | 33 | 20 | 13 | 12 |
| Age (years) | 57.0 ± 11.6 | 55.8 ± 12.8 | 58.8 ± 9.7 | 52.9 ± 17.7 |
| Body mass index (kg/m$^2$) | 21.5 ± 3.6 | 21.7 ± 3.9 | 21.2 ± 3.3 | 21.5 ± 4.7 |
| Systolic blood pressure (mmHg) | 116.3 ± 17.9 | 115.9 ± 20.2 | 116.9 ± 14.5 | 111 ± 15.0 |
| Diastolic blood pressure (mmHg) | 67.8 ± 14.1 | 67.3 ± 16.9 | 68.6 ± 8.9 | 68.8 ± 13.7 |
| Mean arterial pressure (mmHg) | 84.0 ± 14.7 | 83.5 ± 17.4 | 84.7 ± 9.9 | 82.7 ± 13.5 |
| Triglycerides (mg/dl) | 150.8 ± 83.4 | 136.85 ± 67.6 | 172.4 ± 102.4 | 181.8 ± 81.6 |
| LDL cholesterol (mg/dl) | 120.6 ± 39.0 | 116.5 ± 40.4 | 127.8 ± 37.4 | 127.8 ± 25.0 |
| HDL cholesterol (mg/dl) | 65.6 ± 23.2 | 67.5 ± 24.9 | 62.9 ± 21.3 | 59.4 ± 10.9 |
| Disease duration (years) | 11.3 ± 10.0 | 8.4 ± 7.4 | 15.8 ± 12.1 | – |
| ACA positivity (%) | 36.4 | 5.0 | 84.6 | – |
| Topo-I positivity (%) | 51.5 | 80.0 | 7.7 | – |

*SSc* systemic sclerosis, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *ACA* anti-centromere antibody, *Topo-I* anti-topoisomerase I antibody



computed tomography. The degree of ILD was evaluated by the percentage of predicted vital capacity (%VC) and the percentage of predicted diffusion lung capacity for carbon monoxide (%DLco) on the pulmonary function test. Elevated right ventricular systolic pressure (RVSP) was defined as 35 mmHg or more on an echocardiogram [23]. Esophageal hypomotility was defined as distal esophageal hypomotility on barium-contrast radiography.

### Statistical analysis

Statistical analysis was carried out with the Mann–Whitney *U* test for two-group comparison and with a Fisher's exact probability test for the analysis of frequency. Correlations with clinical data were assessed by Spearman's rank correlation coefficient. Statistical significance was defined as a $p$ value of $<0.05$.

## Results

### FMD values in SSc patients

Consistent with previous reports [11–18], FMD values were significantly decreased in SSc patients compared with healthy controls (6.1 ± 3.5 versus 7.7 ± 2.0 %, $p < 0.05$). Since the degrees of dermal, visceral, and vascular involvements are quite different between dcSSc and lcSSc, we next focused on these subgroups and further evaluated them. As shown in Fig. 1, lcSSc patients had significantly lower FMD values than healthy controls (5.3 ± 2.7 versus 7.7 ± 2.0 %, $p < 0.05$), while the values in dcSSc patients (6.7 ± 4.0 %) were comparable to those in lcSSc patients and healthy controls. Given that dcSSc is characterized by extensive fibrosis especially in skin and lung, these results

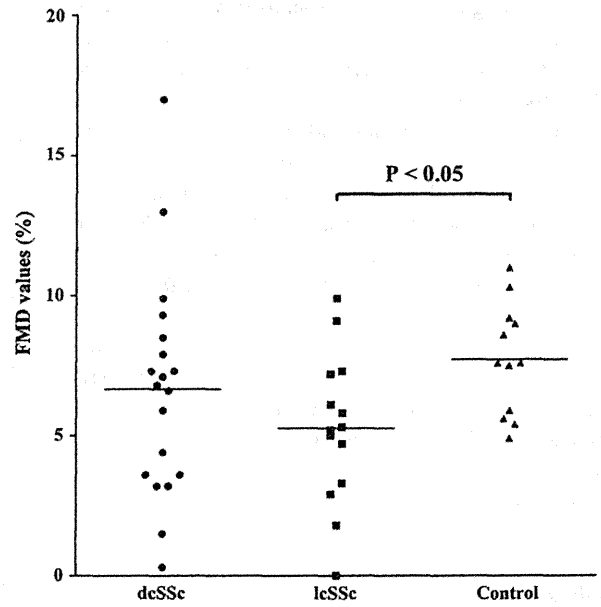


**Fig. 1** The values of FMD in SSc patients and healthy controls. The values of FMD were significantly lower in lcSSc patients than in healthy controls, while there were no significant differences between dcSSc patients and lcSSc patients or healthy controls. The *horizontal bars* indicate the mean values of each group

suggest that FMD values do not reflect the degree of fibrosis in SSc. Supporting this notion, we failed to detect any correlation of FMD values with MRSS, %VC, and %DLco in total SSc patients ($r = 0.14$, 0.14, and 0.15, respectively).

### Clinical correlation of FMD in SSc patients

To further evaluate the clinical correlation of FMD in SSc patients, we set the cut-off value at 3.7 % (mean − 2SD of normal controls) and classified SSc patients into 4 groups:

**Table 2** Clinical correlation of FMD in dcSSc and lcSSc patients

| | dcSSc | | lcSSc | |
|---|---|---|---|---|
| | Decreased FMD values ($n = 6$) | Normal FMD values ($n = 13$) | Decreased FMD values ($n = 4$) | Normal FMD values ($n = 10$) |
| Age (years old) | 58.0 ± 15.6 | 55.4 ± 12.3 | 55.8 ± 8.7 | 60.0 ± 9.7 |
| Disease duration (years) | 7.7 ± 5.6 | 9.4 ± 8.2 | 29.0 ± 3.7* | 9.2 ± 9.4 |
| RP | 67 | 92 | 100 | 90 |
| Nailfold bleeding | 100 | 75 | 75 | 90 |
| Telangiectasia | 67 | 77 | 100 | 90 |
| Digital ulcers | 33 | 23 | 75** | 10 |
| MRSS | 6.8 ± 7.5 | 12.0 ± 6.2 | 3.3 ± 2.3 | 4.7 ± 1.9 |
| ILD | 83 | 85 | 0 | 30 |
| Decreased %VC | 67 | 38 | 25 | 0 |
| Decreased %DLco | 67 | 31 | 75 | 15 |
| Elevated RVSP | 33 | 50 | 75** | 10 |
| Esophageal dysfunction | 100 | 85 | 50 | 70 |

Unless noted otherwise, values are percentages

*RP* Raynaud's phenomenon, *MRSS* modified Rodnan total skin thickness score, *ILD* interstitial lung disease, *VC* vital capacity, *DLco* diffuse capacity for carbon monoxide, *RVSP* right ventricular systolic pressure

* $p < 0.01$ and ** $p < 0.05$ versus lcSSc patients with normal FMD values



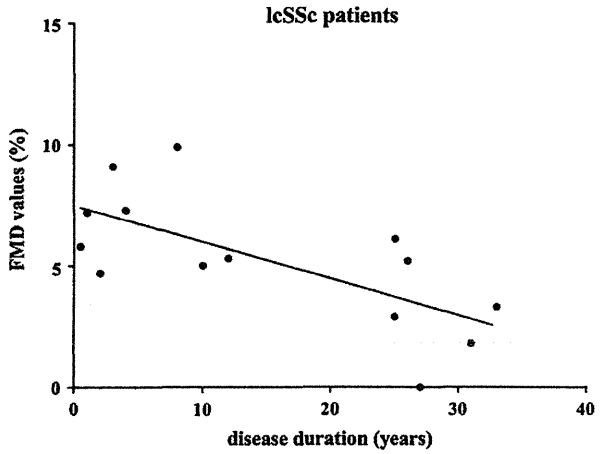


**Fig. 2** The values of FMD inversely correlated with disease duration in lcSSc. There was a significant inverse correlation between FMD values and disease duration in lcSSc ($r = -0.64, p < 0.05$). The *solid line* represents the regression line

dcSSc with decreased FMD values, dcSSc with normal FMD values, lcSSc with decreased FMD values, and lcSSc with normal FMD values. The clinical features of SSc patients in each group are shown in Table 2. In dcSSc patients, there were no significant differences between patients with decreased FMD values and those with normal values in terms of age or disease duration. Regarding the prevalence of cutaneous and visceral involvements in dcSSc patients, we failed to detect any difference between the two subgroups. By contrast, in the lcSSc patient group, patients with decreased FMD values had much longer disease duration than those with normal values (29.0 ± 3.7 versus 9.2 ± 9.4 years, $p < 0.01$), while no significant difference in the age of the two groups was observed. Consistently, there was a greater inverse correlation between FMD values and disease duration in lcSSc patients ($r = -0.64$, $p < 0.05$; Fig. 2). As for clinical symptoms, lcSSc patients with decreased FMD values showed the significantly higher prevalence of digital ulcers and elevated RVSP than those with normal values (for each; 75 versus 10 %, $p < 0.05$). In addition, there was a trend towards the increase in the frequency of decreased %DLco, but not decreased %VC, in lcSSc patients with decreased FMD values as compared to those with normal values (75 versus 15 %, $p = 0.053$), supporting the data for the prevalence of elevated RVSP. Regarding other clinical symptoms, we did not find any difference between the two groups. These results suggest that the reduction in FMD values represents the progression of vascular involvements along with disease duration and the severity of vascular damage leading to digital ulcers and elevated pulmonary arterial pressure in lcSSc patients.

## Discussion

This study was undertaken to evaluate the clinical significance of FMD in SSc. Consistent with previous reports [11–18], FMD values were significantly reduced in SSc patients compared with healthy controls. Importantly,

lcSSc patients, but not dcSSc patients, revealed the significantly lower FMD values than healthy controls. Supporting this finding, lcSSc patients with decreased FMD values exhibited the significantly higher prevalence of clinical symptoms associated with proliferative obliterative vasculopathy, such as digital ulcers and elevated RVSP, than those with normal values. Furthermore, the values of FMD inversely correlated with disease duration in lcSSc patients. Collectively, FMD measurement is potentially a powerful tool to evaluate the damage of vascular system in SSc, especially in limited cutaneous subtype of this disorder.

Given that FMD is largely caused by shear stress-induced NO release [7], the significant decrease of FMD values in SSc compared with healthy controls indicates the impaired NO production in SSc endothelial cells. There are a couple of possible mechanisms behind this observation. The expression levels of NO synthase (NOS), including endothelial NOS (eNOS) and inducible NOS (iNOS), are altered in SSc endothelial cells; namely, eNOS levels are decreased, while iNOS levels are increased, potentially affecting the NO production in those cells [24]. Furthermore, sera from SSc patients suppress the NOS activity without affecting the expression levels of eNOS and iNOS in cultured human dermal microvascular endothelial cells [25]. Moreover, the activity of NOS is decreased in endothelial cells exposed to pulse perfusion when cultured in less distensible conduits [8, 9]. Given that vascular changes leading to digital ulcers and pulmonary arterial hypertension are histopathologically characterized by intimal fibrosis [26–29], the decrease in vascular compliance [19] may also contribute to the reduction of FMD values by reducing NOS activity in SSc. Collectively, the decrease in endothelial NO production is a characteristic feature of SSc vasculopathy, which largely explains the reduced FMD values in SSc patients.

Vascular changes associated with SSc are pathologically classified into two distinct categories; destructive vasculopathy and proliferative obliterative vasculopathy [5]. Proliferative obliterative vasculopathy is characterized by proliferation of vascular cells resulting in intimal hyperplasia, intimal fibrosis, and luminal narrowing or occlusion of large vessels, such as the pulmonary arteries, the palmar arch, and the radial and ulnar arteries, and often manifests later in the course of the disease as pulmonary arterial hypertension and refractory digital ulcers. Given that decreased vascular compliance due to fibrosis potentially contributes to the low NO production activity in SSc endothelial cells, FMD values may reflect the severity of proliferative obliterative vasculopathy in this disease. Consistent with this idea, the decrease in FMD values was linked to the high prevalence of digital ulcers and elevated RVSP in lcSSc patients. These results suggest that FMD values serve as a useful marker to evaluate the severity of proliferative obliterative vasculopathy in lcSSc patients. In contrast to lcSSc, we failed to detect the correlation of decreased FMD values with clinical symptoms associated with proliferative obliterative vasculopathy in dcSSc. A plausible explanation is that most of dcSSc patients enrolled in this study had been already treated with corticosteroids and/or immunosuppressants since the early stage of the disease, which potentially affect the natural course of the disease including vascular changes [30, 31]. Therefore, further studies are required to evaluate the clinical significance of FMD in dcSSc patients.

A series of studies regarding the serum levels of angiogenic factors has revealed that the angiogenic process is aberrantly activated throughout the disease course of SSc. For instance, Michalska-Jakubus et al. [32] demonstrated that serum levels of angiopoietin-2 (Ang2), a potent pro-angiogenic factor exerting its biological effect through Tie1 and Tie2 tyrosine kinase receptors, are significantly elevated in intermediate/late SSc than in early SSc. Furthermore, serum Ang2 levels correlate positively with MRSS and inversely with %DLco and, more importantly, are independently associated with the European Scleroderma Study Group disease activity index score in multivariate regression analysis. These results suggest that Ang2 serves as a potent biomarker for the activity and severity of SSc, reflecting aberrant angiogenesis underlying the pathogenesis of this disease. The elevation of serum Ang2 levels along with the progression of the disease stage indicates that aberrant angiogenesis is generally magnified along with disease duration in SSc. Consistent with this notion, the decrease in FMD values, which is at least partially attributable to vascular fibrosis caused by aberrant angiogenesis, was exacerbated along with disease duration in lcSSc. Thus, FMD values may be useful as a marker for the severity of vascular involvements, as well as serum markers, in SSc. To address this issue more clearly, further studies with a larger number of cases are required in the future.

In summary, this report is the first demonstrating the association of FMD values with the severity of proliferative obliterative vasculopathy in SSc. Although the present conclusions are still preliminary due to the small number of samples and the drug-induced modification of disease course in dcSSc, the present data further support the previous finding that FMD values serve as a marker of vascular complication in certain pathological conditions, including SSc.

**Conflict of interest** None.

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# Elevated plasma homocysteine level is possibly associated with skin sclerosis in a series of Japanese patients with systemic sclerosis

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## ABSTRACT

**Homocysteine is a sulfhydryl-containing amino acid that is derived from dietary methionine, and there has been increasing evidence that elevated plasma homocysteine levels are associated with increased risk of cardiovascular diseases, including carotid, coronary and peripheral arterial disease (PAD). The association of plasma homocysteine levels with peripheral vascular involvements, such as Raynaud phenomenon (RP), digital ulcers (DU) in systemic sclerosis (SSc) patients has not been well studied. The objective of this study was to examine plasma homocysteine levels and their clinical associations in patients with SSc. Plasma homocysteine levels in 151 Japanese patients with SSc and 20 healthy controls were examined. No significant differences were observed in plasma homocysteine levels between SSc patients and healthy individuals. Demographic and clinical features of the SSc patients revealed that severe skin sclerosis, anti-topoisomerase I antibody positivity, complications of DU, acro-osteolysis (AO) and interstitial lung disease (ILD) were significantly more prevalent among the patients with elevated plasma homocysteine levels. The plasma homocysteine levels were positively correlated with modified Rodnan total skin score. The plasma homocysteine levels in the SSc patients with DU, AO and ILD were significantly higher than those in the SSc without DU, AO and ILD, respectively. Plasma homocysteine levels did not correlate with either the mean or max intima-media thickness (IMT) or plaque score, suggesting that plasma homocysteine levels might not be associated with carotid artery atherosclerosis in SSc patients. The measurement of plasma homocysteine levels in SSc patients might be useful for the risk stratifications of severe skin sclerosis, DU and AO.**

Key words: carotid intima-media thickness, digital ulcers, homocysteine, skin sclerosis, systemic sclerosis.

## INTRODUCTION

Homocysteine is a sulfhydryl-containing amino acid derived from dietary methionine. Homocystinuria is a rare genetic disorder characterized by hyperhomocysteinemia that is caused by mutations in cystathione-β-synthase, and leads to early onset atherosclerosis and arterial and venous thromboses. Experimental studies have shown that homocysteine induces vascular damage through mechanisms that include endothelial injury and modifications in circulating mediators that regulate vascular tone.[1–3] Additionaly, several studies have reported that elevated plasma homocysteine levels are associated with increased risks of cardiovascular diseases, including carotid, coronary and peripheral arterial disease (PAD).[4–7] Furthermore, many pathophysiological considerations and several clinical studies suggest that elevations in homocysteine plasma level might be relevant to the pathogenesis of the Raynaud phenomenon (RP).[1,8] These findings suggest that homocysteine might play roles in the pathogeneses of both central and peripheral vascular disorders.

Systemic sclerosis (SSc) is a connective tissue disease that is characterized by fibrosis of the skin and internal organs and vascular dysfunction. Patients with SSc typically reveal RP and persistent digital ischemia and occasionally develop digital ulcers (DU).[9] We recently reported that the demographic and clinical features of the SSc patients with young age, male sex, anti-topoisomerase I antibody positivity, severe skin sclerosis, interstitial lung disease complication and cardiac involvements are significantly more prevalent in patients with DU.[10]

With respect to SSc and homocysteine, it has been reported that plasma homocysteine levels are higher in SSc patients compared to healthy controls.[11–13] Only one study has shown the correlation between plasma homocysteine levels and nailfold videocapillaroscopic patterns in SSc.[11] However, the associations of plasma homocysteine levels with both central and peripheral vascular involvements, such as RP, DU and carotid

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artery stenosis in SSc patients have not been well studied. Furthermore, only one study has shown that higher homocysteine levels were found in patients with moderate skin involvement in comparison with mild skin involvement,[11] suggesting that the association of homocysteine levels with skin sclerosis in SSc patients has also not been well studied. In the present study, we analyze the demographic and clinical features of SSc patients with elevated plasma homocysteine levels and the associations of plasma homocysteine levels with skin sclerosis, central and peripheral vascular disorders.

## METHODS

### Patients

We analyzed 151 Japanese patients with SSc who visited Gunma University Hospital from 2011 to 2014. All of the patients fulfilled the criteria for SSc proposed by the American College of Rheumatology.[14] The patients were classified as having the limited cutaneous type (lcSSc) or diffuse cutaneous type (dcSSc) of SSc according to the classification of LeRoy *et al.*[15] This study was approved by the local research ethics committee of Gunma University. The patients provided written informed consent before participation. Twenty age- and sex-matched healthy Japanese individuals (18 female and 2 male; age 50.1 ± 5.24 years) were used as healthy controls.

### Clinical and laboratory assessments

Skin sclerosis was measured using the modified Rodnan total skin score (mRTSS). Interstitial lung disease (ILD) was detected as the presence of bibasilar interstitial fibrosis or a ground-glass shadow on high-resolution computed tomography (HRCT) scans. Pulmonary artery hypertension (PAH) was defined as an elevated right ventricular systolic pressure (>45 mmHg) as assessed echocardiography and subsequently as an elevated mean pulmonary artery pressure (>25 mmHg) as assessed by cardiac catheterization. Homocysteine levels were determined by the high-performance liquid chromatography method with fluorescent detection as previously described.[16]

### Measurements of carotid IMT and plaques

The wall thickness and plaques of the bilateral carotid arteries were measured with an ultrasound instrument (Aplio TUS-A400; Toshiba, Tokyo, Japan) using a 7.5-MHz linear type B-mode probe. Intima-media thickness (IMT) was defined as the distance from the leading edge of the lumen-intima interface to the media-adventitia interface of the far wall, and averaged to obtain the mean IMT. The max IMT was defined as the single thickest wall. The presence of a plaque was defined as a thickening of the IMT >1.1 mm. According to previous reports, the plaque score was calculated by summing the maximum axial thickness of all plaques in both carotid systems.[17] All scans were evaluated by ultrasonographic physicians who were blinded to the clinical features of patients.

### Statistics

The Mann–Whitney *U* test was used to compare homocysteine levels. Analyses of variance (ANOVAs) followed by Bonferroni's post tests were used for multiple comparisons. Chi-square analysis was used to compare frequencies. Spearman's rank correlation coefficients were used to examine the relationship between two continuous variables. The error bars represent the standard errors of the means.

## RESULTS

### Plasma homocysteine levels in SSc patients

In total, 151 SSc patients were enrolled in this study, including 42 (27.8%) dcSSc and 109 (72.2%) lcSSc patients. It has been reported that plasma homocysteine levels are elevated in SSc patients compared to healthy controls.[11–13] Therefore, the plasma homocysteine levels in our patients and healthy individuals were also assessed. The plasma homocysteine levels in the SSc patients were not significantly different from those in the healthy individuals (SSc 9 ± 0.3 μmol/L vs healthy individuals 8.2 ± 0.6 μmol/L). Additionally, plasma homocysteine levels in both dcSSc (9.7 ± 0.6 μmol/L) and lcSSc (8.7 ± 0.3 μmol/L) patients were also comparable to those in the healthy individuals. There was no difference in the plasma homocysteine levels of the two SSc subgroups (Fig. 1).

### Demographic and clinical features of SSc patients with and without elevated serum homocysteine levels

The demographic and clinical features of the SSc patients with and without elevated serum homocysteine levels are summarized in Table 1. Elevated levels were defined as values greater than the mean + 2 SD (13.2 μmol/L) of the plasma samples from the healthy controls. We identified 14 SSc patients with elevated plasma homocysteine levels (9.3%, mean age, 65.4) and 137 SSc patients with normal plasma homocysteine levels (90.7%) (mean age, 62.8). The mRTSS was significantly higher

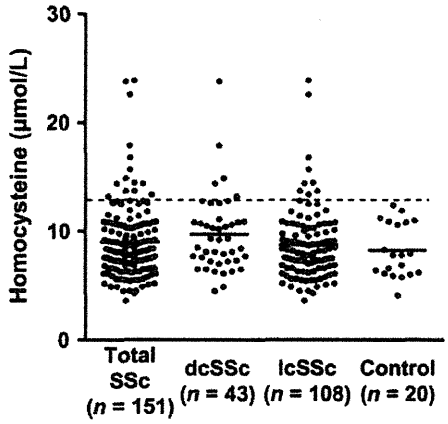


**Figure 1.** Plasma homocysteine levels in patients with systemic sclerosis (SSc), diffuse cutaneous type SSc (dcSSc), limited cutaneous type SSc (lcSSc) and in healthy individuals (control). Bars indicate mean values in each group. Dotted line indicates the cut-off value (mean + 2 SD of control values: 13.2 μmol/L).

**Table 1.** Demographic, clinical features of SSc patients with or without elevated serum homocysteine levels

| | Elevated homocysteine ($n$ = 14) | Normal homocysteine ($n$ = 137) | $P$ |
|---|---|---|---|
| Age (years; mean ± SE) | 65.4 ± 3 | 62.8 ± 0.9 | 0.31 |
| mRTSS (mean ± SE) | 10.9 ± 1.9 | 6.8 ± 0.7 | <0.01 |
| Sex | | | |
| Male (%) | 0 (0/14) | 11.7 (16/137) | 0.18 |
| Female (%) | 100 (14/14) | 88.3 (121/137) | |
| Type | | | |
| lcSSc (%) | 64.3 (9/14) | 73 (100/137) | 0.49 |
| dcSSc (%) | 35.7 (5/14) | 27 (37/137) | |
| Autoantibody | | | |
| ANA (%) | 100 (14/14) | 85.4 (117/137) | 0.13 |
| Topo I (%) | 50 (7/14) | 20.4 (28/137) | <0.05 |
| RNP (%) | 0 (0/14) | 13.1 (18/137) | 0.15 |
| Centromere (%) | 28.6 (4/14) | 46 (63/137) | 0.21 |
| RNAP (%) | 14.3 (2/14) | 3.6 (5/137) | 0.07 |
| Complication | | | |
| RP (%) | 85.7 (12/14) | 83.9 (115/137) | 0.86 |
| DU (%) | 64.3 (9/14) | 23.4 (32/137) | <0.01 |
| AO (%) | 35.7 (5/14) | 8.8 (12/137) | <0.01 |
| PAH (%) | 14.3 (2/14) | 6.6 (9/137) | 0.29 |
| Cardiac involvements (%) | 7.1 (1/14) | 17.3 (24/137) | 0.32 |
| ILD (%) | 78.6 (11/14) | 39.4 (54/137) | <0.01 |

ANA, antinuclear antibody; AO, acro-osteolysis; Centromere, anti-centromere antibody; CI, cardiac involvements; dcSSc, diffuse cutaneous type of SSc; DU, digital ulcers; ILD, interstitial lung disease; lcSSc, limited cutaneous type of SSc; mRTSS, modified Rodnan total skin score; PAH, pulmonary artery hypertension; RNAP, anti-RNA polymerase III antibody; RNP, anti-U1 RNP antibody; RP, Raynaud phenomenon; Topo I, anti-topoisomerase I antibody.

in the SSc patients with elevated plasma homocysteine levels (10.9 vs 6.8, $P < 0.01$). Consistent with the association between elevated homocysteine levels and skin sclerosis, the plasma homocysteine levels were positively correlated with mRTSS ($P < 0.01$, Fig. 2). The SSc patients with elevated plasma homocysteine levels were more frequently positive for anti-topoisomerase I Ab (Topo I) than the SSc patients with normal homocysteine levels (50% vs 20.4%, $P < 0.05$). Acro-osteolysis (AO; i.e., resorption of the terminal tuft of the digit) is a characteristic feature of SSc, and it is thought that AO is associated with severe digital ischemia due to severe skin sclerosis, the Raynaud phenomenon (RP) and chronic vasculopathy.[18] The prevalence of digital ulcer (DU), AO and ILD in the SSc patients with elevated plasma homocysteine levels were significantly greater than those in the SSc patients with normal homocysteine levels (DU 64.3% vs 23.4%, $P < 0.01$, AO 35.7% vs 8.8%, $P < 0.01$, ILD 78.6% vs 39.4%, $P < 0.01$, respectively). In contrast, no significant difference in the prevalence of RP, PAH or cardiac involvement were observed between the two groups. These results suggest that severe skin sclerosis, Topo I positivity, and the complication of DU, AO and ILD were significantly more frequent among the SSc patients with elevated plasma homocysteine levels than among the SSc patients with normal homocysteine levels.

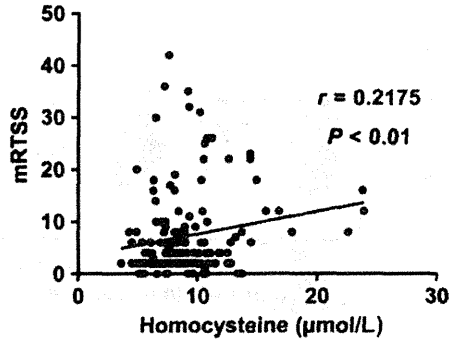


**Figure 2.** Correlation of plasma homocysteine levels with modified Rodnan total skin thickness score (mRTSS) in SSc patients. Solid line represents the regression line. Plasma homocysteine levels significantly correlated with mRTSS ($r$ = 0.2175, $P < 0.01$, Spearman's rank correlation test).

### Plasma homocysteine levels in the presence and absence of complications in the SSc patients

Next, we analyzed the plasma homocysteine levels in the presence or absence of complications in SSc. The plasma homocysteine levels of the SSc patients with DU were significantly higher than those of the SSc patients without DU (DU+ 10.7 ± 0.8 μmol/L vs DU− 8.4 ± 0.2 μmol/L, $P < 0.05$, Fig. 3b). The plasma homocysteine levels in the SSc patients

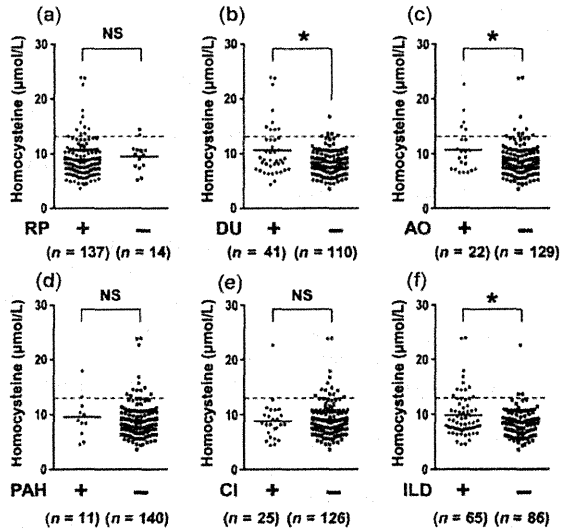


**Figure 3.** Plasma homocysteine levels in patients with systemic sclerosis with and without (a) Raynaud phenomenon (RP), (b) digital ulcers (DU), (c) acro-osteolysis (AO), (d) pulmonary artery hypertension (PAH), (e) cardiac involvements (CI), and (f) interstitial lung disease (ILD). Bars indicate mean values in each group. Dotted line indicates the cut-off value (mean + 2 SD of control values: 13.2 μmol/L). *$P < 0.05$. NS, not significant.

with AO were significantly higher than those in the SSc patients without AO (AO+ 10.7 ± 0.9 μmol/L vs AO− 8.7 ± 0.3 μmol/L, $P < 0.05$, Fig. 3c). However, the plasma homocysteine levels did not significantly differ between the patients with and without RP (RP+ 8.9 ± 0.3 μmol/L vs RP− 9.5 ± 0.7 μmol/L, $P = 0.25$, Fig. 3a).

Consistent with the results shown in Table 1, the plasma homocysteine levels of the SSc patients with ILD were higher than those of the patients without ILD (ILD+ 9.8 ± 0.5 μmol/L vs ILD− 8.4 ± 0.3 μmol/L, $P < 0.05$, Fig. 3f), however, the plasma homocysteine levels in the SSc patients with and without PAH and those with and without cardiac involvements (CI) were not different (PAH+ 9.5 ± 1.2 μmol/L vs PAH− 9 ± 0.3 μmol/L, $P = 0.6$, CI+ 8.8 ± 0.7 μmol/L vs CI− 9.1 ± 0.3 μmol/L, $P = 0.7$, Fig. 3d, e). These results suggest that elevated plasma homocysteine levels might be associated with the presence of DU, AO and ILD in SSc patients.

### Relationship between serum homocysteine level and the extent of carotid artery atherosclerosis in SSc patients

It has been reported that elevated plasma homocysteine levels are associated with increased risks of PAD and carotid artery stenosis.[4–7] Patients with PAD frequently have hyperlipidemia and atherosclerosis including carotid artery stenosis.[19] To analyze the association between hyperlipidemia and plasma homocysteine in the SSc patients, the serum cholesterol and TG were compared. The levels of serum cholesterol and TG were not significantly different between the SSc patients with elevated plasma homocysteine and those with normal plasma homocysteine levels (cholesterol [mg/dL] 199.2 ± 3 vs 204.8 ± 11.9, TG [mg/dL] 135.6 ± 5.7 vs 154.6 ± 14.4). Among the SSc patients, 7.1% (1/14) of the patients with elevated plasma homocysteine and 8.8% (12/137) of the patients with normal plasma homocysteine levels underwent treatment for hyperlipidemia ($P = 0.84$).

Next, we analyzed the relationship between plasma homocysteine level and the extent of carotid artery atherosclerosis in the SSc patients. Carotid IMT and plaques are associated with traditional risk factors for atherosclerosis and are predictors of future cardiovascular events. Additionally, it has been reported that carotid IMT and plaques are markedly higher in patients with PAD and that PAD is a meaningful risk factor for carotid artery stenosis.[19] We found that plasma homocysteine levels were not correlated with either the mean or max IMTs or the plaque scores (mean IMT, $P = 0.737$, max IMT, $P = 0.195$, plaque score, $P = 0.847$) (Fig. 4). These results suggest that plasma homocysteine levels might not be associated with carotid artery atherosclerosis in SSc patients.

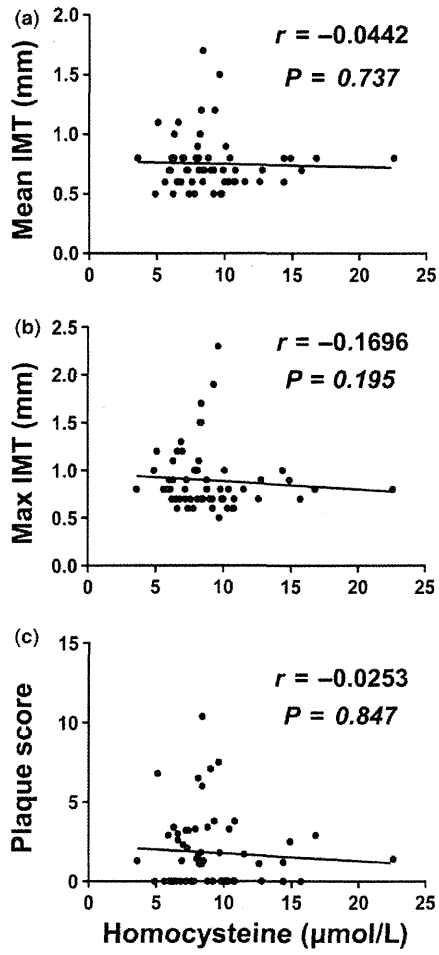


**Figure 4.** Correlation of plasma homocysteine levels with (a, b) mean and max carotid IMT and (c) plaque score in SSc patients. Solid line represents the regression line.

## DISCUSSION

Several studies have reported that plasma homocysteine levels are elevated in SSc patients compared to healthy controls.[11–13] In contrast, two studies have reported no significant difference in homocysteine levels between SSc patients and controls.[20] In our study, plasma homocysteine levels were comparable between SSc patients and healthy individuals. However, we identified that plasma homocysteine levels were correlated positively with mRTSS, and that Topo I was associated with elevated plasma homocysteine levels in SSc, suggesting that elevated plasma homocysteine levels may be associated with the extent of skin sclerosis in SSc.

In addition, we identified the complication of DU, AO and ILD were significantly associated with elevated plasma homocysteine levels in SSc patients. Our findings are consistent with those of previous studies of SSc and homocysteine that have shown that homocysteine plasma level is related to severity of lung impairment in SSc.[12] However, there has been no report that assessed the associations of plasma homocysteine with DU and AO. Regarding RP and plasma homocysteine levels, several studies have investigated plasma homocysteine levels in patients with RP, and the result remains a controversial issue.[1,8,13] In our study, elevated plasma homocysteine levels

were not associated with the presence of RP in SSc patients, suggesting that homocysteine might not be associated with peripheral vasculopathy. Since DU and AO are caused by severe digital ischemia due to severe skin sclerosis and/or chronic peripheral vascular disorders, the severe skin sclerosis may be involved in the pathogenesis of the high prevalence of DU and AO in the SSc patients with elevated plasma homocysteine levels.

Evidence from case-control studies and prospective studies supports the associations between elevated plasma homocysteine levels and increased cardiovascular risks, such as ischemic heart disease and stroke.[21] In our study, neither cardiovascular involvement nor PAH were associated with elevated plasma homocysteine levels in SSc patients. Furthermore, we found no associations of plasma homocysteine levels with either hyperlipidemia or atherosclerosis based on measurements of carotid IMT and plaques. These findings suggest that the mechanisms of cardiovascular involvement in SSc might be different from those of atherosclerotic diseases, including PAD and hyperlipidemia. Additionally, these findings suggest that plasma homocysteine levels might not be associated with central and peripheral vascular disorders.

The mechanisms that cause sclerosis in SSc patients with hyperhomocysteinemia are unknown. However, some reports using animal models of homocysteinemia suggested that the oxidative stress might play a role on inflammation and fibrosis in heart or liver.[22–26] In addition, Raaf *et al.*[26] showed that TGF-β levels were enhanced in hyperhomocysteinemic rats, suggesting that elevated plasma homocysteine level is possibly associated with skin sclerosis in patients with SSc.

Plasma homocysteine levels are affected by the amounts of folic acid and vitamin B in the blood. It has been reported that the elevated plasma homocysteine levels in SSc patients was due to nutritional deficiency rather than inherited factors.[13] However, another study indicated that folate levels in SSc patients were adequate.[11] In the present study, we were unable to assess plasma levels of folic acid and vitamin B, therefore further investigations are required. It has been reported that folic acid and vitamin B supplementation reduce plasma homocysteine levels, however, these reductions failed to significantly affect cardiovascular involvement, which suggests that homocysteine may be a marker rather than a cause of cardiovascular involvement.[27] The clarification of this issue requires further investigation.

**CONFLICT OF INTEREST:** The authors declare there are no conflicts of interest.

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Original Article

# Role of endothelin-1/endothelin receptor signaling in fibrosis and calcification in nephrogenic systemic fibrosis

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**Abstract:** Nephrogenic systemic fibrosis (NSF) is characterized by systemic fibrosis and abnormal calcification in patients with severe renal dysfunction. It is considered that gadolinium (Gd)-containing contrast agents used for magnetic resonance imaging trigger the development of NSF. However, the causative role of Gd and the mechanism of Gd-induced fibrosis and calcification in NSF are unknown. Recently, it has been known that endothelin-1 (ET-1)/ET receptor (ETR) signalling regulates fibrosis and calcification. The objective was to elucidate the role of ET-1/ETR signalling in Gd-induced fibrosis and calcification in NSF. First, we demonstrated that Gd enhanced proliferation and calcification of human adipose tissue-derived mesenchymal stem cells (hMSC) *in vitro*. Next, we examined the expression of ET-1 and ETR-A in hMSC using proliferation or calcification assay. ET-1 and ETR-A expression in hMSC treated with Gd were elevated. ET-1/ETR signalling inhibitor, bosentan, inhibited Gd-induced proliferation and calcification of hMSC. In addition, bosentan inhibited Gd-induced phosphorylation of ERK and Akt in hMSC. Plasma ET-1 levels of the patients were significantly higher than these of normal individuals and systemic sclerosis patients. In immunofluorescence staining, the expression of ETR-A in fibroblasts in dermal fibrosis lesion of NSF was increased. We conclude that Gd induces proliferation and calcification of hMSC via enhancement of ET-1/ETR signalling. Our results contribute to understand the pathogenesis of NSF.

**Key words:** calcification – endothelin-1 – fibrosis – mesenchymal stem cells – nephrogenic systemic fibrosis

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## Introduction

Nephrogenic systemic fibrosis (NSF), also known as nephrogenic fibrosing dermopathy, is a systemic fibrosing disorder primary in patients with chronic renal failure undergoing dialysis (1–6). The development of NSF is strongly associated with the administration of gadolinium (Gd)-based contrast agents (GBCAs) during magnetic resonance imaging (MRI) in the patients with chronic renal failure (4). Gd deposition has been observed in the skin of NSF patients in many reports, suggesting that free Gd ions directly affect cells in skin and other organs (7,8). Histologically, $CD34^+$ and procollagen-I-producing spindle cells were frequently observed in the middle to deep dermis. These cells have been considered as circulating fibrocytes, which belong to mesenchymal stem cells (MSCs) derived from bone marrow, suggesting that these cells might be involved in the pathogenesis of fibrosis in NSF (2,9–11).

Patients with NSF have been reported showing an abnormal calcification in the dermis and subcutis, including dystrophic calcinosis cutis, metastatic calcinosis, osseous metaplasia and vascular calcification (12–14). Abnormal calcification of NSF might be associated with osteogenic differentiation of MSCs, leading to the mineralization of the extracellular matrix. In our previous studies, we demonstrated that $GdCl_3$ induced calcium deposition in human adipocyte MSC *in vitro*, suggesting that $CD34^+$ fibrocytes (and/or MSC) in dermis of lesion might account for the calcification in NSF (15).

Endothelin-1 (ET-1) is mainly produced by endothelial cells and other cells such as monocytes and macrophages (16,17). ET-1 exerts biological effects through ET receptor A (ETR-A) and B (ETR-B). ETR-A is mainly present in vascular smooth muscle cells (VSMC) and regulates vascular contraction and cell growth (17). ETR-B is mainly localized in endothelial cells (EC). ET-1 has been implicated in the pathogenesis of fibrosis in many organ systems, including skin, lung and heart (18,19). ET-1 induces the production of collagen type I and III and fibronectin via ET receptor A and B on fibroblasts (19). In addition, ET-1 is recognized to be chemotactic for fibrocytes (18). Plasma ET-1 levels were higher in patients with systemic sclerosis (SSc) than in normal controls (20,21), suggesting that ET-1 is associated with the pathogenesis of fibrosis in SSc (22). Bosentan, a non-specific ETR antagonist, can block the effect of ET and has been shown to be effective in the treatment of pulmonary arterial hypertension (PAH) in patients with SSc (23). Bosentan was also reported to be effective to reduce skin fibrosis in patients with SSc (24).

Regarding calcification and ET-1, it has been reported that expression of mRNA and protein levels of ET-1 were increased in calcified VSMC (25). ET-1 induces the intra-cellular calcium concentration and stimulates both proliferation and the formation of bone nodules in osteoblastic progenitor cells (26,27). These data indicate that ET-1/ETR signalling is involved in the regulation of fibrosis and calcification. Therefore, we hypothesized that ET-1/ETR signalling might play a role in Gd-induced fibrosis and calcification in NSF. Herein, we demonstrated that $GdCl_3$ induces proliferation and calcification of hMSC via enhancement of ET-1/ETR signalling, that bosentan can inhibit Gd-induced proliferation and calcification of hMSC and that serum ET-1 level and the expression of ETR-A in lesional skin in patients with NSF were increased.

## Materials and methods

### Cell culture and calcification assay

Adult human adipose tissue-derived mesenchymal stem cells (hMSC) (Zen-Bio, Research Triangle Park, NC, USA) was maintained Mesenchymal Stem Cell Growth Medium (Lonza, Walkersville, MD, USA) and used before passage 10. HUVEC were maintained in EBM-2 basal medium (Lonza) supplemented with EGM-2 Single Quot Kit Suppl. & Growth Factors (Lonza). Calcification assay was performed as described before (15). Briefly, hMSC was plated on 96-well plates at the density of 1000 cells per well and incubated with Differentiation Basal Medium–Osteogenic supplemented with hMSC Osteogenic SingleQuots (Lonza) with or without $GdCl_3$ and/or bosentan (Actelion Pharmaceuticals Ltd., Allschwil, Switzerland) for 2 weeks. The medium was changed twice a week. To examine the extent of calcification, alizarin red S staining was performed. After incubation of hMSC with osteogenic medium, cells were washed once with phosphate-buffered saline (PBS), fixed with cold 70% ethanol for 60 min and washed with distilled water. Cells were stained with 40 mM alizarin red S (pH 4.2) for 15 min. Non-specific staining was removed by five times washes with water and 5 min incubation in PBS, and then stained cells were photographed. For quantification, cells were incubated with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 15 min with shaking. The concentration of alizarin red S were analysed by measurement of absorbance at 562 nm using microplate reader.

### Proliferation assay

Cell proliferation was measured using the MTS assay. hMSC was treated with trypsin–EDTA and plated at a density of 5000 cells per well in 96-well plates. Cells were treated with or without ET-1 (1 μM), $GdCl_3$ (50 μM) and/or bosentan. After 48 h of incubation at 37°C, 20 μl of CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI, USA) was added. After an additional incubation at 37°C for 4 h, the absorbance at 490 nm was measured using an ELISA plate reader.

### RNA extraction and real-time RT-PCR

Total RNA was isolated using RNeasy Mini Kits (Qiagen, Valencia, CA, USA) and was subjected to reverse transcription with the use of a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative RT-PCR was performed via the TaqMan system (Applied Biosystems, Foster City, CA, USA) using 7300 Real-Time PCR systems (Applid Biosystems) according to the manufacturer's instructions. TaqMan probes and primers for ET-1, ETR-A and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems. As an internal control, levels of GAPDH were quantified in parallel with target genes. Normalization and fold changes were calculated using the comparative Ct method.

### Flow cytometry analysis

For examination of surface expression of CD34, hMSC was incubated in normal medium with or without $GdCl_3$ (50 μM) for 24 or 48 h, then hMSC was washed and incubated consecutively at 4°C with Alexa 488-conjugated anti-human CD34 Ab or isotype control Ab (BioLegend, San Diego, CA, USA) before flow cytometric analysis with a FACS Calibur instrument and CellQuest software (BD Biosciences, San Jose, CA).

### Immunoblot assay

hMSC was incubated in normal medium with or without $GdCl_3$ (50 μM) or bosentan (50 μM) for 30 min. After washing with ice-cold PBS, cells were disrupted in lysis buffer (20 mM Tris-HCl pH 7.6, 140 mM NaCl, 1% Nonidet P-40) containing 1 mM phenylmethylsulfonylfluoride, aprotinin (10 mg/ml) and 1 mM sodium vanadate. Lysates were centrifuged at 10 000 × g for 15 min at 4°C and the resulting supernatants were subjected to SDS-PAGE, followed by immunoblot analysis using anti-phospho-Akt (Ser473) pAb, anti-Akt pAb, anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) pAb, anti-44/42 MAPK (Erk1/2) pAb (Cell Signalling, Danvers, MA, USA). Anti-rabbit HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used with ECL (Thermo Scientific, Rockford, IL, USA) to image immunoblots. Densitometric analysis of blots was accomplished using ImageJ software version 1.46r (NIH, Bethesda, MD, USA). Images were obtained and saved using 16-bit scanner. The relative density of the contents of the rectangle, which was drawn around each lane, was represented and calculated using gel analyse tool in Image J.

### Patients of NSF

Patient 1: One of author (O. I.) previously reported a case of NSF in a 50-year-old Japanese man, who had a skin sclerosis on his extremities. He had chronic renal failure and had been treated with haemodialysis. He had undergone magnetic resonance angiography using gadodiamide. Histologically, increased collagen fibres were observed in the deep dermis, and thickened fascia was also noted, as well as osseous metaplasia under the fascia. Computed tomography of the whole body revealed multiple calcification of the fascia in many muscles (13). Patient 2: One of author (F. M.) previously reported a case of NSF in a 14-year-old Japanese man, who had a skin sclerosis on his hands, lower legs and feet. He had chronic renal failure due to hydronephrosis and had been treated with haemodialysis. He had undergone magnetic resonance imaging using gadodiamide. The part of skin sclerosis in lower legs and hand was improved at 6 years after onset; however, strong skin sclerosis with stiffening of fingers in left foot and left hand was still remained (28).

### Human serum and skin

The serum was obtained from Japanese patients with systemic sclerosis (SSc) or NSF, and normal healthy volunteers. SSc patients (11 diffuse cutaneous type; 11 women; mean age 57.2 ± 2.8 years; mean disease duration 7.6 ± 1.2 years) fulfilled the criteria for SSc proposed by American College of Rheumatology (29). All SSc patients had severe skin sclerosis. Skin sclerosis was measured using the modified Rodnan total skin score (mRTSS), and mean mRTSS was 18.5 ± 3.1. Skin specimens were obtained from a hard massive nodule in right lower extremity in NSF patient and forearm in healthy volunteers. The study was approved by the institutional review board and the local research ethics committee of Gunma University and Yamagata University. All patients and volunteers provided written informed consent before participation. This study was conducted according to the Declaration of Helsinki principles.

### Enzyme-linked immunosorbent assay

The serum levels of human ET-1 were quantified by ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

### Immunofluorescence and immunohistological staining

hMSC was seeded in 8-well culture slides (BD Bioscience, San Jose, CA, USA), and cells were treated with or without Gd (50 $\mu$M) for 24 h and then fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 30 min. After being blocked with 3% dry milk–PBS supplemented with 5% normal goat serum for 1 h at room temperature, cells were stained with rabbit anti-ETR-A pAb (5 $\mu$g/ml; Abcam, Cambridge, UK) followed by Alexa 488-conjugated secondary Ab (Invitrogen). Cells were mounted in ProLong Gold antifade reagent (Invitrogen).

Human tissue was embedded in paraffin and sectioned 3 $\mu$m thick. The sections were deparaffinized with xylene for 30 min. Tissue sections were treated for antigen retrieval with a pressure cooker for 10 min at 120°C. After blocking using Peroxidase Blocking (Dako, Copenhagen, Denmark) for 5 min and Protein Block (Dako) for 10 min, sections were incubated with anti-ETR-A pAb (4 $\mu$g/ml; Abcam) for 2 h at room temperature. After washing, the sections were incubated with a horseradish peroxidase-labelled polymer-conjugated anti-mouse secondary antibody (ENVISION+; Dako) for 1 h at room temperature. Finally, colour was developed with 3,3′-diaminobenzidine tetrahydrochloride.

### Statistics

*P* values were calculated using the Student's *t*-test (two-sided) or by analysis of one-way ANOVA followed by Bonferroni's post-test as appropriate. Error bars represent standard errors of the mean, and numbers of experiments (n) are as indicated.

## Results

### Increase in proliferation and the expression of ET-1 and ET receptor A in hMSC by $GdCl_3$

To examine the involvement of ET-1/ETR signalling in $GdCl_3$-induced proliferation of hMSC, we analysed the expression of ET-1 and ETR-A in hMSC treated with or without $GdCl_3$. Proliferation of hMSC treated with $GdCl_3$ was 1.5-fold higher than that with no treatment (Fig. 1a). This result was consistent with our previous result (15). In this condition, mRNA level of ET-1 expression in hMSC was significantly increased by $GdCl_3$ treatment (Fig. 1b). In addition, mRNA level of ETR-A expressions in hMSC were significantly increased by $GdCl_3$ treatment in a dose-dependent manner (Fig. 1c and d). In immunofluorescence staining, the expression of ETR-A on the surface of hMSC was also increased by $GdCl_3$ treatment (Fig. 1e). These results suggest that increases in ET-1 and ETR-A expression are associated with $GdCl_3$-induced proliferation of hMSC.

### Increase in calcium deposition and the expression of ET-1 and ET receptor A in hMSC by $GdCl_3$

Next, we analysed the involvement of ET-1/ETR signalling in $GdCl_3$-induced calcium deposition in hMSC. As we previously reported, $GdCl_3$ enhanced calcium deposition in hASC incubated with osteogenic differentiation medium (Fig. 2a). In this condition, mRNA level of ET-1 expression in hMSC incubated in osteogenic medium with $GdCl_3$ was increased by 1.4-fold compared with that without $GdCl_3$ (Fig. 2b). In addition, mRNA level of ETR-A expression in hMSC treated with $GdCl_3$ was increased by 1.8-fold compared with that without $GdCl_3$ (Fig. 2c). These results suggest that increases in ET-1 and ETR-A expression are associated with $GdCl_3$-induced calcium deposition in hMSC.

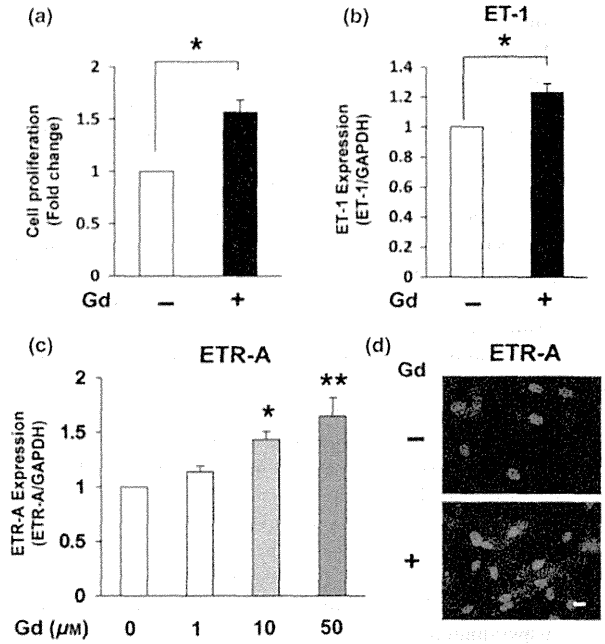


**Figure 1.** Increase in proliferation and the expression of ET-1 and ET receptor A in hMSC by $GdCl_3$. (a) Proliferation in hMSC treated with or without $GdCl_3$. hMSC was incubated in normal medium with or without $GdCl_3$ (50 $\mu$M) for 48 h. (b) ET-1 mRNA levels in hMSC using quantitative RT-PCR (normalized to GAPDH mRNA levels). hMSC was incubated in normal medium with or without $GdCl_3$ (50 $\mu$M) for 24 h. (c) ETR-A mRNA levels in hMSC using quantitative RT-PCR (normalized to GAPDH mRNA levels). hMSC was incubated in normal medium with or without $GdCl_3$ (0, 1, 10 and 50 $\mu$M) for 24 h. All values represent means ± SEM from n = 3 experiments and relative to mRNA levels of hMSC incubated in medium without $GdCl_3$. *$P$ < 0.05, **$P$ < 0.01. (d) ETR-A expression on the surface of hMSC. hMSC was incubated in normal medium with or without $GdCl_3$ (50 $\mu$M) for 24 h. Scale bar = 20 $\mu$m.

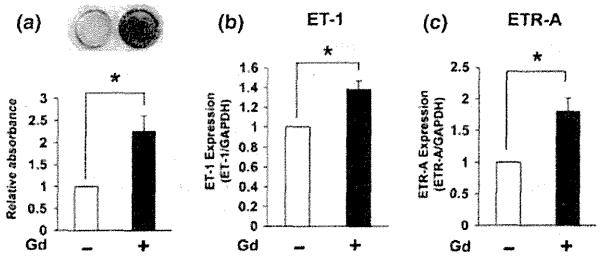


**Figure 2.** Increase in calcium deposition and the expression of ET-1 and ET receptor A in hMSC by $GdCl_3$. (a) The amount of calcium deposition in hMSC treated with or without $GdCl_3$. hMSC was incubated in osteogenic differentiation medium with or without $GdCl_3$ (50 $\mu$M) for 2 weeks. Calcium deposition was visualized by alizarin red S staining. Concentration of Alizarin red S was determined by absorbance measurement. Data are means ± SEM from n = 3 experiments and relative to the amount in hMSC incubated in medium without $GdCl_3$. (b, c) ET-1 (b) and ETR-A (c) mRNA levels in hMSC using quantitative RT-PCR (normalized to GAPDH mRNA levels). hMSC was incubated in osteogenic medium with or without $GdCl_3$ (50 $\mu$M) for 2 weeks. All values are means ± SEM from n = 3 experiments and relative to mRNA levels in hMSC incubated in medium without $GdCl_3$. *$P$ < 0.01.

We also examined the expression of CD34 in hMSC with or without $GdCl_3$ treatment by flow cytometry analysis (Figure S1). CD34 expression in hMSC was not changed by $GdCl_3$ treatment.

### Inhibition of $GdCl_3$-induced proliferation and calcium deposition in hMSC by ET receptor antagonist, bosentan

To assess the role of ET-1/ETR signalling in Gd-induced proliferation and calcium deposition in hMSC, we next examined the influence of ET receptor antagonist, bosentan, on $GdCl_3$-induced proliferation and calcium deposition in hMSC. To confirm the effect of bosentan, we analysed the inhibition effect of bosentan on ET-1-induced MSC proliferation (Figure S2). ET-1-induced proliferation was significantly inhibited by bosentan at 20, 50 and 100 $\mu$M. Therefore, we decided the concentration (50 $\mu$M) of bosentan. $GdCl_3$-induced hMSC proliferation was significantly inhibited by bosentan (Fig. 3a). In addition, $GdCl_3$-induced hMSC proliferation was significantly inhibited by bosentan in a dose-dependent manner, with maximal inhibition occurring at 50 to 100 $\mu$M (Figure S3). Furthermore, $GdCl_3$-induced calcium deposition in hMSC was significantly inhibited by bosentan (Fig. 3b). Both proliferation and calcium deposition in hMSC treated without $GdCl_3$ were not inhibited by bosentan (Fig. 3a and b). These results suggest that ET-1/ETR signalling might involve in $GdCl_3$-induced proliferation and calcium deposition in hMSC.

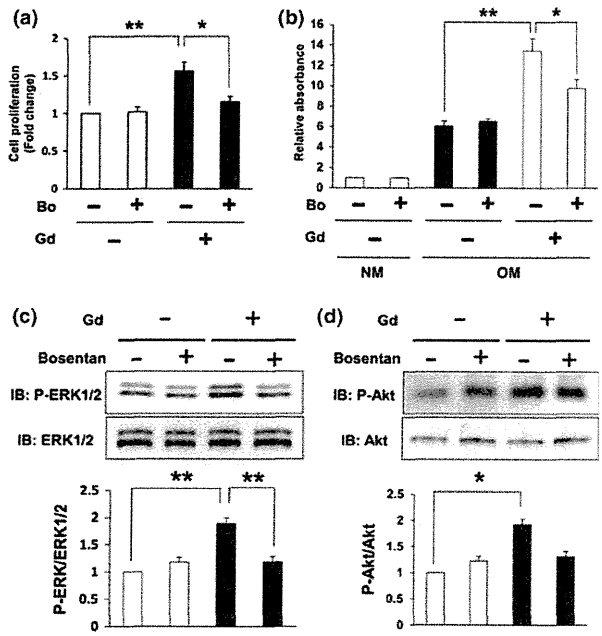


**Figure 3.** Inhibition of $GdCl_3$-induced proliferation and calcium deposition in hMSC by ET receptor antagonist, bosentan. (a) Proliferation in hMSC treated with or without $GdCl_3$ and/or bosentan. hMSC was incubated in normal medium with or without $GdCl_3$ (50 $\mu$M) or bosentan (50 $\mu$M) for 48 h. Values are means ± SEM from n = 3 experiments and normalized to the extent of proliferation in hMSC incubated in medium without $GdCl_3$ and bosentan. (b) The amount of calcium deposition in hMSC treated with or without $GdCl_3$ and/or bosentan. hMSC was incubated in normal medium (NM) or osteogenic differentiation medium (OM) with or without $GdCl_3$ (50 $\mu$M) or bosentan (50 $\mu$M) for 2 weeks. Concentration of Alizarin red S was determined by absorbance measurement. All values represent means±SEM from n = 3 experiments and relative to the amount in hMSC incubated in normal medium without $GdCl_3$ and bosentan. Inhibition of $GdCl_3$-induced phosphorylation of ERK1/2 and Akt in hMSC by ET receptor antagonist, bosentan. Phosphorylation of ERK1/2 (c) and Akt (d) in hMSC treated with or without $GdCl_3$ and/or bosentan by immunoblotting. hMSC was incubated in normal medium with or without $GdCl_3$ (50 $\mu$M) or bosentan (50 $\mu$M) for 30 min. Quantification of relative phosphorylation levels of ERK1/2 and Akt was accomplished via densitometry using ImageJ. The levels of ERK1/2 or Akt phosphorylation in hMSC incubated in medium without $GdCl_3$ and bosentan were assigned a value of 1. All values are means ± SEM from n = 3 experiments. $^*P < 0.05$, $^{**}P < 0.01$.

### Inhibition of $GdCl_3$-induced phosphorylation of ERK1/2 and Akt in hMSC by ET receptor antagonist, bosentan

It has been reported that activation of ERK signal promoted osteogenic differentiation, and ERK inhibitor inhibited osteogenic differentiation in MSC, suggesting that phosphorylation of ERK plays an important role in calcium deposition in MSC (30,31). Therefore, we next analysed the extent of phosphorylation of ERK1/2 in hMSC treated with or without $GdCl_3$. Phosphorylation of ERK1/2 was significantly enhanced by $GdCl_3$ treatment (Fig. 3c). Akt activation has been reported to be important for cell proliferation; therefore, we also analysed the effect of $GdCl_3$ on phosphorylation of Akt in hMSC. Phosphorylation of Akt was also significantly enhanced by $GdCl_3$ treatment (Fig. 3d). These results suggest that $GdCl_3$-induced phosphorylation of ERK1/2 and Akt might be involved in the mechanism of $GdCl_3$-induced proliferation and calcium deposition in hMSC.

Next, we examined the influence of ET receptor antagonist, bosentan, on Gd-induced phosphorylation of ERK1/2 and Akt to determine whether ET-1/ETR signalling involves in Gd-induced phosphorylation of ERK1/2 and Akt. Interestingly, $GdCl_3$-induced phosphorylation of ERK1/2 and Akt in hMSC was inhibited by bosentan (Fig. 3c and d). These results suggest that ET-1/ETR signalling might be responsible for $GdCl_3$-induced phosphorylation of ERK1/2 and Akt in hMSC.

### Increase in plasma ET-1 levels and the expression of ET-1 in fibrous and calcified lesions in nephrogenic systemic fibrosis

To examine the role of ET-1 in the pathogenesis of fibrosis and abnormal calcification in NSF, we analysed plasma ET-1 levels in 2 patients with NSF. Similar to previously reported results (18,19), plasma ET-1 levels were higher in patients of diffuse cutaneous type systemic sclerosis (SSc) with severe skin sclerosis than those in normal controls (Fig. 4a). Plasma ET-1 levels were higher in 2 patients with NSF (patient 1: 4.98 pg/ml, patient 2: 3.07 pg/ml) than SSc (1.82 ± 0.32 pg/ml) and normal controls (1.20 ± 0.04 pg/ml). The serum from patient 1 was obtained at the onset of skin sclerosis; however, the serum from patient 2 was obtained at 6 years after onset. In patient 2, the part of skin sclerosis in lower legs and hand was improved at 6 years after onset; however, strong skin sclerosis with stiffening of fingers in left foot and left hand was still remained. Next, we analysed the expression of ETR-A in the skin lesions in NSF patient using immunofluorescence staining. ETR-A expressions in fibroblasts in the dermis of sclerotic lesions were significantly increased compared with that in normal dermis (Fig. 4b). These results suggest that activation of ET-1/ETR signalling might account for the pathogenesis of NSF. In addition, we found that mRNA level of ET-1 expression in human endothelial cells (EC) (HUVEC) was significantly increased by $GdCl_3$ treatment *in vitro* (Figure S4), suggesting that Gd-induced ET-1 from human EC might activate ETR signalling and involve in the pathogenesis of NSF.

## Discussion

The histopathological examination of lesional skin in NSF shows prominent dermal fibrosis and occasional calcium deposition. Of note, numerous $CD34^+$ and procollagen-$I^+$ spindle cells are observed in fibrous dermis and around calcium deposition (2,9,10). These observations suggest that circulating fibrocytes, which are

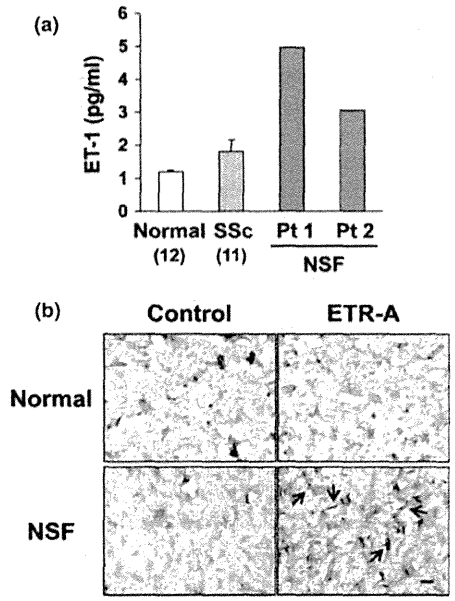


**Figure 4.** Increase in plasma ET-1 levels and the expression of ET-1 in fibrous and calcified lesions in nephrogenic systemic fibrosis. (a) Plasma ET-1 levels in 12 normal control, 11 patients of systemic sclerosis with severe skin sclerosis and 2 patients with NSF (Pt 1 and Pt 2). (b) Expression of ETR-A in normal skin and the fibrous lesions in NSF by immunohistological staining. Arrows indicate ETR-A staining in dermal fibroblasts. Scale bar = 20 μm.

bone marrow-derived MSC, might be responsible for dermal fibrosis and calcium deposition in NSF. As Gd depositions are identified in lesional skin (7,8), we hypothesized that Gd might directly act on fibrocytes/MSC and induce proliferation and calcium deposition in fibrocytes/MSC, which results in dermal fibrosis and abnormal calcification in NSF. At first, we confirmed that $GdCl_3$ directly induced cell proliferation and calcium deposition in hMSC, as we previously reported (15). Interestingly, we found that ET-1 and ETR-A expressions in hMSC were significantly increased by $GdCl_3$ treatment during the proliferation and calcification of hMSC. Furthermore, $GdCl_3$-induced hMSC proliferation and calcium deposition were significantly inhibited by ET receptor antagonist, bosentan. It has been reported that ET-1 increases intra-cellular $Ca^{2+}$ concentration and DNA synthesis and enhances proliferation and calcium deposition in MSC via ETR-A *in vitro* and *in vivo* (25,26,32). Collectively, these results suggest that Gd might induce ET-1 production from hMSC and also induce ETR-A expression on hMSC, resulting in proliferation and calcium deposition via activation of ET-1/ETR signalling in autonomous action. In contrast, both proliferation and calcium deposition in hMSC treated without $GdCl_3$ were not inhibited by bosentan, suggesting that ET-1/ETR signalling might specifically involve in $GdCl_3$-induced proliferation and calcium deposition in hMSC.

We found that phosphorylation of ERK1/2 and Akt was significantly enhanced by $GdCl_3$ treatment, and bosentan inhibited these $GdCl_3$-induced phosphorylation of ERK1/2 and Akt. It has been reported that activation of ETR-A results in phosphorylation of ERK1/2 and Akt in vascular smooth muscle cells (33,34), suggesting that Gd-induced phosphorylation of ERK1/2 and Akt in hMSC might be partially mediated via activation of ET-1/ETR signalling. ERK signal regulates calcium deposition in hMSC (30,31) and Akt is well known as a regulator of cell proliferation, indicating that Gd-induced phosphorylation of ERK1/2 and Akt in hMSC might participate in the mechanisms of Gd-induced proliferation and calcium deposition.

Serum ET-1 level in patient 1 with NSF which was obtained at the onset of skin sclerosis was significantly higher than those of SSc patients with severe skin sclerosis and normal controls. In addition, we found that mRNA level of ET-1 expression in human EC (HUVEC) was significantly increased by $GdCl_3$ treatment *in vitro*. It has been known that serum ET-1 level is elevated in the patients in chronic renal dysfunction with dialysis (35,36), suggesting that both dialysis-induced ET-1 and $GdCl_3$-induced ET-1 from MSC and EC might be responsible for the elevation of serum ET-1 level in patient 1 of NSF. We also found that ETR-A expression in fibroblasts in the dermis of sclerotic lesions was increased compared with that in normal dermis. This result is consistent with previous findings that the expression of ETR-A was enhanced in stenotic aortic valves with fibrosis and calcification (37) and that ETR-A expression of dermal fibroblasts in SSc patients was markedly increased (38).

Taken together with our findings, we propose a model for the role of ET-1/ETR signalling in $GdCl_3$-induced proliferation and calcium deposition in hMSC (Figure S5). $GdCl_3$ enhances the production of ET-1 in hMSC and hEC, and the expression of ETR-A in hMSC, leading to the activation of ET-1/ETR-A signalling in hMSC. This $GdCl_3$-induced activation of ET-1/ETR signalling might consequently enhance phosphorylation of ERK1/2 and Akt, as well as proliferation and calcium deposition in hMSC. These regulations by ET-1/ETR signalling might provide new insights into the pathogenesis of NSF. ETR antagonist, bosentan inhibited $GdCl_3$-induced phosphorylation of ERK1/2 and Akt as well as proliferation and calcification of hMSC *in vitro*, suggesting that bosentan can be a choice of treatments on fibrosis and abnormal calcification in NSF.

The use of imatinib mesylate, which inhibits both c-Abl and platelet-derived growth factor receptor (PDGFR) signalling, was reported to be an effective treatment of skin fibrosis in NSF (39,40), suggesting that activation of PDGFR signalling might be involved in the pathogenesis of NSF. Interestingly, it has been reported that ET-1 induced the transactivation of the tyrosine kinase receptors, such as PDGFR, vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) signalling (34,41,42). ET-1 receptor stimulation causes activation of metalloproteinase that sheds ligands such as heparin biding epidermal-like growth factor (HB-EGF), leading to activate tyrosine kinase receptors (41). An increase in $Ca^{2+}$ by ET-1 also might be involved in the mechanisms of transactivation of tyrosine kinase receptors. These findings suggest that $GdCl_3$-induced ET-1/ETR signalling might involve in the mechanisms of NSF, including the activation of PDGFR signalling.

The present study implicates $GdCl_3$ promotes MSC proliferation and calcium deposition via potentiation of ET-1/ETR signalling. Further investigations such as studies of animal models of NSF treated with ETR antagonist are required to elucidate the precise role of ET-1/ETR signalling in NSF.

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## Author contributions

S.M., E.O., A.U., K.Y., S.O., Y.Y., Y.T. and F.M. performed the experiments. S.M. designed the research. S.M., T.S. and O.I. wrote the manuscript. All authors discussed the results and edited the manuscript.

## Conflict of interests

The authors have declared no conflicting interests.

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## Supporting Information

Additional supporting data may be found in the supplementary information of this article:

**Figure S1.** No changes of CD34 expression on hMSC treated with or without $GdCl_3$.

**Figure S2.** Dose-dependent of inhibition of ET-1-induced hMSC proliferation by bosentan.

**Figure S3.** Dose-dependent of inhibition of $GdCl_3$-induced hMSC proliferation by bosentan.

**Figure S4.** Increase in the expression of ET-1 and ET in human EC by $GdCl_3$.

**Figure S5.** Model for the role of ET-1/ETR signalling in $GdCl_3$-induced proliferation and calcium deposition in hMSC.

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ORIGINAL ARTICLE

# No association of atherosclerosis with digital ulcers in Japanese patients with systemic sclerosis: Evaluation of carotid intima-media thickness and plaque characteristics

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## ABSTRACT

**Patients with systemic sclerosis (SSc) usually develop Raynaud's phenomenon, persistent digital ischemia and sometimes develop digital ulcers (DU). Several studies have reported an association of carotid artery atherosclerosis with SSc by evaluating carotid intima-media thickness (IMT) in SSc patients. However, none of those studies analyzed the association between DU and carotid artery atherosclerosis in SSc patients. We examined the association of carotid artery atherosclerosis with digital ulcers by comparing SSc patients with (*n* = 48, 29.5%) and without (*n* = 206, 70.5%) DU. The demographic and clinical features of the SSc patients showed that young age, male sex, anti-topoisomerase I antibody positivity, severe skin sclerosis, interstitial lung disease complication and cardiac involvements were significantly prevalent in patients with DU. In addition, diffuse cutaneous type, anti-RNA polymerase III antibody positivity and severe skin sclerosis are more frequent in SSc patients with DU at the extensor surface of joints than SSc patients with DU at the digital tip. There were no differences in serum lipid level, carotid IMT or plaque score between SSc patients with and without DU, suggesting that atherosclerotic changes are not primarily involved in the development of DU.**

**Key words:** atherosclerosis, carotid intima-media thickness, digital ulcers, systemic sclerosis.

## INTRODUCTION

Peripheral arterial disease (PAD) is an atherosclerotic disease with severe peripheral circulation disturbance that results in digital ulcers (DU). PAD is also associated with significant mortality caused by cardiovascular diseases. Recently, it has been reported that carotid intima-media thickness (IMT), the thickness of the intima-media complex in the carotid artery, and plaques were markedly higher in patients with PAD and that PAD is a meaningful risk factor for carotid artery stenosis.[1]

Systemic sclerosis (SSc) is a connective tissue disease characterized by fibrosis of the skin and internal organs and vascular dysfunction. Patients with SSc usually develop Raynaud's phenomenon and persistent digital ischemia and sometimes develop DU. Several studies have reported elevated IMT in SSc patients compared to normal controls,[2–6] however, other studies reported that SSc was not associated with increased IMT.[7–12] The association between DU and carotid artery atherosclerosis in SSc patients has not been studied. In this study, we analyzed the association of carotid artery atherosclerosis with DU by comparing SSc patients with and without DU. In addition, demographic and clinical features of SSc patients with or without DU were analyzed.

## METHODS

### Patients

We analyzed 254 Japanese patients with SSc who visited Gunma University Hospital from 2006 to 2013. All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology.[13] Patients were classified as having the limited cutaneous type (lcSSc) or diffuse cutaneous type (dcSSc) of SSc according to the classification by LeRoy *et al.*[14] This study was approved by the local research ethics committee of Gunma University. Patients provided written informed consent before participation.

### Clinical and laboratory assessments

Skin sclerosis was measured using the modified Rodnan total skin score (mRTSS). Interstitial lung disease (ILD) was detected as bibasilar interstitial fibrosis or a ground-glass shadow visible

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on high-resolution computed tomography scans. Pulmonary artery hypertension (PAH) was defined as an elevated right ventricular systolic pressure (>45 mmHg) by echocardiography, and subsequently, as an elevated mean pulmonary artery pressure (>25 mmHg) by cardiac catheterization.

### Measurement of carotid IMT and plaques

The wall thickness and plaques of the bilateral carotid arteries were measured by an ultrasound instrument (Aplio TUS-A400; Toshiba, Tokyo, Japan) using a 7.5-MHz linear type B-mode probe. IMT was defined as the distance from the leading edge of the lumen–intima interface to the media–adventitia interface of the far wall, and averaged to obtain the mean IMT. The maximum IMT was defined as the single thickest wall. The presence of a plaque was defined as a thickening of the IMT of more than 1.1 mm. According to the previous reports, the plaque score was calculated by adding the maximum axial thickness of all plaques in both carotid systems.[15] All scans were evaluated by ultrasonographic physicians who were blinded to the clinical features of patients.

### Statistics

*P*-values were calculated using Student's *t*-test or $\chi^2$-test analysis. Error bars represent standard errors of the mean.

## RESULTS

### Demographic and clinical features of SSc patients with or without DU

In total, 254 SSc patients were enrolled in this study, including 75 (29.5%) dcSSc and 179 (70.5%) lcSSc patients. The demographic, clinical characteristics and treatments of all SSc patients and SSc patients with or without DU are summarized in Table 1. We identified 48 SSc patients with prior or current DU (18.9%) (mean age, 60.4 years) and 206 SSc patients who had not been affected by DU (mean age, 65.8 years). The SSc patients with DU were significantly younger than those without DU (60.4 vs 65.8 years old, $P < 0.05$). The proportion of male patients was significantly higher in the SSc patients with DU than in the SSc patients without DU (22.9% vs 10.2%, $P < 0.05$). DU occurred more frequently in dcSSc patients compared with lcSSc patients (52.1 vs 24.3, $P < 0.01$). mRTSS was significantly higher in SSc patients with DU (13.3 vs 5.4, $P < 0.01$). SSc patients with DU were more frequently positive for anti-topoisomerase I antibody (Ab) and anti-RNA polymerase III Ab (RNAP) than SSc patients without DU (anti-topoisomerase I: 35.4% vs 20.4%, $P < 0.05$; RNAP: 8.3% vs 2.4%, $P < 0.05$). The prevalence of ILD in SSc patients with DU was significantly higher than that in SSc patients without DU

**Table 1.** Demographic, clinical characteristics and treatments of all SSc patients and SSc with or without DU

| | SSc ($n$ = 254) | SSc with DU ($n$ = 48) | SSc without DU ($n$ = 206) | $P$ |
|---|---|---|---|---|
| Age (years; mean ± SE) | 64.6 ± 0.8 | 60.4 ± 2.1 | 65.8 ± 0.8 | <0.05 |
| mRTSS (mean ± SE) | 6.9 ± 0.5 | 13.3 ± 1.3 | 5.4 ± 0.5 | <0.01 |
| Sex | | | | |
| Male (%) | 12.6 (32/254) | 22.9 (11/48) | 10.2 (21/206) | <0.05 |
| Female (%) | 87.4 (222/254) | 77.1 (37/48) | 89.8 (185/206) | |
| Type | | | | |
| lcSSc (%) | 70.5 (179/254) | 47.9 (23/48) | 75.7 (156/206) | <0.01 |
| dcSSc (%) | 29.5 (75/254) | 52.1 (25/48) | 24.3 (50/206) | |
| Autoantibody | | | | |
| ANA (%) | 89.4 (213/254) | 93.8 (45/48) | 88.3 (182/206) | 0.274 |
| Topo I (%) | 23.2 (59/254) | 35.4 (17/48) | 20.4 (42/206) | <0.05 |
| RNP (%) | 13.8 (35/254) | 14.6 (7/48) | 13.6 (28/206) | 0.858 |
| Centromere (%) | 41.7 (106/254) | 37.5 (18/48) | 42.7 (88/206) | 0.509 |
| RNAP (%) | 3.5 (9/254) | 8.3 (4/48) | 2.4 (5/206) | <0.05 |
| Complication | | | | |
| ILD (%) | 44.9 (106/236) | 60.9 (28/46) | 41.1 (78/190) | <0.05 |
| PAH (%) | 10.2 (23/225) | 9.3 (4/43) | 10.4 (19/182) | 0.825 |
| Cardiac involvements (%) | 17.3 (44/254) | 27.1 (13/48) | 15 (31/206) | <0.05 |
| Treatment | | | | |
| Corticosteroids (%) | 27.2 (69/254) | 54.2 (26/48) | 20.9 (43/206) | <0.01 |
| Other immunomodulators (%) | 6.7 (17/254) | 8.3 (4/48) | 6.3 (13/206) | 0.614 |
| Antiplatelet agent (%) | 33.1 (84/254) | 64.6 (31/48) | 25.7 (53/206) | <0.01 |
| Oral prostanoid (%) | 63.8 (162/254) | 89.6 (43/48) | 57.8 (119/206) | <0.01 |
| Intravenous prostanoid (%) | 9.1 (23/254) | 47.9 (23/48) | 0 (0/206) | <0.01 |
| Bosentan (%) | 6.3 (16/254) | 25 (12/48) | 1.9 (4/206) | <0.01 |

ANA, antinuclear antibody; centromere, anticentromere antibody; dcSSc, diffuse cutaneous type of SSc; DU, digital ulcers; ILD, interstitial lung disease; lcSSc, limited cutaneous type of SSc; mRTSS, modified Rodnan total skin score; PAH, pulmonary artery hypertension; RNAP, anti-RNA polymerase III antibody; RNP, anti-U1 RNP antibody; SSc, systemic sclerosis; SE, standard error; Topo I, anti-topoisomerase I antibody. Other immunomodulators include methotrexate, cyclosporin, azathioprine, tacrolimus and tocilizumab. Antiplatelet agents include aspirin, sarpogrelate hydrochloride (serotonin receptor antagonist) and cilostazol (phosphodiesterase inhibitor). Oral prostanoid includes beraprost sodium (prostaglandin I2 analog). Intravenous prostanoid includes lipoprostaglandin E1.