Original Research

Serum-free Quality and Quantity Control Culture Improves the Angiogenic Potential of Peripheral Blood Mononuclear Cells Harvested from Patients with Connective Tissue Diseases

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Abstract

Objectives: The number and quality of endothelial progenitor cells decrease in patients with connective tissue diseases. This limits the efficacy of mononuclear cell therapy for ischemic ulcers associated with connective tissue diseases. To overcome these problems, we developed a serum-free quality and quantity control culture method that potentially improves the function of endothelial progenitor cells and expands their numbers. Here, we show the effect of quality and quantity control culture on mononuclear cells from patients with connective tissue diseases.
Methods: Peripheral blood mononuclear cells were isolated from C57BL/6JJmsSlc-*lpr/lpr* mice with systemic lupus erythematosus, patients with connective tissue diseases, and healthy volunteers. Mononuclear cells were cultured using the quality and quantity control culture method, and the number of endothelial progenitor cells was analyzed using flow cytometry, an endothelial progenitor cell culture assay, and an endothelial progenitor cell colony-forming assay. Flow cytometry was also used to examine mononuclear cell subpopulations. A human umbilical vein endothelial cell tube-forming assay was used to examine the function of quality and quantity control cultured mononuclear cells.
Results: Mice with systemic lupus erythematosus showed a significantly lower number of endothelial progenitor cells, which increased to the same levels as those of the control mice after quality and quantity control culture. In humans, the numbers of endothelial progenitor cells and M2 macrophages were significantly increased and the number of proinflammatory cells was decreased after quality and quantity control culture in both healthy volunteers and patients with connective tissue diseases. The human umbilical vein endothelial cell tube formation assay showed higher angiogenic potential in quality and quantity control cultured mononuclear cells from patients with connective tissue diseases than that in quality and quantity control cultured mononuclear cells from healthy controls.
Conclusions: Our study suggests that the quality and quantity control culture method is effective in recovering the angiogenic ability of mononuclear cells from patients with connective tissue diseases.

Introduction

Autologous mononuclear cells (MNCs) have been used for clinical vascular regenerative therapy since the discovery of endothelial progenitor cells (EPCs) by Asahara et al. in 1997[1,2]. EPCs can differentiate into the endothelium of blood vessels. EPCs are one of the components of the MNCs in bone marrow (BM) and peripheral blood (PB), which are distinguished by the expression of CD34 and CD133. For regenerative cell therapy using EPCs, MNCs have been harvested from BM or PB treated with granulocyte-colony stimulating factor (G-CSF). Autologous BM cell therapy and G-CSF-mobilized PB CD34+ cell therapy have been shown to be safe and clinically effective in

ischemic diseases[2,3]. However, autologous EPC therapy is limited for some diseases, such as diabetes and connective tissue diseases (CTDs), because of the lack of the number and angiogenic function of EPCs[4-6].

To overcome these problems, a novel technique to promote the angiogenic function of EPCs and to expand their number, which is called serum-free quality and quantity control culture (QQc) method, was developed[7]. We also reported that the population of EPCs significantly increased in PBMNCs from patients with diabetes and their vasculogenic capability substantially improved through QQc. Moreover, MNC-QQ cells from patients with diabetes promoted wound healing in mice with diabetes[8]. Phase I and II clinical trials to investigate the safety and efficacy of autologous MNC-QQ cell therapy in patients with diabetes and chronic non-healing ischemic extremity wounds were conducted from 2014 to 2017. PB (200 mL) was collected from the patients, and 2×10^7 MNC-QQ cells were injected into the area surrounding the ulcer. No death and other serious adverse events were observed during the 12-week follow-up after transplantation. In all 10 cases, vascular perfusion and skin perfusion pressure were increased and pain intensity was decreased[9].

Autoimmune diseases and vasculitis account for approximately 20%-23% of refractory chronic wounds[10]. Because the number of EPCs is decreased and their angiogenic capability is impaired in patients with CTD and in patients with diabetes, QQc could be helpful as an effective cell therapy for limb salvage. Here, we compared the angiogenic potential of MNC-QQ cells from patients with CTD with that of MNC-QQ cells from healthy controls *via* an *in vitro* study.

Methods

1 Participants

This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee and review board of Juntendo University. All participants provided informed consent. Patients with CTD were selected from the outpatient department. As the controls, healthy volunteers were recruited (**Table 1**).

2 Collection of PBMNCs

PB samples were collected from healthy volunteers and patients with CTD, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and systemic sclerosis (SSc), using BD Vacutainer cell preparation tubes (CPTs; BD, Franklin Lakes, NJ, USA). PBMNCs were isolated according to the manufacturer's protocol. Briefly, whole blood in BD Vacutainer CPTs was centrifuged at 1800 g for 20 min and MNC layers were collected into 50-mL tubes. After washing with ethylenediaminetetraacetic acid-phosphate-buffered saline (EDTA-PBS), cells were treated with ammonium chloride-potassium lysis buffer (Gibco, Thermo Fisher Scientific, USA) to lyse the remaining red blood cells. After washing twice with EDTA-PBS, the cells were suspended in PBS.

Table 1. Summary of the Profiles of the Patients with CTD and Healthy Subjects.

	Patients with CTD	Healthy subjects
N	15	16
Age (years)	57.3 ± 12.7	46.7 ± 13.5*
BMI	23.0 ± 5.7	20.2 ± 3.1
CTD duration (years)	19.9 ± 13.6	N/A
Diagnosis		
Rheumatoid arthritis	8	N/A
Systemic lupus erythematosus	3	N/A
Systemic sclerosis	3	N/A
Other CTDs	2	N/A
Diabetes mellitus	4	N/A
CRP	0.84 ± 0.93	N/A

Notes: Values are expressed as mean ± SD. *p < 0.05, patients with CTD vs. healthy subjects.

BMI, body mass index; CTD, connective tissue disease; N/A, not applicable

3 Ex vivo expansion culture for MNC-QQ cells

PBMNCs were processed in an *ex vivo* serum-free expansion culture system named QQc as previously described[7]. PBMNCs were seeded at a density of 2×10^6 cells/2 mL/well in six-well Primaria plates (Corning Inc., NY, USA) with Stemline II medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with recombinant human (rh) Fms-related tyrosine kinase-3 ligand (rh FLT-3L, 100 ng/mL), rh vascular endothelial growth factor (rhVEGF, 50 ng/mL), rh thrombopoietin (rhTPO, 20 ng/mL), rh stem cell factor (rhSCF, 100 ng/mL), rh interleukin-6 (rhIL-6, 20 ng/mL) (all from Pepro Tech, Rocky Hill, NJ, USA), and penicillin-streptomycin (Invitrogen) and were cultured for 7 days at 37°C in a 5% carbon dioxide (CO_2) atmosphere. After 7 days of not changing the medium, the MNC-QQ cells were harvested and washed with EDTA-PBS.

4 Flow cytometry

PBMNCs and MNC-QQ cells in flow cytometry staining (FACS) buffer (EDTA-PBS with 2% fetal bovine serum [FBS]) were treated with the FcR blocking reagent (Miltenyi Biotec, Auburn, CA, USA) and stained with specific antibodies as follows: Fluorescein isothiocyanate (FITC) antihuman CD19 (clone: HIB19, BioLegend, San Diego, CA, USA), PerCP/Cy5.5 antihuman CCR2 (CD192, clone: K036C2, BioLegend), BV421 antihuman CD56 (NCAM, clone: HCD56, BioLegend), PE antihuman CD34 (clone: 581, BioLegend), PE/Cy7 antihuman CD206 (MMR, clone: 15-2, BioLegend), APC antihuman CXCR4 (CD184, clone: 12G5, BioLegend), Alexa Fluor-700 antihuman CD3 (clone: UCHT1, BioLegend), APC/Cy7 antihuman CD14 (clone: HCD14, BioLegend), FITC antihuman CD4 (clone: RPA-T4, BioLegend), PerCP/Cy5.5 antihuman CD25 (clone: M-A251, BioLegend), BV421 antihuman CD127 (IL-7Rα, clone: A019D5, BioLegend), PE antihuman CD133/1 (clone: AC133, Miltenyi Biotec), PE/Cy7 antihuman CD31 (clone:

WM59, BioLegend), APC/Cy7 antihuman CD8a (clone: HIT8a, BioLegend), and appropriate isotype controls. After 30 min of incubation at 4°C and washing with FACS buffer, the cells were analyzed using a BD LSRFortessa Flow Cytometer (BD Biosciences) and the FlowJo software.

5 Tube formation assay

Human umbilical vein endothelial cells (HUVECs; Lonza, Basel, Switzerland) were cultured in microvascular endothelial cell growth medium-2 (EGM-2 MV, Lonza) according to the manufacturer's protocol, and the medium was replaced with endothelial cell growth basal medium (EBM-2, Lonza) for 1 h. PBMNCs and MNC-QQ cells were labeled with Alexa Fluor-488 acetylated low-density lipoprotein (Ac-LDL, Invitrogen, Thermo Fisher Scientific) at 37°C for 1 h. HUVECs (2×10^4) and PBMNCs (4×10^3) or MNC-QQ cells (4×10^3) were mixed in 200 μL of PBS(−) and seeded in 24-well plates precoated with 200 μL/well Matrigel matrix (Corning Inc.). The plates were incubated for 2 h at 37°C in a 5% CO_2 atmosphere before being photographed using an Olympus IX83 microscope (Tokyo, Japan). The number of closed circles was counted and normalized to that of the controls, which contained only HUVECs.

6 EPC culture assay

The EPC culture assay was performed as described previously[8]. PBMNCs and MNC-QQ cells were seeded at 1×10^5 cells/well in human fibronectin-coated 96-well plates with EGM-2 MV medium (Lonza) and were cultured at 37°C with 5% CO_2 for 7 days. After 7 days, the attached cells were stained with Alexa Fluor-488 Ac-LDL (Invitrogen) and rhodamine-labeled *Ulex europaeus* agglutinin I (UEA-I, Vector Lab., Burlingame, CA, USA) for 4 h at 37°C in 5% CO_2. After fixation with 4% paraformaldehyde phosphate-buffered solution (PFA; Wako, Osaka, Japan) at 4°C for 30 min, the cells were mounted using Vectashield (Vector Lab.) with 4', 6-diamidino-2-phenylindole. Three to five fields in each well were photographed using a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan), and Alexa Fluor-488 Ac-LDL and rhodamine UEA-I double-positive cells were counted as early EPCs.

7 EPC colony-forming assay

An EPC-colony-forming assay (EPC-CFA) semisolid culture medium using MethoCult SF H4236 (Stem Cell Tech.) supplemented with 30% FBS (CCB, Nichirei Biosci., Tokyo, Japan), rhEGF (50 ng/mL), rhVEGF (50 ng/mL), rhSCF (100 ng/mL), rhIGF-1 (50 ng/mL), rhFGF-2 (50 ng/mL), rhIL-3 (20 ng/mL) (all chemicals obtained from Pepro Tech.), heparin (2 IU/mL) (Ajinomoto, Tokyo, Japan), and penicillin-streptomycin was diluted in 30% FBS-Iscove's Modified Dulbecco's Medium. MNCs or MNC-QQ cells were mixed with the medium and were seeded using blunt-end 18 G needles (Nipro, Osaka, Japan) in a 35-mm Primaria culture dish (2×10^5 cells/1 mL/dish). After 2 weeks of culture, the number of adherent colonies per dish was measured using a phase-contrast light microscope (Nikon,

Tokyo, Japan). EPCs were classified as either definitive EPC colony-forming units (dEPC-CFUs) or primitive EPC (pEPC)-CFUs, as previously described[7].

8 Isolation and culture of MNCs from mice with SLE

C57BL/6J and C57BL/6JJmsSlc-*lpr/lpr* male mice aged 10 weeks were purchased from Japan SLC Inc. All animal experiments were approved by the institutional guidelines of the Care and Use Committee of Juntendo University, School of Medicine, and followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. PBMNCs were isolated by density gradient centrifugation using Histopaque-1083 (Sigma-Aldrich). MNCs were cultured for 5 days at 37°C in a 5% CO_2 atmosphere in Stemline II medium supplemented with 100 ng/mL mouse FLT-3L, 50 ng/mL mouse VEGF, 20 ng/mL mouse TPO, 100 ng/mL mouse SCF, 20 ng/mL mouse IL-6 (Pepro Tech), and penicillin-streptomycin. For flow cytometry analysis, the PBMNCs were incubated with anti-Sca-1 and anti-c-Kit antibodies for 20 min. Sca-1$^+$/c-Kit$^+$ cells, presumably representing circulating EPCs, were evaluated using LSRFortessa (BD). The vasculogenic potential of the PBMNCs was assessed using EPC-CFA[5,7]. A total of 2×10^5 PBMNCs per dish were seeded into a 35-mm Primaria tissue culture dish (BD Falcon). After 7 days, the EPC-CFUs were counted.

9 Statistics

The Mann-Whitney test (a nonpaired nonparametric test) was performed to compare the healthy and CTD groups. The Wilcoxon matched pairs signed-rank test (a paired nonparametric test) was performed to compare the pre- and post-QQc groups. All analyses were performed using the GraphPad Prism 8 software. Mean ± standard deviation was used to represent all data.

Results

1 Numbers of EPCs in PB from healthy mice and mice with SLE

There was no difference in the number of MNCs collected from PB between healthy mice and mice with SLE ($1.32 \pm 0.64 \times 10^6$ cells vs. $1.37 \pm 0.76 \times 10^6$ cells, p = 0.93) (**Figure 1A**). PBMNCs from healthy mice included more circulating EPCs (Sca-1$^+$/c-Kit$^+$ cells) (0.06% ± 0.019% vs. 0.04% ± 0.009%, p < 0.05) than those from mice with SLE. Following QQc, the numbers of EPCs were significantly increased and there was no difference between healthy mice and mice with SLE (0.20% ± 0.03% vs. 0.18% ± 0.01%, p = 0.20) (**Figure 1B**). Pre-QQc cells from healthy mice contained slightly more dEPC-CFUs than those from mice with SLE (1.87 ± 1.94 counts per well vs. 0.56 ± 0.47 counts per well, p = 0.077). After QQc, the number of dEPC-CFUs was significantly increased in both healthy mice and mice with SLE. The numbers of dEPC-CFUs derived from post-QQc cells from healthy mice and mice with SLE were not significantly different (30.0 ± 7.00 counts per
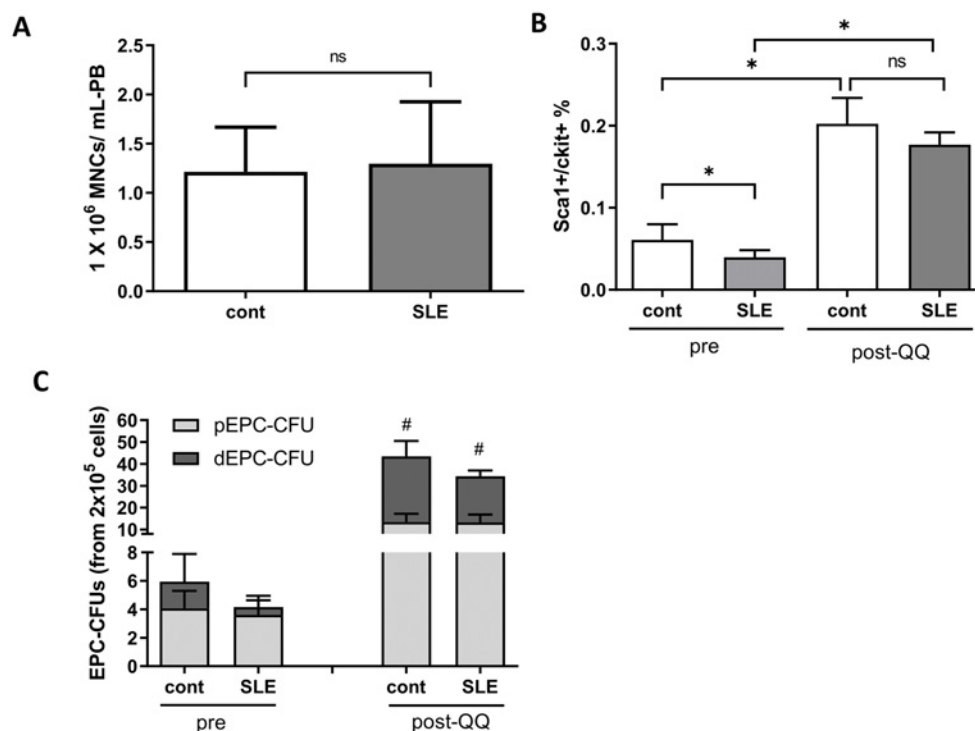
Figure 1. Depletion of EPCs in C57BL/6JJmsSlc-lpr/lpr (SLE) mice PBMNCs was recovered by QQ culture.

(A) The number of PBMNCs in control mice and mice with SLE (n = 12). (B) Flow cytometry analysis detected EPCs that were double positive for Sca-1 and c-kit in control mice and mice with SLE pre- and post-QQc PBMNCs (pre-QQ, n = 6; post-QQ, n = 3). (C) The number of pEPC and dEPC colonies in control mice and mice with SLE pre- and post-QQc PBMNCs (pre-QQ, n = 11; post-QQ, n = 3).

*p < 0.05, #p < 0.05 versus pre-MNCs.

ns, not significant; EPCs, endothelial progenitor cells; pEPC, primitive EPC; dEPC, definitive EPC; SLE, systemic lupus erythematosus; PBMNCs, peripheral blood mononuclear cells

well vs. 21.0 ± 2.65 counts per well, p = 0.10) (**Figure 1C**). These results suggest that the reduced number of EPCs in mice with SLE was recovered by QQc.

2 Significant increase in the number of EPCs after QQc

The number of MNCs in the PB did not differ significantly between healthy controls and patients with CTD ($0.78 \pm 0.22 \times 10^6$ cell/mL vs. $1.07 \pm 0.53 \times 10^6$ cells/mL, p = 0.25) (**Figure 2A**). The number of EPCs was analyzed using flow cytometry. The percentages of CD34 and CD133 double-positive cells, presumably representing EPCs, were significantly increased in MNCs from both healthy controls and patients with CTD after QQc compared to before QQc (0.03% ± 0.03% vs. 0.14% ± 0.09%, p < 0.05; 0.03% ± 0.04% vs. 0.22% ± 0.24%, p < 0.05, respectively), whereas there was no difference in the percentage of those cells between healthy controls and patients with CTD both before and after QQc (**Figure 2B**). Concordant with the FACS results, the EPC culture assay results showed that the numbers of Ac-LDL+/lectin+ cells, representing early EPCs, derived from MNCs were significantly increased in both healthy controls and patients with CTD after QQc (37.66 ± 28.38 cells vs. 145.3 ± 30.32 cells, p < 0.05; 25.96 ± 18.59 cells vs. 100.3 ± 53.9 cells, p < 0.05, respectively), whereas there

was no difference in their numbers between healthy controls and patients with CTD (**Figure 2C**). Moreover, QQc significantly increased the total EPC-CFUs and dEPC-CFUs that were derived from the MNCs from patients with CTD and each of those from healthy controls (**Figure 2D**).

3 Cell populations change toward anti-inflammation after QQc in healthy controls and patients with CTD

We performed FACS analysis to examine the effects of QQc on cell populations involved in inflammation. The percentage of CD14-positive monocytes or macrophages was significantly higher in MNCs from patients with CTD than in those from healthy controls. This difference was lost because of the slight decrease in the percentage of CD14-positive cells in MNCs from patients with CTD after QQc, whereas there was no change in that from healthy controls. Among the macrophages, the CD206-positive M2 macrophage population was larger in MNCs from patients with CTD than in those from healthy controls, although the difference was not significant. The percentages of CD206-positive cells were significantly increased both in MNCs from healthy controls and in those from patients with CTD after QQc. The percentages of proinflammatory cells, such as CCR2-positive M1 macrophages, CD19-positive B cells,
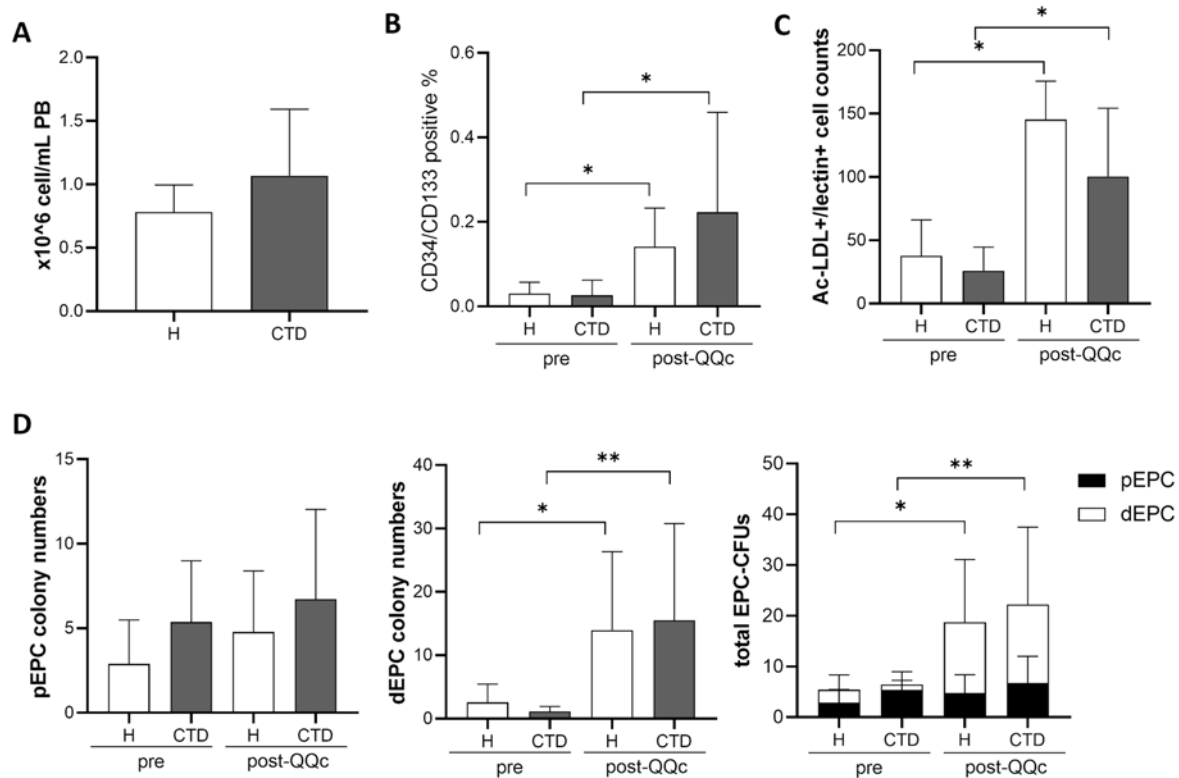
Figure 2.   Number of EPCs in PBMNCs was increased by QQ culture in patients with connective tissue disease.
(A) The number of PBMNCs was counted in healthy controls and patients with CTD (healthy, n = 5; CTD, n = 12). (B) Flow cytometry analysis detected EPCs that were double positive for CD34 and CD133 in healthy and CTD pre- and post-QQc PBMNCs (healthy, n = 7; CTD, n = 12). (C) The EPCs stained with Ac-LDL and lectin were counted after the EPC culture was counted (n = 6). (D) The number of pEPC and dEPC colonies in pre- and post-QQc PBMNCs from healthy controls and patients with CTD (healthy, n = 6; CTD, n = 9).
*p < 0.05, **p < 0.01. PBMNCs, peripheral blood mononuclear cells; QQc, serum-free quality and quantity control culture; H, healthy subjects; CTD, connective tissue disease; pEPC, primitive EPC; dEPC, definitive EPC; Ac-LDL, acetylated low-density lipoprotein

and CD56-positive natural killer (NK) cells, were significantly decreased both in MNCs from healthy controls and in those from patients with CTD after QQc, whereas there were no differences between those from healthy controls and patients with CTD. Although the percentage of angiogenic T cells in MNCs from patients with CTD was likely to be lower than that in MNCs from healthy controls, QQc significantly expanded the population of angiogenic T cells from patients with CTD (**Figure 3**).

4 Significant improvement in angiogenic potential after QQc in patients with CTD

Next, we examined the angiogenic potential of MNC-QQ cells from patients with CTD and healthy controls using the HUVEC tube formation assay. There was no significant difference in tube formation between healthy MNCs and CTD MNCs cocultured with HUVECs for 2 h. QQc significantly promoted the tube formation capacity of MNCs from patients with CTD compared with that from healthy controls (1.84 ± 0.77-fold vs. 6.66 ± 5.2-fold upon HUVEC only, p < 0.05) (**Figure 4**).

Discussion

Chronic refractory ulcers often occur in CTDs such as RA, SSc, and SLE. Impaired wound regeneration is mainly caused by poor vasculogenesis, which partly results from the dysfunction of EPCs[6,11]. In this study, we showed the effect of the QQc method on PBMNCs from patients with CTD. In both mice and humans, after QQc, the percentages of the EPC population both in MNCs from healthy controls and in those from mice with SLE/patients with CTD were significantly increased. Among the total EPC population, the percentages of the definitive EPC-CFUs, which represent mature EPCs and have high vasculogenic potential, were increased after QQc. Consistent with the EPC-CFU assay, *in vitro* tube formation assays showed the increased angiogenic ability of both MNC-QQ cells from healthy controls and those from patients with CTD conditions compared with those of pre-QQc MNCs.

In mice with SLE, the number and angiogenic function of EPCs in PB were decreased, in agreement with a previous report[11]. However, there was no significant difference in the number of EPCs in human healthy controls and patients with CTD, although many studies have reported a decrease
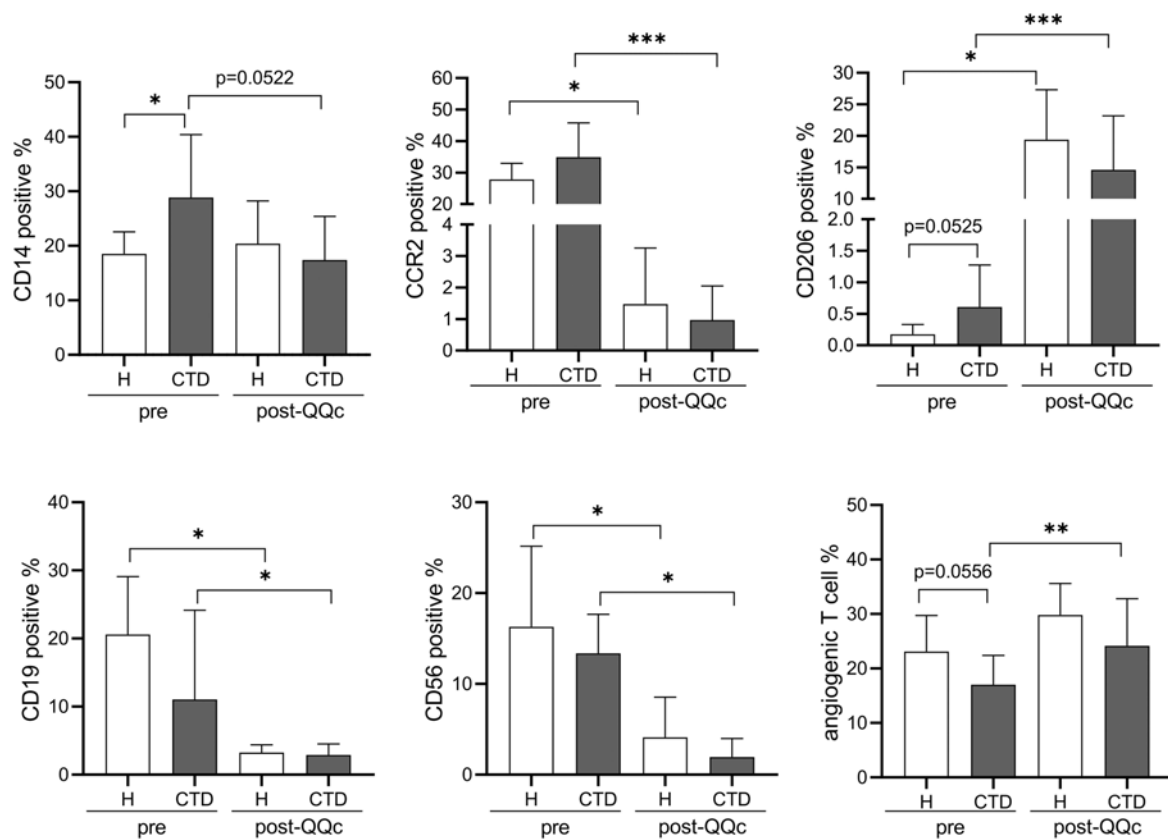
Figure 3. Flow cytometry analysis of the PBMNC subpopulation after QQc in healthy controls and patients with CTD.

The percentages of CD14+, CCR2+, CD206+ macrophage markers, CD19+ B cell markers, CD56+ NK cell markers, and angiogenic T cell markers in PBMNCs and MNC-QQ cells were analyzed by flow cytometry (healthy, n = 7; CTD, n = 12).

*p < 0.05, **p < 0.01, ***p < 0.001. H, healthy subjects; CTD, patients with connective tissue disease; PBMNCs, peripheral blood mononuclear cells; QQc, serum-free quality and quantity control culture; NK, natural killer

in the number of EPCs in patients with CTD[6,11,12]. One reason for this inconsistency would be that we included patients with CTD with a variety of disease levels and medications in this study. Grisar et al.[13] reported the effects of medication and activity on circulating angiogenic cells in patients with RA. They found that the number of EPCs was much lower in patients with active RA than in those with inactive RA or those receiving antitumor necrosis factor therapy[13,14]. Thus, the EPC-expanding effects of QQc on patients in the active stage of CTD remain to be seen in the future.

Our results showed a tendency for a reduction in angiogenic T cells in CTD MNCs compared with healthy MNCs, whereas no significant difference in EPC percentage was found between them. Similar to EPCs, the number of angiogenic T cells is also known to be decreased in patients with RA[15]. Angiogenic T cells are a subset of T cells that enhance endothelial function by cooperating with EPCs. The decrease in the number of angiogenic T cells and higher levels of IFN alpha in patients with CTD were reported to be associated with the occurrence of cardiovascular events[15], and this association might be mediated by an impaired endothelial repair mechanism. QQc increased the percentage of

angiogenic T cells in MNCs from patients with CTD to a level similar to that observed in those from healthy controls. These results also show the promising applicability of potentially angiogenic MNC-QQ cells harvested from patients with CTD.

M2 macrophages are anti-inflammatory macrophages that are important in wound healing. We showed that the number of CD206-positive cells, which represent M2 macrophages, was slightly increased in PBMNCs from patients with CTD than in those from healthy controls. Some reports have demonstrated an increase in the number of CD14-positive monocytes/macrophages and M2 macrophages in patients with CTD[16-18]. The number of M2 macrophages was significantly increased after QQc, whereas the numbers of immune system cells, CD56-positive NK cells, CD19-positive B cells, and proinflammatory CCR2-positive M1 macrophages were decreased. These results suggest that QQc could also contribute to the creation of an anti-inflammatory environment in MNC transplantation therapy.

In this study, we only performed an *in vitro* analysis of MNC-QQ cells obtained from patients with CTD. The wound healing and angiogenic effects of MNC-QQ cells from healthy mice and mice with diabetes mellitus *in vitro*
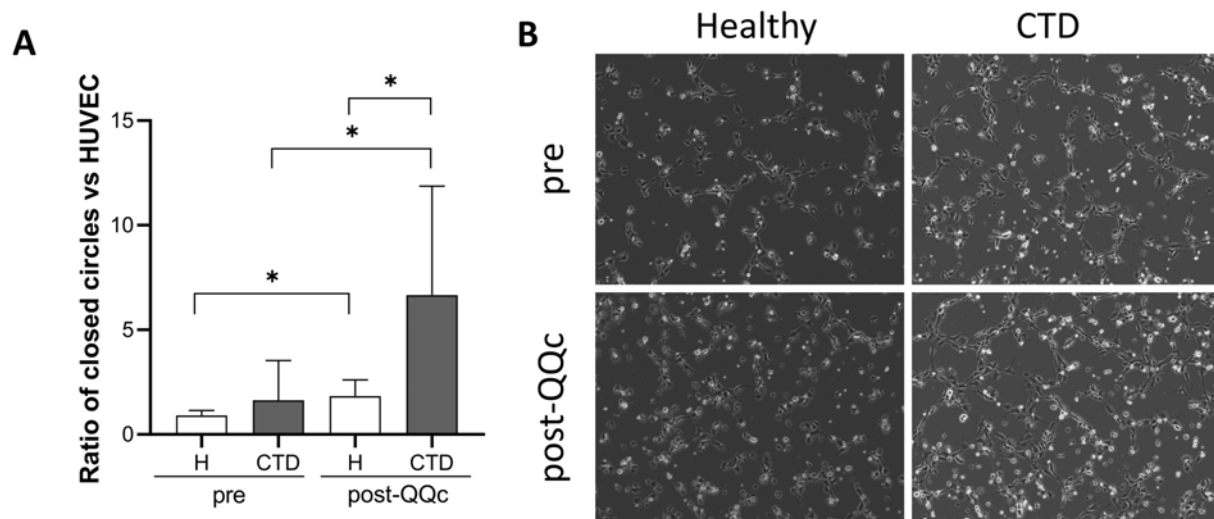
Figure 4. Improvement in angiogenic ability after QQc in CTD PBMNCs was analyzed using the HUVEC tube formation assay.

(A) The number of closed-circle formations on the Matrigel was counted. The graph shows the ratio of the combination of MNCs, MNC-QQ cells, and HUVECs to the HUVEC-only control group (n = 6). (B) Representative images of tube formation.

*$p < 0.05$. H, healthy; CTD, connective tissue disease; PBMNCs, peripheral blood mononuclear cells; QQc, serum-free quality and quantity control culture; HUVEC, human umbilical vein endothelial cell

and *in vivo* were previously reported[7,8,19]. Recently, phase I and IIa clinical trials were conducted to investigate the safety and efficacy of autologous MNC-QQ cell therapy in patients with chronic nonhealing ischemic extremity wounds[9]. In these trials, two patients with SSc were included and MNC-QQ cell transplantation improved vascular perfusion and pain in patients with SSc as effectively as it did in other patients with diabetes mellitus. Thus, we believe that MNC-QQ cell therapy serves as a noninvasive and effective therapy for collagen tissue diseases with refractory chronic ulcers.

This study had some limitations. First, the number of participants with CTD was small and the study included patients with various diseases, such as RA and SLE. As far as we could obtain information, most patients were well controlled with medication. Thus, further analysis with a larger number of participants is required to determine the effects of QQc methods on each type and pathological stage of CTDs. Second, we only performed *in vitro* analyses. Although we confirmed that there was no difference in the cell population and the angiogenic potential of MNC-QQ cells between healthy controls and patients with CTD, the *in vivo* function of MNC-QQ cells in CTD model mice should be examined in more detail in the future.

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