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Therapeutic vascular angiogenesis for intractable macroangiopathy-related digital ulcer in patients with systemic sclerosis: a pilot study

Gen Takagi[1], Masaaki Miyamoto[1], Shuhei Tara[1], Sonoko Kirinoki-Ichikawa[1], Yoshiaki Kubota[1], Tomohito Hada[1], Ikuyo Takagi[1] and Kyoichi Mizuno[1]

Abstract

Objective. SSc causes intractable ischaemic ulcers. To avoid major amputation, we examined the safety and efficacy of therapeutic vascular angiogenesis for digital ulcers due to SSc.

Methods. A single-centre, open-label pilot study was conducted in patients with an ischaemic digital ulcer [$n = 40$, mean age 65 years (S.D. 8), Rutherford class III-5 or III-6) due to lcSSc ($n = 11$) or arteriosclerosis obliterans (ASO; $n = 29$). Bone marrow mononuclear cells (0.4–5.1 $\times 10^{10}$ cells in total) were administered into the ischaemic limbs. We evaluated short-term safety and efficacy by means of a pain scale, ^{99m}Tc-tetrofosmin scintigraphy and transcutaneous oxygen tension ($TcPO_2$) before and 4 weeks after treatment. Also, the 2-year outcome was compared.

Results. There was a case of amputation in each group within 4 weeks after therapy. The pain scale significantly decreased in both groups [lcSSc 93 mm (S.D. 9) to 11 (S.D. 16), $P < 0.01$; ASO 77 mm (S.D. 22) to 16 (S.D. 13), $P < 0.01$] and $TcPO_2$ significantly improved [lcSSc 9.0 mmHg (S.D. 9) to 35 (S.D. 14), $P < 0.01$; ASO 18 mmHg (S.D. 10) to 29 (S.D. 21), $P < 0.05$). At the 2-year follow-up, the limb amputation rate was 9.1% in lcSSc and 20.7% in ASO ($P = 0.36$), while the recurrence rate was 18.2% in lcSSc and 17.2% in ASO ($P = 0.95$). All-cause mortality was 27.3% in lcSSc and 17.2% in ASO ($P = 0.65$).

Conclusion. In patients with lcSSc, bone marrow mononuclear cell implantation provides clinical benefit and is safe, without major adverse reactions, and may become an effective strategy.

Trial registration: UMIN-CTR, http://www.umin.ac.jp/ctr/index-j.htm, no. UMIN000004112.

Key words: scleroderma and related disorders, cell transplantation, stem cell, haematopoietic, vasculitis.

Introduction

SSc is a connective tissue disease characterized by cutaneous and visceral fibrosis, as well as vascular involvement of the peripheral circulation, often causing intractable peripheral digital ulcers. The standard approach for digital ulcers, including analgesics, antibiotics, vasodilators and topical hydrocolloid dressings, is often ineffective. Thus one of the pitfalls in the management of digital ulcers is the limitation of therapeutic options. Angiography shows severely stenotic or occluded arteries at the digital level in most patients. Since there is no indication for catheter intervention or bypass graft surgery in this peripheral artery disease (PAD), major limb amputation has been performed, with deterioration of quality of life and a poor outcome [1]. Thus there remains a clear need for further research on an effective approach to avoid limb amputation. Our previous study showed that autologous bone marrow mononuclear cell implantation (BMCI) is effective for PAD due to atherosclerosis [2]. The aim of this study was to salvage patients with impending risk of major amputation. Therefore we examined the safety, incidence of recurrence and efficacy, including the effect on long-term event-free survival, of autologous bone marrow mononuclear cell implantation in patients with digital ulcers due to SSc, and the data were

[1]Department of Internal Medicine, Division of Cardiovascular and Regenerative Medicine, Nippon Medical School, Tokyo, Japan.

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Correspondence to: Gen Takagi, Department of Internal Medicine, Division of Cardiovascular and Regenerative Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan. E-mail: gen52@nms.ac.jp

CLINICAL SCIENCE

compared with those in PAD due to arteriosclerosis obliterans (ASO).

Patients and methods

Forty consecutive patients [mean age 65 years (S.D. 8)] with critical limb ischaemia who developed an ischaemic digital ulcer or gangrene (Fontaine class 4, Rutherford class III-5 or III-6) with rest pain after >3 months of standard local treatment for ulcers, including antibiotic ointment, prostanoid ointment and topical hydrocolloid dressings, and standard medication such as analgesics, antibiotics, calcium channel blockers, prostanoids, endothelin receptor antagonists and steroids (see supplementary Table S1 for detailed information, available at *Rheumatology* Online) were enrolled in this study. The patients were divided by original disease, such as lcSSc ($n = 11$) or ASO ($n = 29$) as an age-matched control, defined by the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee [3, 4] and the diagnostic guidelines for PAD [Trans-Atlantic Inter-Society Consensus Document on Management of Peripheral Arterial Disease II (TASC II)] [5]. The study was approved by the ethical committee of the Nippon Medical School, Tokyo, Japan, and written informed consent was obtained from each patient. The protocol followed the Declaration of Helsinki and was registered with the University Hospital Medical Information Network Clinical Trial Registry (no. UMIN000004112).

The study was a single-centre, open-label pilot study. The data were compared with those in ASO. The primary endpoint of this study was the occurrence of a major adverse event within 4 weeks of follow-up, which was defined as any unfavourable and unintended sign, symptom or disease temporally associated with the use of a medical treatment or procedure that may or may not be considered related to the medical treatment or procedure, according to Common Terminology Criteria for Adverse Events (CTCAE). Secondary endpoints were blood flow recovery at 4 weeks after treatment and long-term survival of the limb and life at 2 years. Exclusion criteria were (i) no evidence of digital ulcer, (ii) no evidence of angiological stenosis in the affected limbs confirmed by digital subtraction angiography, (iii) the existence of untreated significant coronary artery disease determined by coronary angiography, (iv) a history of vascular surgery (within 30 days), (v) the presence of any malignant disease or history of its treatment within 5 years (determined by fiberscopy, tumour marker, or faecal occult blood), (vi) untreated proliferative diabetic retinopathy, (vii) smoker unable to quit smoking, (viii) drug addiction of any kind (including alcohol), (ix) evidence of viral infection (hepatitis B virus, hepatitis C virus or HIV) and (x) complication of any serious disease affecting the patient's general condition (heart, lung, kidney or liver failure, etc.). In order to avoid the effect of additional conflictive effect, medication was not changed throughout the study period. Baseline characteristics were determined on admission with a routine blood exam. Renal function was evaluated by estimating the creatinine clearance calculated by the Cockcroft–Gault method [6].

After the screening study, all patients were treated with bone marrow mononuclear cell implantation. This method has been described previously [2, 7]. Briefly, bone marrow (400–600 ml, $0.4–5.1 \times 10^{10}$ cells in total) was collected from the bilateral iliac bones under general anaesthesia. The mononuclear cell fraction was sorted and 60–100 ml of cell suspension was processed using an AS-TEC 204 cell separator (Fresenius, Bad Homburg, Germany). While processing bone marrow aspirates, necrotic tissue was surgically debrided under sterile conditions. Thereafter the cell suspension (1 ml/point) was intramuscularly injected at about 70 points/ischaemic area using a 1-ml syringe with a 26-gauge needle. Finally, skin grafting was performed to cover the ulcers unless the wound margin spontaneously epithelialized (patient 5).

For quality analysis of the mononuclear cell count including endothelial progenitor cells (EPCs), flow cytometry was performed from bone marrow cells. EPCs were analysed for the expression of CD34, CD45, CD133 and vascular endothelial growth factor receptor 2 (VEGFR-2) with four-colour flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Two millilitres of bone marrow aspirates were obtained. Red cells were lysed by the addition of ammonium chloride–based lysing reagent. The samples were washed in 0.2% PBS with BSA. FcR-blocking reagent (1% human gamma globulin; Sigma-Aldrich, St Louis, MO, USA) was added and incubated for 15 min at room temperature in the dark. The samples were incubated with anti-CD34 FITC (Beckman Coulter, Marseille, France), anti-CD45-PerCP (BD Biosciences), anti-CD133/2(293C3)-APC (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-VEGFR-2-phycoerythrin conjugated (R&D Systems) for 40 min at 4°C, followed by erythrolysis by the addition of lysing reagent and then washed once with 0.2% PBS with BSA. The cells were resuspended in 0.2% PBS with BSA for flow cytometric analysis. As a control for analysis, cells in a separate tube were treated with PE-labelled mouse IgG1 antibody. $CD34^+$ cells were analysed using sequential gating strategies. The CD45 *vs* side-scatter dot plot was set to include all $CD45^+$ events (supplementary Fig. S1A, available at *Rheumatology* Online). The $CD45^+$ events were established to include all nucleated white blood cells and to exclude red blood cells, nucleated red blood cells, platelets and other cellular debris that do not express CD45. $CD45^+$ cells were gated on a forward-scatter *vs* side-scatter dot plot to confirm the mononuclear cell fraction (supplementary Fig. S1B, R1, available at *Rheumatology* Online). Mononuclear cells formed a cluster with low side scatter and low to intermediate forward scatter. $CD34^+$ and $CD45^{dim}$ cells in the mononuclear cell fraction (supplementary Fig. S1C, R2, available at *Rheumatology* Online) were gated on a forward-scatter *vs* side-scatter dot plot to obtain a cluster of true $CD45^{dim}CD34^+$ cells (supplementary Fig. S1D, R3, available at *Rheumatology* online). True $CD45^{dim}$ and $CD34^+$ events were displayed on a CD133 *vs* VEGFR-2

dot plot and the resulting population was examined for the dual expression of VEGFR-2 and CD133. $CD45^{dim}CD34^{+}$ $CD133^{+}$ VEGFR-2^{+} cells were enumerated in the upper right quadrant of plot (supplementary Fig. S1E, available at *Rheumatology* Online). At least 2 000 000 events were measured in the $CD45^{+}$ gate. Data were analysed using CELLQuest (BD Biosciences). The EPC values were defined as the percentage of $CD34^{+}$, $CD45^{dim}$, $CD133^{+}$ and VEGFR-2^{+} cells per $CD34^{+}CD45^{dim}$ cells fraction.

As a quantifying measurement of symptoms, a visual analogue pain scale (VAS) indicating maximum pain as 100 mm and minimum pain as 0 was used. As a quantifying measurement of local blood flow recovery, the following parameters were evaluated. The ankle-brachial index (ABI; Omron Healthcare, Kyoto, Japan) was measured by standard methods and calculated as the ratio of ankle-to-brachial pressure. Tissue oxygen content was measured with a TCM 400 [transcutaneous oxygen tension ($TcPO_2$); Radiometer Medical, Brønshøj, Denmark]. The transducer was placed on the dorsum of the ischaemic limb, including one control site, and warmed to 43.5°C to increase the permeability of the skin to oxygen molecules at the measurement site. Tissue blood flow was determined by ^{99m}technetium tetrofosmin (^{99m}TcTF) scintigraphy [2, 7]. ^{99m}TcTF (555–740 MBq) was injected intravenously. Approximately 10 min after injection of the radiotracer, whole-body scintigraphy was performed with a dual-head large field of view gamma camera (Vertex, ADAC, Milpitas, CA, USA). For quantitative analysis, regions of interest (ROIs) of equal size were drawn around the appropriate muscle group (e.g. calf muscles). Additionally, brain uptake was calculated as the background. The muscle-to-brain (M/B) ratio was defined as the average counts per pixel in each muscle/average counts per pixel in the brain. Evaluation was performed before and 4 weeks after therapy.

For safety evaluation, long-term limb survival, recurrence of ischaemia and all-cause mortality were determined by Kaplan–Meier analysis and the data were compared with those in ASO.

Statistical analysis

All data are presented as mean (S.D.). Statistical analysis was performed using SPSS statistics version 20 software (IBM, Armonk, NY, USA). Repeated measures analysis of variance (ANOVA) was used to test for differences in blood flow and VAS as continuous variables. Within-treatment analyses of changes were performed using the Wilcoxon rank-sum test. The probability of the risk factor was compared by chi-squared test. Time-to-event curves were compared using stratified log-rank tests. Hazard ratios were calculated using Cox regression models. A P-value <0.05 was taken as the minimum level of significance.

Results

Representative pictures of an ulcer (supplementary Fig. S2, available at *Rheumatology* Online), angiograms (supplementary Fig. S3, available at *Rheumatology* Online), individual lcSSc data (supplementary Table S1, available at *Rheumatology* Online) and the Kaplan–Meier analysis (supplementary Fig. S4, available at *Rheumatology* Online) are shown in the supplementary data available at *Rheumatology* Online. Baseline characteristics of objects are shown in Table 1. The lcSSc patients were all female and had a lower body weight and normal ABI values. Risk factors, except dyslipidaemia, showed a lower prevalence in lcSSc. Blood examination data showed lower haemoglobin A1c, creatinine and white blood cell count in lcSSc. ACA was positive in all lcSSc patients. Regarding medication, the frequency of prednisolone use was higher in lcSSc. Other medication was not significantly different between the groups. Regarding the primary endpoint, adverse events occurred in two cases during the 4-week follow-up period, consisting of major limb amputation (one case from each group) due to pre-existing osteomyelitis, but this was not related to technical failure. Also, two lcSSc patients received removal of pre-existing osteomyelitis. Fig. 1A shows the time course of the VAS. The VAS significantly decreased after BMCI [lcSSc 93 mm (S.D. 9) to 11 (S.D. 16), $P < 0.01$; ASO 77 mm (S.D. 22) to 16 (S.D. 13), $P < 0.01$] in both groups. ^{99m}TcTF scintigraphy, reflecting tissue blood flow (Fig. 1B), showed improvement in the ASO group [0.8 (S.D. 0.3) to 0.9 (S.D. 0.4) count ratio/pixel, $P < 0.05$], but it did not reach statistical significance in the lcSSc group [0.6 (S.D. 0.3) to 0.7 (S.D. 0.2), NS] 4 weeks after treatment. Also, tissue oxygen content, determined by $TcPO_2$ in the dorsum position, was significantly increased 4 weeks after bone marrow mononuclear cell implantation in both groups [lcSSc 9.0 mmHg (S.D. 9) to 35 (S.D. 14), $P < 0.01$; ASO 18 mmHg (S.D. 10) to 29 (S.D. 21), $P < 0.05$; Fig. 1C]. At the 2-year follow-up, Kaplan–Meier analysis (the number at risk is shown under the graph) of the limb amputation rate was 9.1% in lcSSc and 20.7% in ASO ($P = 0.36$; supplementary Fig. S4A, available at *Rheumatology* Online). Recurrence of limb ischaemia occurred in 18.2% of patients in lcSSc and 17.2% in ASO ($P = 0.95$; supplementary Fig. S4B, available at *Rheumatology* Online). All-cause mortality at 2 years of follow-up was not different between the groups (lcSSc 27.3%, ASO 17.2%, $P = 0.65$; supplementary Fig. S4C, available at *Rheumatology* Online).

Discussion

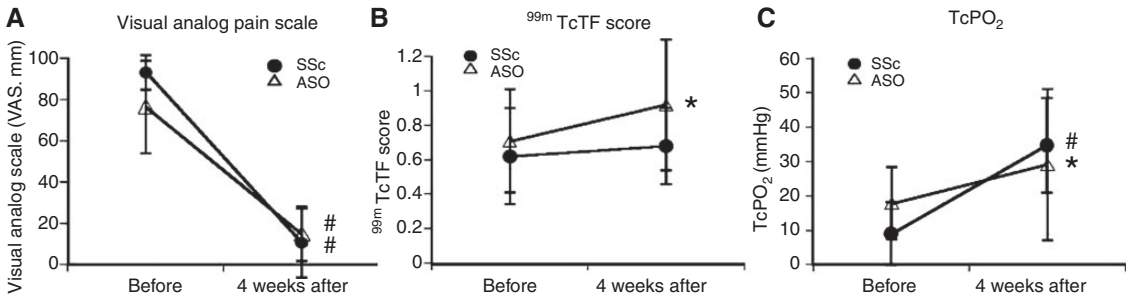
The pathogenesis of vascular involvement is defined by the presence of an inflammatory process in the vessel wall, with reactive damage to mural structures and defective vasculogenesis [8], resulting in digital ulcers. Thus direct incision for wound debridement often worsens wound healing. There are no established guidelines for ischaemic ulcer with lcSSc. One of the recent topics is vascular regenerative therapy for PAD [2, 9]. In this context, the susceptibility to the treatment option has been extended to secondary PAD due to collagen disease. Among vascular regenerative medicine, cell therapy is an effective strategy. It is reported that bone marrow includes several cytokines that promote angiogenesis,

Table 1 Baseline characteristics of subjects

	All patients ($n = 40$)	ASO ($n = 29$)	lcSSc ($n = 11$)	*P*-value
Clinical characteristics				
Age, mean (S.D.), years	65.1 (8.2)	64.9 (7.9)	65.0 (9.3)	0.92
Sex, *n* (%), female	20 (50)	9 (31)	11 (100)	<0.01
Body mass index, mean (S.D.), kg/m^2	22.0 (3.5)	21.9 (2.6)	21.8 (5.8)	0.98
Body weight, mean (S.D.), kg	55.9 (10.2)	58.2 (9.5)	48.2 (10.6)	0.02
ABI, mean (S.D.)	0.83 (0.3)	0.68 (0.2)	1.13 (0.2)	<0.01
Risk factors, *n* (%)				
Smoking history	17 (48)	16 (55)	1 (9)	<0.01
Coronary heart disease	16 (40)	16 (55)	0 (0)	<0.01
Diabetes mellitus	25 (63)	25 (86)	0 (0)	<0.01
Hypertension	25 (63)	24 (83)	1 (9)	<0.01
Dyslipidaemia	25 (53)	20 (69)	5 (45)	0.16
CKD (CCr <30 ml/min/1.73 m^2)	12 (30)	12 (38)	0 (0)	0.02
Clinical chemistry				
Total cholesterol, mean (S.D.), mmol/l	4.21 (1.10)	4.18 (1.21)	4.29 (0.80)	0.97
Triglyceride, mean (S.D.), mmol/l	1.20 (0.76)	1.31 (0.86)	0.95 (0.25)	0.24
HDL cholesterol, mean (S.D.), mmol/l)	1.17 (0.41)	1.12 (0.40)	1.32 (0.44)	0.33
LDL cholesterol, mean (S.D.), mmol/l	2.45 (0.87)	2.51 (0.93)	2.39 (0.65)	0.54
Fasting blood glucose, mean (S.D.), mmol/l)	5.88 (2.00)	6.16 (2.25)	5.00 (0.61)	0.10
Haemoglobin A1c, mean (S.D.), % (NGSP)	6.23 (1.1)	6.43 (1.2)	5.63 (0.4)	0.04
Creatinine, mean (S.D.), μmol/l	221.0 (265.2)	302.3 (315.6)	79.6 (28.3)	0.04
ACA positive, no. (%)	11 (28)	0 (0)	11 (100)	<0.01
CRP, mean (S.D.), nmol/l	21.0 (24.8)	23.7 (26.6)	14.9 (18.4)	0.19
White blood cell count, mean (S.D.)	6340 (1819)	6731 (1614)	5018 (1773)	<0.01
Haemoglobin, mean (S.D.), g/l	108.0 (19.2)	108.0 (20.3)	104.1 (13.0)	0.64
Bone marrow mononuclear cells, mean (S.D.), $\times 10^9$	5.08 (4.5)	5.12 (4.5)	4.93 (4.5)	0.90
Medication on admission, *n* (%)				
Aspirin	22 (55)	17 (59)	5 (46)	0.46
Thienopyridines	11 (28)	9 (31)	2 (18)	0.43
Cilostazol	17 (43)	10 (35)	7 (64)	0.10
Renin–angiotensin system antagonist	25 (63)	20 (69)	5 (46)	0.16
Prednisolone	11 (28)	3 (10)	8 (73)	<0.01
Statins	25 (63)	19 (66)	6 (55)	0.51

CKD: chronic kidney disease; CCr: creatinine clearance; HDL: high-density lipoprotein; LDL: low-density lipoprotein; NGSP: National Glycohaemoglobin Standardization Program.

Fig. 1 Quantitative analysis of efficacy



(**A**) The visual analogue pain scale (VAS) was significantly improved in both groups. (**B**) Muscle blood flow estimated by ^{99m}technetium tetrofosmin (^{99m}TcTF) scintigraphy [^{99m}TcTF perfusion index: muscle-to-brain (M/B) ratio of mean counts per pixel] improved in ASO. (**C**) Skin perfusion was determined by transcutaneous oxygen tension ($TcPO_2$). $TcPO_2$ was significantly improved in both groups. $^*P < 0.05$, $^\#P < 0.01$ *vs* before administration value.

which is the main source of vascular angiogenesis [10]. Even bone marrow consists of crude cell types and cytokines, and a recent meta-analysis supported the clinical impact of bone marrow cell implantation in ischaemic heart disease [11]. Recent evidence has also demonstrated that bone marrow mononuclear cells can produce anti-fibrotic growth factors and cytokines, including hepatocyte growth factor, IL-10 and adrenomedullin [12–14]. These factors have been shown to attenuate fibrosis and scar formation through down-regulation of the expression of TGF-β1 [15] and to promote extracellular matrix restoration by down-regulating the expression of collagens and up-regulating the expression of MMPs [16]. To confirm the mechanism of action, we previously investigated the clinical effects of a single cytokine, controlled-release basic fibroblast growth factor [17, 18] derived from bone marrow cells in response to ischaemia [19].

Regarding vascular angiogenesis therapy, other cell therapies such as embryonic stem cells or induced pluripotent stem cells are not ready for clinical use in PAD. An alternative strategy is to use proteins or genes. An optimal delivery strategy has not been established, possibly because of factors such as the selection and formulation of the growth factor, duration of exposure, route of administration, selection of patients and timing of observation [20]. There has been a pilot study regarding bone marrow–derived mononuclear cell implantation for autoimmune disease–related peripheral ischaemia [21–23]. However, those reports did not confirm angiogenesis by quantitative blood flow analysis and also included subjects with various diseases. In order to explore bone marrow mononuclear cell implantation as a universal approach for intractable digital ulcers, we focused on specific diseases such as lcSSc and ASO, and the effect was compared with quantitative blood flow analysis. The primary endpoint of short-term safety was comparable to that in ASO. Secondary endpoints of limb amputation rate, recurrence of ischaemic insult and all-cause mortality as long-term efficacy were also comparable to those in ASO. To support the mechanism, quantitative analysis of recovery of ischaemia is essential. Because ischaemia is mainly distal to the wrist or ankle joint, including the finger level, ABI is in the normal range in lcSSc. ^{99m}TcTF is referenced to palm or foot level as a ROI, however it also detects tissue inflammation, thus detection of digital ischaemia is difficult. Although quantitative blood flow analysis is limited, tissue oxygen content assessed by $TcPO_2$ showed that vascular angiogenesis initiated skin perfusion by autologous bone marrow mononuclear cells implantation at 4 weeks after treatment, consistent with our previous investigation [7].

Conclusion

These results support that bone marrow mononuclear cell implantation is safe and effective for intractable digital ulcers in lcSSc and ASO and is a promising therapeutic option for peripheral digital ulcer patients.

Limitations

This investigation was designed as an observational study. However, all subjects were consecutively enrolled, thus there was no bias in patient selection. Medications varied at the time when patients were referred to our hospital, and this may have affected the outcome. Vascular angiogenesis is a quite promising strategy for wound healing of local digital ulcers in patients with lcSSc; however, this therapy is only effective for local ischaemia. Systemic management may be needed to maintain the original disease condition along with this local therapy.

Rheumatology key messages

- Peripheral artery disease causes digital ulcers in patients with SSc.
- Vascular regeneration therapy is safe and effective for digital ulcers in patients with SSc.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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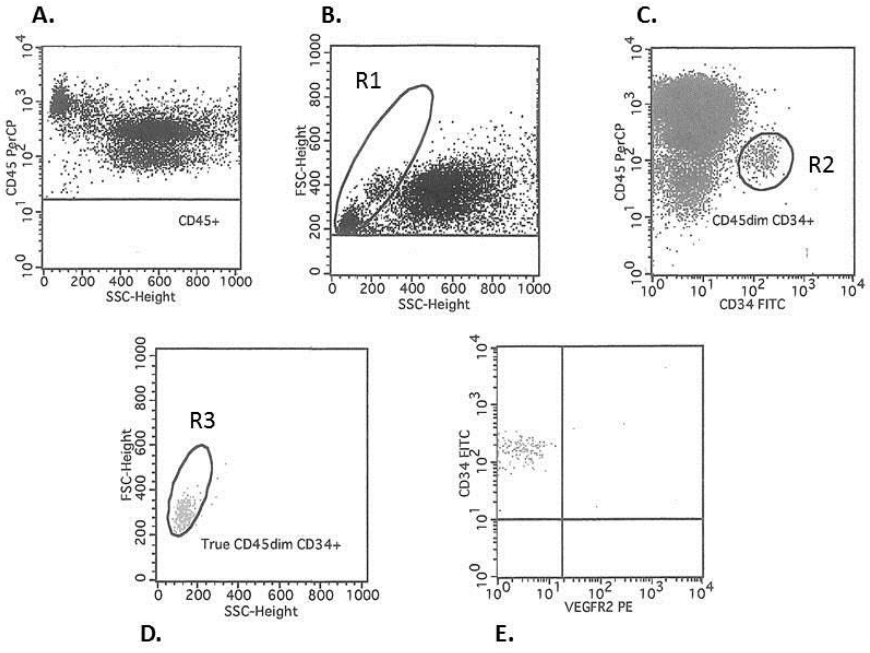
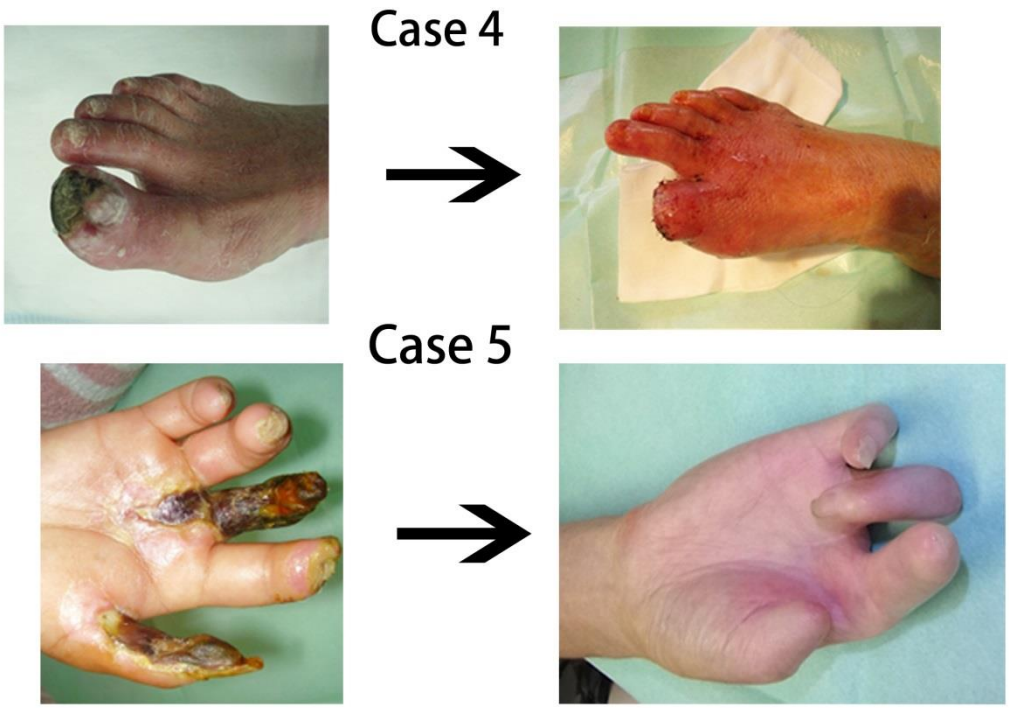
Supplementary data

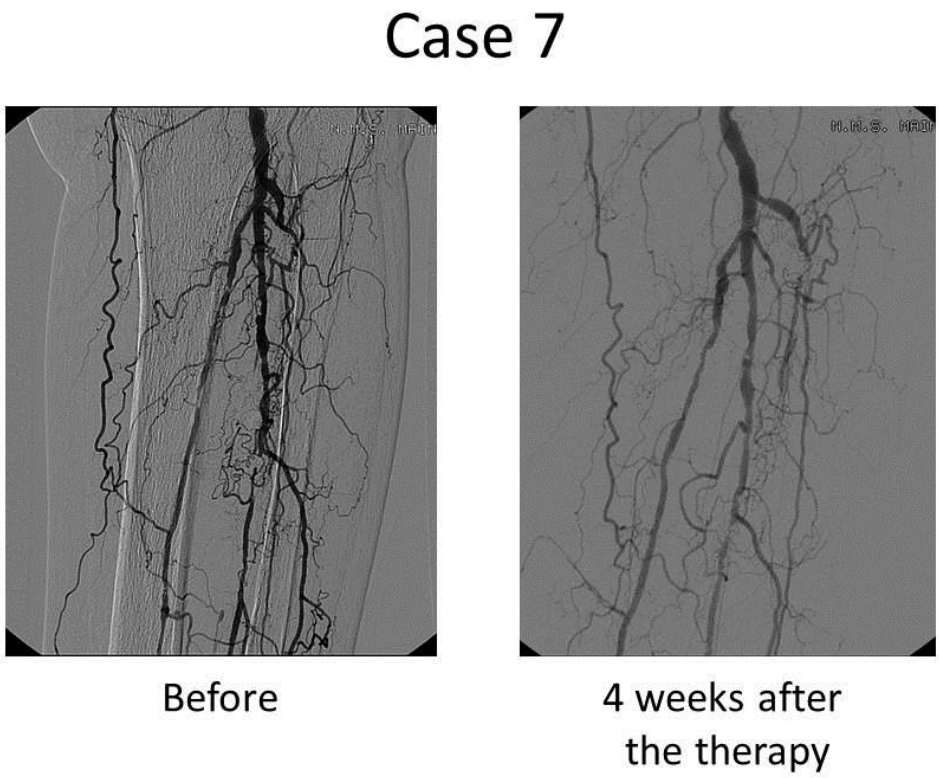
Supplementary Figure S1. (A) CD45 versus side scatter dot plot included all $CD45^+$ events. (B) $CD45^+$ cells were gated on a forward scatter versus side scatter dot plot to confirm mononuclear cell fraction (R1). (C) $CD34^+$ and $CD45^{dim}$ cells in the mononuclear cell fraction (R2). (D) A cluster of true $CD45^{dim}CD34^+$ cells was gated on a forward scatter versus side scatter dot plot (R3). (E) $CD45^{dim}CD34^+CD133^+$ vascular endothelial growth factor receptor-2^+ cells were enumerated in upper right quadrant of plot.



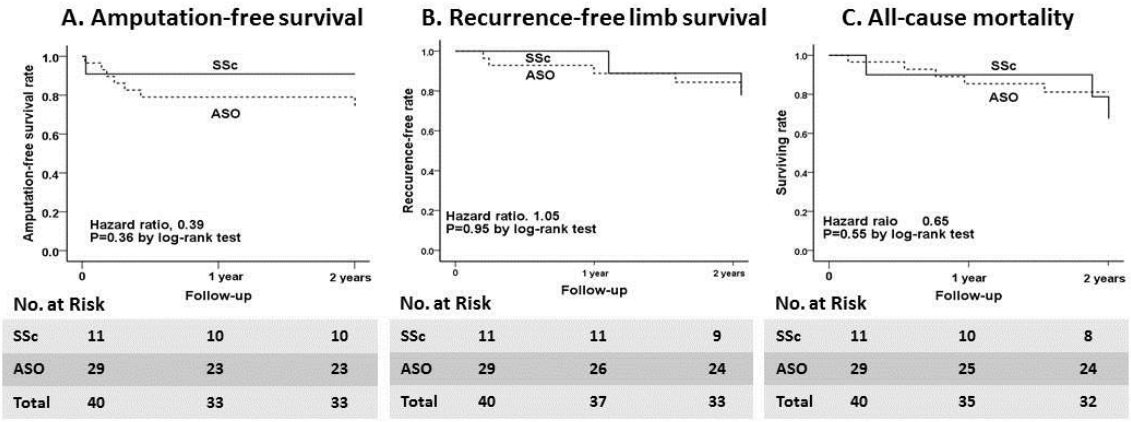
Supplementary Figure S2. Representative photographs of the patients.



Supplementary Figure S3. Representative digital subtraction angiographic image.



Supplementary Figure S4. Prognosis assessment by Kaplan-Meier analysis curves.



No. at Risk			
SSc	11	10	10
ASO	29	23	23
Total	40	33	33

No. at Risk			
SSc	11	11	9
ASO	29	26	24
Total	40	37	33

No. at Risk			
SSc	11	10	8
ASO	29	25	24
Total	40	35	32

Supplementary Table S1. Characteristics of individual lcSSc patients

Case	Age	Sex	Location of DU	Systemic treatment	Local treatment	Duration of disease, yr	Duration of RF, yr	Duration of DU, months	Organ involvement	Telangiec-tasia	ANA	ACA	$CD34^+$ cells (x 10^7)	2 year outcome
1	56	F	Bilateral hands	P, AB	P, AB	13	16	158	Lung fibrosis	None	×640	71.3	7.56	Recurrence
2	57	F	Bilateral hands	P, AB	P, AB	14	17	182	Lung fibrosis	None	×1280	148.8	19.2	Complete wound closure
3	73	F	Left foot	CCB, A, P	P, AB	32	10	10	RA, pericarditis Sjögren	Yes	×1280	222.8	8.0	Complete wound closure
4	60	F	Right foot	P	AB	20	35	4	Sjögren, AIH, HCM	None	×2560	144.1	10.0	Complete wound closure
5	52	F	Right hand	A, P	P, AB	19	22	246	PH, lung fibrosis	Yes	×1280	195.7	4.86	Complete wound closure
6	56	F	Right hand	CCB, A, P	P, AB	7	10	8	Lung fibrosis, RA	None	×1280	204.7	1.62	Complete wound closure
7	65	F	Left foot	CCB, A, P	P, AB	21	21	6	Lung fibrosis, CKD	None	×640	239	3.91	Complete wound closure

8	74	F	Left foot	AB, P	P, AB	0	45	7	PBC, GERD	None	×2560	213.9	1.82	Complete wound closure
9	77	F	Right foot	CCB, A, P	AB	36	14	6	RA, CKD	Yes	×640	185.7	0.47	Complete wound closure
10	75	F	Right foot	CCB, A, P	AB	34	12	6	RA, CKD	Yes	×640	190.8	1.6	Below-knee amputation
11	71	F	Bilateral hands	A, P, ETA	AB	3	12	4	Type B1 thymoma	None	×1280	320	0.56	Complete wound closure

yr: year, F: female, RF: Raynaud's phenomenon, DU: digital ulcer, P: prostanoid, AB: antibiotics, CCB: calcium channel blocker, A: analgesics, ETA: endothelin receptor antagonist, RA: rheumatoid arthritis, Sjögren: Sjögren syndrome, AIH: autoimmune hepatitis, HCM: hypertrophic cardiomyopathy, PH: pulmonary hypertension, CKD: chronic kidney disease, PBC: primary biliary cirrhosis, GERD: gastroesophageal reflux disease, ANA: anti-nuclear antibody, ACA: anti-centromere antibody.