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RHEUMATOLOGY

Rheumatology 2010;49:2375–2380
doi:10.1093/rheumatology/keq259
Advance Access publication 19 August 2010

Concise report

Mobilization of endothelial progenitor cells by intravenous cyclophosphamide in patients with systemic sclerosis

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Abstract

Objective. To evaluate the effects of i.v. CYC on the number of circulating endothelial progenitor cells (EPCs) in patients with SSc, and the potential association of the EPC response with CYC's effect for treating interstitial lung disease (ILD).

Methods. This open-label, prospective study involved 12 patients with SSc and alveolitis (CYC group). All patients received six courses of i.v. CYC (0.5 g/m^2) at 4-week intervals in combination with low-dose prednisolone. Ten patients were followed for 24 months. Seven SSc patients treated with low-dose prednisolone alone were used as a control for the EPC measurement (control group). Five patients with non-SSc CTD who received i.v. CYC and prednisolone also served as disease controls. EPCs were quantified by the partial enrichment of CD34$^+$ cells followed by three-colour flow cytometry. The circulating levels of vascular injury markers were measured by immunoassay.

Results. The EPC count was significantly increased at 2 weeks after treatment in the CYC group ($P = 0.02$), but not in the control group, while CYC increased EPC count in all disease controls. The SSc patients in the CYC group were divided into five EPC responders and seven EPC non-responders. Circulating vascular injury markers were reduced in the responders, but not in the non-responders. During the 24-month follow-up, 3 of 10 patients developed end-stage lung disease, and all of them were EPC non-responders.

Conclusion. A low-dose i.v. CYC induces EPC mobilization, which may contribute to the efficacy for treating SSc-associated ILD.

Key words: Scleroderma, Respiratory, Biomarkers, Immunosuppressants, Outcome measures.

CLINICAL SCIENCE

Introduction

Interstitial lung disease (ILD) is the leading cause of mortality in patients with SSc [1]. The pathogenesis of SSc-associated ILD is thought to be chronic inflammation in the lung parenchyma, which causes lung injury and resultant fibrosis. Based on this theory, immunosuppressive agents, such as CYC, are used for treating SSc-associated ILD. In a placebo-controlled, double-blind, randomized trial in SSc patients with ILD and alveolitis, 1 year of treatment with oral CYC resulted in significant beneficial effects on lung function, although a significant proportion of CYC-treated patients showed a deterioration of lung function [2]. Another clinical trial, in which six courses of i.v. CYC (0.6 g/m^2) were followed by AZA in combination with low-dose CSs, demonstrated a potential effect on stabilizing lung function; a statistical trend towards preventing a decline of forced vital capacity [3]. Therefore, the beneficial effects of CYC on SSc-associated ILD seem limited, with only a subset of patients obtaining a substantial treatment benefit [4].

Recently, accumulating lines of evidence have indicated that bone marrow-derived progenitors contribute to tissue

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Submitted 27 September 2009; revised version accepted 7 July 2010.

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repair and remodelling of the lung [5]. Such progenitors include endothelial progenitor cells (EPCs), which play an important role in vascular formation and healing in response to vascular injury, by homing to the site of injury and working in concert with existing endothelial cells [6]. Human EPCs, also termed circulating endothelial precursors, are identified as non-haematopoietic cells with a characteristic phenotype positive for CD34, CD133 and VEGF receptor type 2 (VEGFR2) [7]. We recently reported that EPCs in SSc patients are reduced in number and deficient in their capacity to mature into endothelial cells compared with those of healthy individuals [8], although whether the number of EPCs is reduced is a matter of debate [9]. In haematopoietic stem cell transplantation (HSCT), mobilization and conditioning regimens that include high-dose CYC ($\geqslant 4\,g/m^2$) mobilize a variety of bone marrow-derived progenitors [10]. Thus, we hypothesized that EPCs are also mobilized, to some extent, even by the low-dose CYC regimen used to treat SSc-associated ILD, and that increasing the circulating EPCs would contribute to CYC's clinical benefits. To test this hypothesis, we conducted a pilot study to evaluate the effect of low-dose CYC regimen on the EPC count in circulation, and its association with clinical efficacy for SSc-associated ILD.

Materials and methods

Study design

This open-label, prospective study was conducted at Keio University, Nagasaki University and Kanazawa University, Japan, during the period from June 2004 to June 2010. Twelve consecutive patients with SSc who received i.v. CYC for ILD were enrolled. All the patients fulfilled the ACR preliminary classification criteria for SSc [11], and had ILD with alveolitis confirmed by high-resolution CT and/or analysis of bronchoalveolar lavage fluid [2]. Exclusion criteria included end-stage lung disease (ESLD), which was defined by a per cent vital capacity (%VC) <50% or a requirement for continuous oxygen supplementation [12]. The i.v. CYC protocol consisted of six courses of the i.v. infusion of $0.5\,g/m^2$ CYC at 4-week intervals. Prednisolone at a dose of $\leqslant 30$ mg daily was simultaneously started, and then tapered gradually. After completion of the CYC regimen, two patients received AZA as maintenance therapy, but the others did not. As a control for the evaluation of EPC number, we enrolled seven SSc patients who received prednisolone alone ($\leqslant 30$ mg daily) for progressive skin thickening ($n=4$) or inflammatory conditions such as arthritis ($n=3$) during the study period. In addition, five patients with non-SSc CTD who received i.v. CYC ($0.5\,g/m^2$) and moderate- to high-dose prednisolone (>30 mg daily) served as disease controls. These included three patients with SLE, and one each with DM or microscopic polyangiitis (MPA). Peripheral blood samples were obtained at pre-treatment and 2 weeks after the first i.v. CYC course (CYC group) or after the initiation of prednisolone (control group). The study was approved by individual institutional review boards (IRBs) (Keio University IRB, Kanazawa University IRB and Nagasaki University IRB), and written informed consent was obtained from each patient.

Quantification of EPCs

The absolute number of EPCs in circulation was determined using a method described previously [13]. Briefly, a $CD34^+$-cell-enriched fraction was prepared from peripheral blood mononuclear cells using a magnetic-activated cell sorter immunomagnetic technique (Miltenyi Biotech, Bergisch Gladbach, Germany) with Fc receptor blocking reagent, according to the manufacturer's protocol. The $CD34^+CD133^+VEGFR2^+$ cells were detected by flow cytometric analysis on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Finally, the number of EPCs in 20 ml of peripheral blood was calculated based on the ratio of $CD34^+$ cells to FlowCount microbeads (Beckman-Coulter, Fullerton, CA). All procedures were performed by the same experienced operator (Y.O.), who was blinded to the sample identity. This procedure met all the recommendations proposed by the EULAR Scleroderma Trials and Research group [9], except the use of a viability marker. Instead, we excluded dead cells by gating for lymphocytes in the scatter analysis before examining cell surface markers. Our preliminary analysis comparing the use of viability marker 7-AAD with the use of gating strategy in SSc patients and healthy controls revealed a strong correlation between the results obtained from these strategies ($r=0.96$) [Kuwana M. (data not published)]. In SSc patients, an EPC responder was defined as a patient meeting both of the following criteria 2 weeks after treatment: (i) 50% increase in the pre-treatment EPC level: this was shown to result in significant improvement in RP variables and reductions in the up-regulated vascular endothelial injury markers during the atorvastatin treatment [13], and (ii) more than 600 EPCs/20 ml peripheral blood; this is the lower limit of EPC distribution in healthy individuals [8]. The remaining patients were classified as EPC non-responder, although there is currently no definition of the EPC responder in literature.

Circulating levels of vascular injury markers

The level of VEGF and soluble E-selectin in heparinized plasma samples was measured using ELISA kits (Quantikine; R&D Systems, Minneapolis, MN).

Statistical analysis

All continuous variables are shown as the mean (S.D.). Changes in serial variables were analysed by the paired *t*-test. The cumulative rates for no ESLD in two groups were compared by the log-rank test.

Results

Baseline characteristics of the patients

Table 1 shows the baseline clinical characteristics of SSc patients in the CYC and control groups. There was no difference in the disease duration, modified Rodnan

TABLE 1 Baseline characteristics of SSc patients enrolled

Demographic and clinical findings	CYC group ($n = 12$)	Control group ($n = 7$)	P-value
Age, mean (S.D.), years	56.6 (10.3)	47.7 (19.1)	NS
Female, %	67	43	NS
SSc duration, mean (S.D.), years	2.4 (2.8)	3.8 (8.9)	NS
dcSSc, %	83	100	NS
mTSS, mean (S.D.)	21.1 (9.6)	24.3 (10.7)	NS
ILD, %	100	71	NS
VC, mean (S.D.), %	80.8 (13.7)	103.0 (13.0)	0.01
Anti-topo I antibody, %	58	57	NS
Anti-RNA polymerase III antibody, %	17	14	NS
Initial prednisolone dosage, mean (S.D.), mg/day	19.0 (6.2)	22.1 (5.7)	NS

NS: not significant.

total skin thickness score (mTSS) or initial prednisolone dosage, but %VC was significantly lower in the CYC group than in the control group ($P = 0.01$).

Effects of i.v. CYC on the EPC count

All patients in the CYC group completed six courses of i.v. CYC, and 10 of them completed a 24-month follow-up. Flow cytometric analyses for stained CD133 and VEGFR2 on the gated $CD34^+$ cells in a representative SSc patient, in whom the EPCs were increased 2 weeks after i.v. CYC, are shown in Fig. 1A. The 2-week time point was chosen because preliminary serial analyses revealed that the number of EPCs started to increase 1 week after i.v. CYC, and reached the peak at 2 weeks, which lasted for at least a week (data not shown). EPCs were significantly increased from the baseline at 2 weeks [243 (153) to 476 (287), $P = 0.02$)], but only five patients (42%) were classified as EPC responders (Fig. 1B). In one responder, the EPC response following i.v. CYC treatment was reproduced in all six consecutive courses. In contrast, no increase in EPC number was observed in the control group treated with prednisolone alone [207 (116) to 162 (74)]. Interestingly, all disease controls experienced an increase in EPC number after CYC treatment, and this change was statistically significant [685 (277) to 1229 (406), $P = 0.003$; Fig. 1C]. Within the CYC group of SSc patients, there was no difference in the baseline characteristics, including the disease duration, mTSS, %VC and initial prednisolone dosage, between five EPC responders and seven non-responders.

Effects of i.v. CYC on vascular injury markers

In SSc patients, EPC responders in the CYC group showed reduction in the VEGF level and a trend towards the reduced soluble E-selectin level at 2 weeks ($P = 0.04$ and 0.07, respectively), while these trends were not observed in EPC non-responders of the CYC group or in the control group (Fig. 1D).

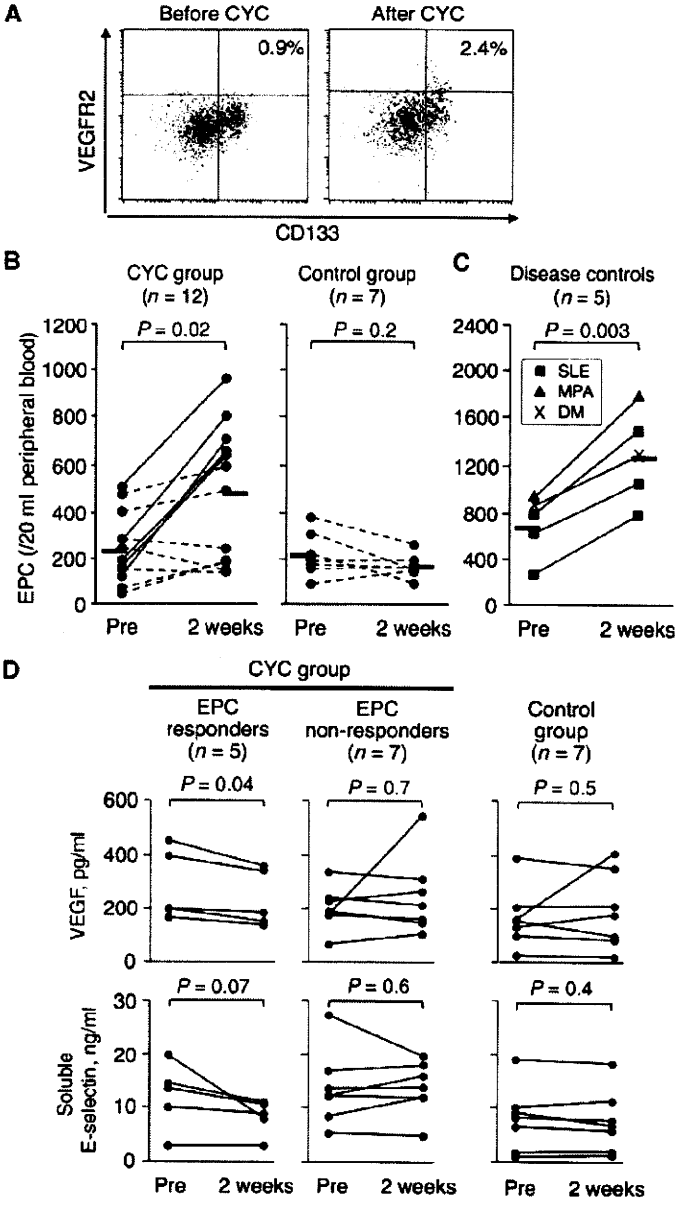
Association between the EPC response and efficacy of i.v. CYC on ILD

In 10 SSc patients who completed the 24-month follow-up period, none of the 4 responders, but 3 (50%) of 6 non-responders developed ESLD. However, life-table analysis to compare the probability of no ESLD showed that this difference did not reach statistical significance ($P = 0.1$). Four patients in the CYC group had digital ulcers at the time of entry. In two EPC responders, the number of new digital ulcers prior to the enrolment was 2 and 6/year, but these decreased to 1 and 1.5/year after the CYC treatment. In contrast, in two EPC non-responders, the number of new digital ulcers did not change before and after the CYC treatment.

Discussion

CYC is an alkylating agent that exerts its immunosuppressive effect mainly through a rapid cytotoxic effect on activated lymphocytes. On the other hand, immature progenitor cells including EPCs are relatively resistant to this drug, and thus are recruited to the circulation in the recovery phase after CYC exposure [14]. This pilot study demonstrated that low-dose i.v. CYC (0.5 g/m^2) plus CSs, but not CSs alone, increased the EPC count in a subset of SSc patients, although CYC-induced EPC recruitment was less efficient in patients with SSc compared with those with other CTDs. This may be explained by impaired differentiation potential of bone marrow progenitors in SSc patients [15]. We found that EPC responders showed trends towards reduced levels of circulating vascular injury markers, and a low probability of developing ESLD, whereas non-responders did not. These findings suggest that EPC mobilization may contribute to the efficacy of i.v. CYC for treating SSc-associated ILD. In this regard, in a recent study of high-dose CYC (50 mg/kg for 4 consecutive days) without HSCT rescue, patients with active dcSSc showed clinically significant improvement in lung function [16]. It is likely that treatment with a higher dose of CYC would result in more prominent EPC

FIG. 1 Circulating EPCs were increased after i.v. CYC in SSc patients with ILD and alveolitis. (**A**) Serial measurements of EPCs before and 2 weeks after i.v. CYC by flow cytometry in a representative SSc patient in the CYC group. EPCs were identified as $CD133^+VEGFR2^+$ cells on gated $CD34^+$ cells, which are shown in the upper-right portion. The number denotes the proportion of $CD133^+VEGFR2^+$ cells in the total $CD34^+$ cells. (**B**) The number of EPCs at pre-treatment and 2 weeks after treatment in 12 SSc patients in the CYC group and 7 SSc patients in the control group. EPC responders are shown as solid lines, while non-responders are shown as broken lines. The bar denotes the mean. (**C**) The number of EPCs at pre-treatment and 2 weeks after CYC treatment in five patients with SLE, MPA or DM. The bar denotes the mean. (**D**) Circulating levels of VEGF and soluble E-selectin at pre-treatment and 2 weeks after the treatment in EPC responders and non-responders of the CYC group, and in the control group.



mobilization in many patients than the low-dose treatment we used in the present study.

It has been reported that EPCs are mobilized during massive tissue damage and recruited to the lung of patients with acute lung injury [17, 18]. Moreover, in a rat model of pulmonary hypertension, therapeutic application of bone marrow progenitors containing EPCs resulted in remodelling of the lung and heart [19]. In these studies,

it has been hypothesized that bone marrow EPCs contribute to the repair of the damaged tissue by homing to the site of injury, replacing the injured vascular endothelium and promoting regeneration processes. The pathogenesis of SSc-associated ILD mainly involves the excessive fibrotic process in response to the alveolar epithelial injury [20], and one of the histological characteristics is prominent reduction of pulmonary vascular density [21]. Since failure of re-endothelialization and re-epithelialization of the alveolar-capillary barrier leads to destroyed lung architecture and fibrosis [22], EPCs recruited to the damaged lung may suppress this ongoing pathogenic process. Therefore, it is likely that the clinical benefit of i.v. CYC observed in a subset of SSc patients might have resulted, in part, from repair or remodelling of the lung parenchyma through EPC mobilization. This mechanism is probably unique for the i.v. CYC regimen, since no increase in EPC number was observed during oral CYC treatment (our unpublished observation).

In a retrospective study of i.v. CYC (0.6 g/m^2) followed by AZA in 27 patients with SSc-associated ILD, 22% had improved, 30% were stable and 48% had worsened [23]. This finding suggests that a clinical response to the i.v. CYC was observed only in a subset of patients. In this regard, we found that mobilization of EPCs after the first i.v. CYC administration may predict beneficial effects of the CYC regimen for ILD. Therefore, EPC measurement may be useful for deciding whether a potentially harmful CYC regimen should be continued or discontinued.

A major limitation of this study is a small number of the patients analysed. Thus, it still remains unanswered if increasing the circulating EPCs would contribute to CYC's clinical benefits. Since this is a preliminary study, further prospective studies involving a large number of patients are necessary to confirm our hypothesis.

Rheumatology key messages

- The i.v. CYC used for treatment of SSc-associated ILD increased circulating EPC count in some patients.
- EPC mobilization after i.v. CYC may predict the beneficial effects for ILD.

Acknowledgements

Funding: This work was supported by a research grant for intractable diseases from the Japanese Ministry of Health, Labour and Welfare.

Disclosure statement: The authors have declared no conflicts of interest.

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Yamaguchi et al. Arthritis Research & Therapy 2010, 12:R205
http://arthritis-research.com/content/12/6/R205

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RESEARCH ARTICLE Open Access

Enhanced angiogenic potency of monocytic endothelial progenitor cells in patients with systemic sclerosis

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Abstract

Introduction: Microvasculopathy is one of the characteristic features in patients with systemic sclerosis (SSc), but underlying mechanisms still remain uncertain. In this study, we evaluated the potential involvement of monocytic endothelial progenitor cells (EPCs) in pathogenic processes of SSc vasculopathy, by determining their number and contribution to blood vessel formation through angiogenesis and vasculogenesis.

Methods: Monocytic EPCs were enriched and enumerated using a culture of peripheral blood mononuclear cells and platelets on fibronectin in 23 patients with SSc, 22 patients with rheumatoid arthritis (RA), and 21 healthy controls. To assess the capacity of monocytic EPCs to promote vascular formation and the contribution of vasculogenesis to this process, we used an *in vitro* co-culture system with human umbilical vein endothelial cells (HUVECs) on Matrigel® and an *in vivo* murine tumor neovascularization model.

Results: Monocytic EPCs were significantly increased in SSc patients than in RA patients or healthy controls ($P = 0.01$ for both comparisons). Monocytic EPCs derived from SSc patients promoted tubular formation in Matrigel® cultures more than those from healthy controls ($P = 0.007$). Transplantation of monocytic EPCs into immunodeficient mice resulted in promotion of tumor growth and blood vessel formation, and these properties were more prominent in SSc than healthy monocytic EPCs ($P = 0.03$ for both comparisons). In contrast, incorporation of SSc monocytic EPCs into the tubular structure was less efficient *in vitro* and *in vivo*, compared with healthy monocytic EPCs.

Conclusions: SSc patients have high numbers of aberrant circulating monocytic EPCs that exert enhanced angiogenesis but are impaired in vasculogenesis. However, these cells apparently cannot overcome the anti-angiogenic environment that characterizes SSc-affected tissues.

Introduction

Systemic sclerosis (SSc) is a multi-system connective tissue disease characterized by excessive fibrosis and microvascular abnormalities. SSc vasculopathy mainly affects small arteries, causing reduced blood flow and tissue ischemia, which leads to Raynaud's phenomenon, digital ulcers, and gangrene [1]. The pathogenesis of SSc vasculopathy is not fully understood, but several lines of evidence have shown that the primary mechanism involves enhanced vascular injury, occurring as a result of an inflammatory-immune response and ischemia-reperfusion reactions [2,3]. On the other hand, defective vascular repair machinery has recently been proposed as an alternative mechanism [4].

The formation and repair of blood vessels in adults are mediated through two different processes: angiogenesis is a process of sprouting from pre-existing vessels; it involves the proliferation and migration of mature endothelial cells. Vasculogenesis is mediated through the recruitment and *in situ* differentiation of bone marrow-derived endothelial progenitor cells (EPCs) [5]. Human EPCs, also termed circulating endothelial precursors, are progenitors lacking typical hematopoietic markers that give rise to endothelium and are characterized by a

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unique phenotype: positive for CD34, CD133, and vascular endothelial growth factor (VEGF) receptor type 2 [6]. We recently reported defective vasculogenesis in SSc patients, based on the reduced number of EPCs in circulation and their impaired maturation potential [7]. However, whether the number of EPCs in SSc patients is reduced or not is a matter of debate [8].

A subpopulation of circulating $CD14^+$ monocytes also has EPC-like characteristics, in terms of their expression of endothelial markers upon endothelial induction, formation of tube-like structures *in vitro*, and incorporation into newly formed blood vessels *in vivo* [9]. This EPC subset of myeloid origin, termed monocytic EPCs, is apparently distinct from "classic" EPCs [10] and may share characteristics of early outgrowth cells and circulating angiogenic cells [11]. Monocytic EPCs are now considered oligopotent cells that may differentiate into endothelium as well as into other elements of the vasculature, such as pericytes and smooth muscle cells, but their *in vivo* vasculogenic potential is far inferior to "classic" EPCs [12]. In addition, monocytic EPCs contribute to new vessel formation and vascular repair through angiogenesis by angiogenic factor secretion and other mechanisms [11,12].

We recently reported that primitive cells with the capacity to differentiate into various types of mesenchymal-lineage cells and into endothelial cells can be enriched from a subpopulation of circulating monocytes in an *in vitro* culture system [13-15]. These cultured cells, termed monocyte-derived multipotential cells, have a spindle-shaped morphology and a unique phenotype positive for CD14, CD45, CD34, and type I collagen [13]. Since these monocyte-derived cells are capable of proliferating and differentiating along the endothelial lineage *in vitro* and *in vivo* [15], it is reasonable to say that circulating precursors for monocyte-derived multipotential cells are compatible with or belong among the monocytic EPCs. In this study, we evaluated the potential involvement of monocytic EPCs in SSc vasculopathy by examining their quantity as well as their angiogenic and vasculogenic properties using the procedure to enrich circulating precursors for monocyte-derived multipotential cells.

Materials and methods

Patients and controls

We studied blood samples from 23 patients with SSc, 5 men and 18 women (60.2 ± 14.8 years), who fulfilled the American College of Rheumatology (ACR) preliminary classification criteria [16], and from 21 healthy controls, 4 men and 17 women (63.6 ± 10.4 years). In some analyses, samples from 22 patients with rheumatoid arthritis (RA), 2 men and 20 women (58.8 ± 8.1 years), who fulfilled the ACR classification criteria [17], were used as a disease control. Eighteen SSc patients (78%) were classified as having diffuse cutaneous SSc according to published criteria [18]. Disease duration was 11.1 ± 9.6 years, and 10 (43%) patients had received their diagnosis within five years. Clinical characteristics at the time of blood sampling are summarized in Table 1. A series of SSc-related autoantibodies were determined using indirect immunofluorescence and immunoprecipitation assays [19]. None of SSc patients received cytotoxic drug at anytime in their illness, but six patients were on low-dose corticosteroids (< 10 mg/day) at examination. All samples were obtained after the patients and control subjects gave their written informed consent in accordance with the tenets of the Declaration of Helsinki, as approved by the International Review Boards of Keio University and Yokohama City University.

Preparation and quantification of monocytic EPCs

Monocytic EPCs were enriched using a culture system we developed previously [13] with some modifications. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by Lymphoprep (Fresenius Kabi Norge AS, Halden, Norway) density-gradient centrifugation. Since the number of platelets and microparticles contaminating the PBMC fraction influences the recovery of monocytic EPCs in cultures [20], PBMCs were first subjected to platelet depletion with the MACS® system (Miltenyi Biotec, Bergisch Gladbach, Germany) using anti-CD61 monoclonal antibody (mAb)-coupled magnetic beads. Platelet-depleted PBMCs (3×10^6) were then cultured in duplicate on fibronectin-coated six-well plates with autologous platelets (3×10^7) in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (JRH Bioscience, Lenexa, KS, USA), 2 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (without any additional growth

Table 1 Clinical characteristics at the time of blood sampling in 23 patients with SSc*

Raynaud's phenomenon	23 (100%)
Digital ulcers	9 (39%)
Interstitial lung disease	10 (43%)
Current smoker	1 (4%)
Past smoker	1 (4%)
Hypertension	6 (26%)
Hypercholesterolemia	4 (17%)
Positive anti-nuclear antibody	23 (100%)
Positive anticentromere	8 (35%)
Positive anti-topoisomerase I	5 (22%)
Positive anti-U1RNP	2 (9%)
Positive anti-Th/To	3 (13%)
Positive anti-RNA polymerase III	2 (9%)

* The results are expressed as the number and frequency (%).

factors) at 37°C in a 5% CO_2 humidified atmosphere. The medium, which contained floating cells, was exchanged for fresh medium at Day 3. At 10 days of culture, adherent cells with a spindle-like morphology were counted under an inverted microscope. The number of monocytic EPCs in 1 mL of peripheral blood was calculated as the mean of multiple measurements in proportion to the volume of peripheral blood that yielded 3×10^6 PBMCs at the isolation procedure. The expression of CD1a, CD14 CD34, CD80, CD83, and VEGF receptor type 1 (VEGFR1) and uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (Dil)-labeled acetylated low-density lipoprotein (acLDL) (2.5 mg/ml: Molecular Probes, Eugene, OR, USA) in monocytic EPCs was evaluated by flow cytometry, while expression of CD31, CD144, and VEGFR1 on adherent cells was evaluated by immunohistochemistry [13].

In some instances, monocytic EPCs were cultured on fibronectin-coated plastic plates for 3, 5, 7, 10, and 14 days in endothelial cell basal medium-2 (EBM-2; Clonetics, San Diego, CA, USA) supplemented with EBM-2 MV SingleQuots® (Clonetics) containing 5% fetal bovine serum, VEGF, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor-1, heparin, and ascorbic acid [15]. The medium was exchanged with fresh medium every three to four days. Differentiation into mature endothelial cells was evaluated by immunohistochemistry for expression of VEGF receptor type 2 (VEGFR2) and von Willebrand factor (vWF) [15].

In vitro vascular tube formation in Matrigel® culture

The capacity of monocytic EPCs to promote the formation of tubular structures by mature endothelial cells was examined in Matrigel® culture (BD Biosciences, San Diego, CA, USA) as described previously [15]. Briefly, a suboptimal number (10^4) of human umbilical vein endothelial cells (HUVECs), which formed a small number of short tubular structures when cultured alone, were cultured in duplicate in EBM-2 supplemented with EBM-2 MV SingleQuots® on 12-well Matrigel® plates (BD Biosciences) with or without monocytic EPCs (10^4). Each experiment was conducted by pairing samples of monocytic EPCs derived from SSc patients and from healthy controls. As a control, monocytic EPCs were cultured alone on Matrigel®. After 24 hours, the total tube length in each well was measured. The capacity of monocytic EPCs to enhance tubular formation was assessed as the ratio of the total tube length in the culture of HUVECs plus monocytic EPCs to the length in the culture of HUVECs alone. In some instances, culture supernatants of HUVECs (10^4) plus monocytic EPCs (10^4) in EBM-2 supplemented with EBM-2 MV SingleQuots® on 12-well Matrigel® plates were collected as conditioned medium, and used in the second Matrigel® cultures with HUVECs alone (10^4). The capacity to enhance tubular formation was assessed as the ratio of the total tube length in the culture with conditioned medium of HUVECs plus monocytic EPCs to the length in the culture with conditioned medium of HUVECs alone.

In some experiments, monocytic EPCs and HUVECs were pre-labeled with PKH67 (Sigma, St. Louis, MO, USA), and Dil-acLDL, respectively, and cultured together in Matrigel® [15]. The cells were observed at 24 hours under a fluorescence microscope, and the capacity of the monocytic EPCs to be incorporated into the tubular structure was evaluated as the number of PKH67-labeled monocytic EPC-derived cells within the tubular structure divided by the total tube length (cells/mm).

In vivo tumor neovascularization model

A murine tumor neovascularization model was described previously [15]. Briefly, murine colon carcinoma CT-26 cell line cells (2.5×10^5) were transplanted beneath the skin of the back of severe combined immunodeficient (SCID) mice (Charles River Japan, Yokohama, Japan) in conjunction with or without monocytic EPCs (10^4 or 10^5). Each experiment was conducted using a pair of monocytic EPCs derived from SSc patients and from healthy controls. Ten days later, the mice were sacrificed and the volume of the subcutaneous tumor was calculated as follows: 0.5 × longest diameter × (shortest diameter)^2. Formalin-fixed, paraffin-embedded specimens were stained with hematoxylin and eosin. The number of blood vessels carrying erythrocytes was counted in 10 independent fields at a magnification of x10, and the results were expressed as the mean. Frozen specimens (8-mm thick) were incubated with rat anti-mouse CD31 mAb (BD Biosciences) in combination with mouse anti-human CD31 mAb-fluorescein isothiocyanate (FITC) conjugate (Chemicon, Temecula, CA, USA) or mouse anti-human leukocyte antigen (HLA) class I-FITC conjugate (Sigma), followed by incubation with AlexaFluor®488 anti-FITC and AlexaFluor®568 anti-rat-specific IgG antibodies (Molecular Probes). Negative controls were sections incubated with isotype-matched mouse or rat mAb to an irrelevant antigen, instead of the primary antibody. Nuclei were counter-stained with TO-PRO3 (Molecular Probes). These slides were examined with a confocal laser fluorescence microscope (LSM5 PASCAL; Carl-Zeiss, Göttingen, Germany). The efficiency of monocytic EPC incorporation into the vascular wall was evaluated as the proportion of blood vessels containing human CD31-expressing endothelial cells in at least 100 blood vessel sections.

Statistical analysis

All continuous variables were expressed as the mean ± standard deviation. Comparisons between two groups

were tested for statistical significance using the Mann-Whitney *U* test or Wilcoxon *t*-test as appropriate.

Results

Number of monocytic EPCs

We enriched for monocytic EPCs by culturing PBMCs on fibronectin with autologous platelets. After 10 days, adherent cells with a spindle-shaped morphology made their appearance in all cultures (Figure 1a). Nearly all adherent cells obtained in this culture were positive for both CD14 and CD34 (Figure 2a), as shown in our previous report [13]. These adherent cells expressed VEGFR1 and incorporated Dil-labeled acLDL, but lacked expression for dendritic cell markers CD1a and CD83 or a mature macrophage marker CD80 (Figure 2a). Immunohistochemistry showed expression of a series of endothelial markers, including CD31, CD144, and VEGFR1, by nearly all adherent cells (Figure 2b). These findings indicate that monocytic EPCs enriched in our culture system with fibronectin are a homogeneous cell population in terms of protein expression profiles. When the number of monocytic EPCs was compared among 23 patients with SSc, 22 with RA, and 21 healthy controls, there were significantly more monocytic EPCs in cultures derived from SSc patient samples, than in those from RA patients or healthy controls ($P = 0.01$ for both comparisons; Figure 1b). There was no significant association between the number of monocytic EPCs in culture and the SSc patients' disease duration, disease subset, digital ulcers, interstitial lung disease, SSc-related autoantibodies, or treatment with corticosteroids.

Capacity of monocytic EPCs to promote tubule formation *in vitro*

We first evaluated potentials of monocytic EPCs to differentiate into mature endothelial cells *in vitro*. Monocytic EPCs from seven patients with SSc and seven healthy controls cultured in endothelial induction medium resulted in expression of mature endothelial cell markers VEGFR2 and vWF, and expression of VEGFR2 and vWF was observed after Days 5 and 7, respectively, in all samples irrespective of the presence or absence of SSc (data not shown).

Next, monocytic EPCs from healthy and SSc subjects cultured alone on Matrigel® failed to form tubular structures, but they promoted tubule formation in 24-hour co-culture with HUVECs (Figure 3a). In this short-term culture, tube was formed mainly by cell migration. When we examined monocytic EPCs derived from 15 pairs of SSc patients and healthy controls (Figure 3b),

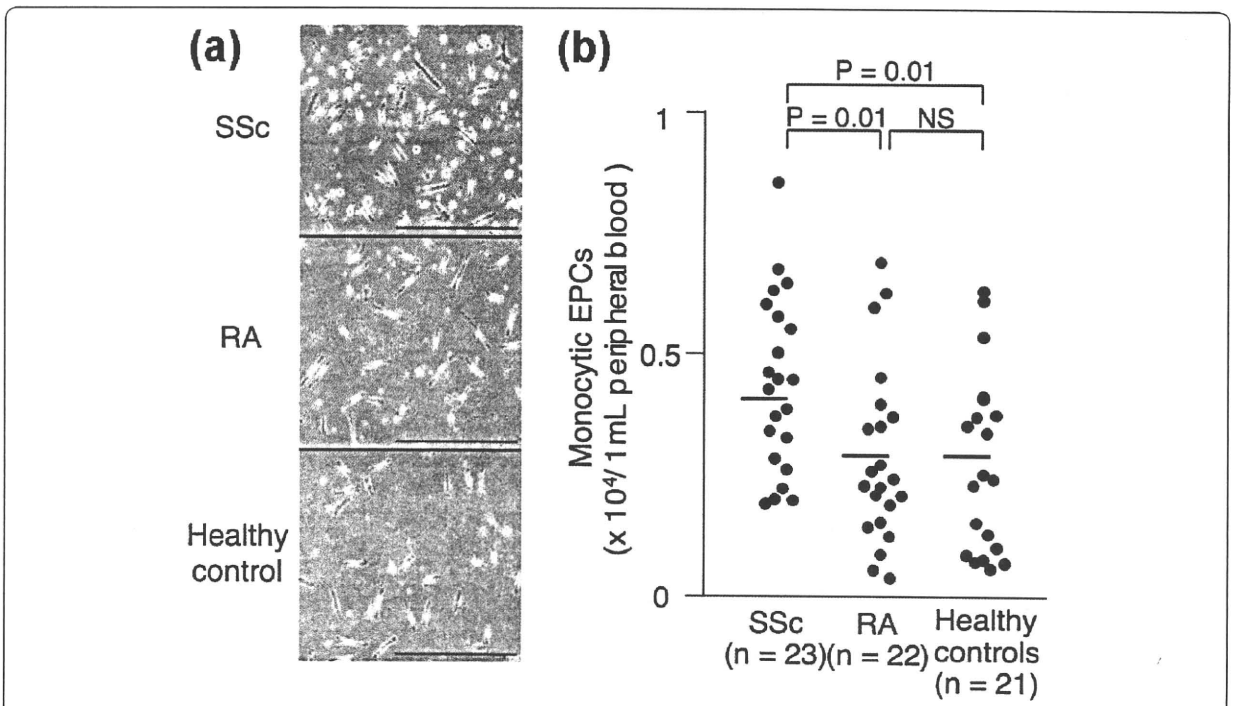

Figure 1 Monocytic EPCs enriched in culture on fibronectin. (a) Representative images of monocytic EPCs cultured for 10 days, from an SSc patient, an RA patient, and a healthy control. Adherent cells with a typical spindle shape are regarded as monocytic EPCs. Scale bars = 500 mm. **(b)** Monocytic EPCs were quantified in SSc patients, RA patients, and healthy controls, and expressed as the number in 1 mL of peripheral blood. Horizontal bars indicate the mean values. NS, not significant.

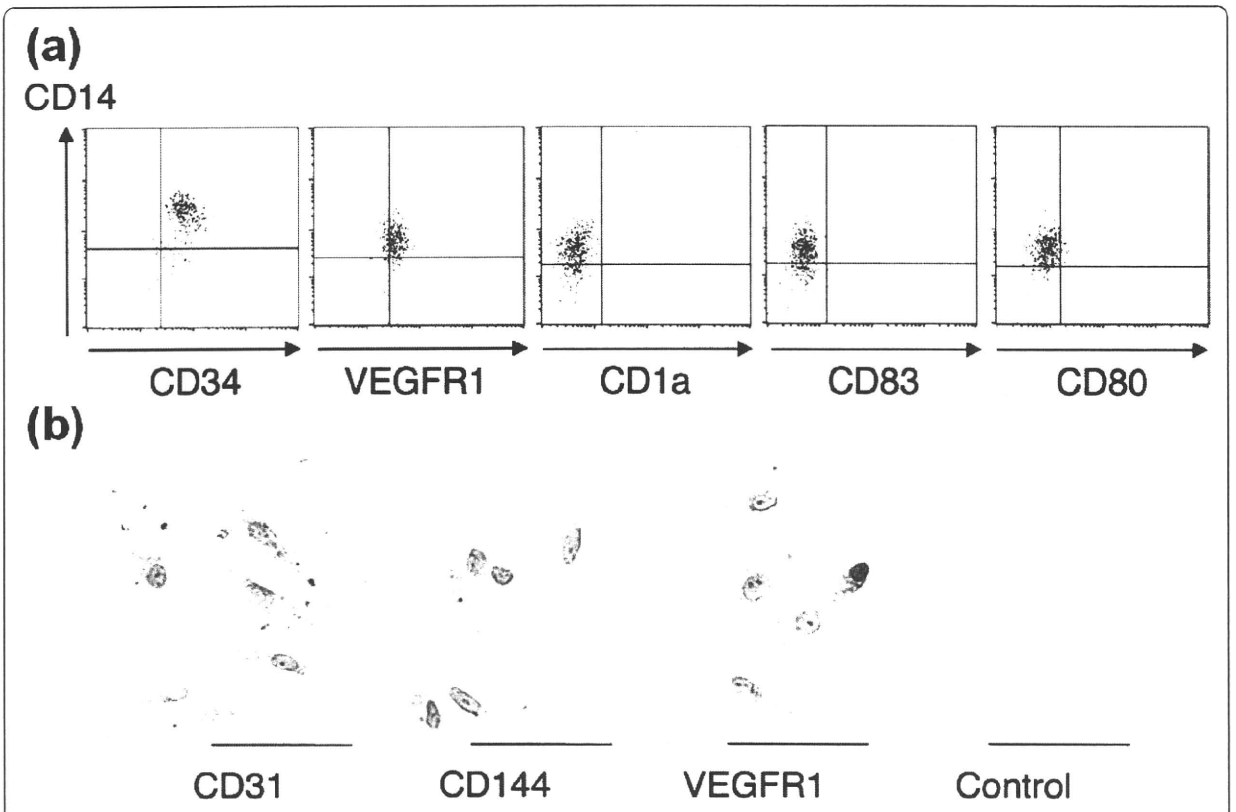

Figure 2 Protein expression profiles of monocytic EPCs. (a) Flow cytometric analysis of monocytic EPCs derived from a healthy control. Cells were stained with anti-CD14 mAb plus mAb to CD34, VEGFR1, CD1a, CD83, or CD80, and analyzed by flow cytometry. **(b)** Immunohistochemical analysis of monocytic EPCs. Cells were stained with a mouse mAb to the endothelial marker, as indicated. Controls were incubated with an isotype-matched mouse mAb to an irrelevant antigen. Nuclei were counterstained with hematoxylin. Bars, 50 μm.

the capacity of the monocytic EPCs to enhance tubular structure formation was significantly greater in cultures from SSc patients than in those from healthy controls (P = 0.0007).

Two potential mechanisms could account for the monocytic EPCs' role in promoting tubule formation *in vitro*: the support of tubule formation by HUVECs and their own incorporation into the tubular structure. To examine whether the former mechanism was involved in this process, we collected supernatants of the Matrigel® cultures as conditioned medium, and examined their capacity to promote tube formation in the second Matrigel® cultures with HUVECs alone (Figure 4). The conditioned medium was prepared from the first cultures with HUVECs plus monocytic EPCs (10^4) derived from seven pairs of SSc patients and healthy controls, and from the cultures with HUVECs alone. The capacity to enhance tubular formation was significantly greater in SSc-derived conditioned medium than in healthy control-derived medium (P = 0.04).

To further evaluate the ability of monocytoc EPCs to be incorporated into the tubular structures, monocytic EPCs were pre-labeled with a green fluorescent cell linker PKH67 and cultured with Dil-acLDL-labeled HUVECs in Matrigel®. We found that a small number of monocytic EPCs were integrated into tubular structures that were primarily formed by HUVECs (Figure 5a). When we tested monocytic EPCs derived from 10 pairs of SSc patients and healthy controls (Figure 5b), their capacity to integrate into tubule formation was less efficient in the SSc-derived cultures than in those from healthy controls (P = 0.01).

Capacity of monocytic EPCs to promote neovascularization *in vivo*

The *in vivo* capacity of monocytic EPCs to promote blood vessel formation was evaluated using a murine tumor neovascularization model [15]. Murine colon carcinoma CT-26 cells were injected beneath the back of SCID mice, either alone or in combination with monocytic EPCs derived from SSc patients or healthy controls. As shown in Figure 6, co-transplantation of CT-26 cells with monocytic EPCs promoted tumor growth, and the amount of growth depended on the number of

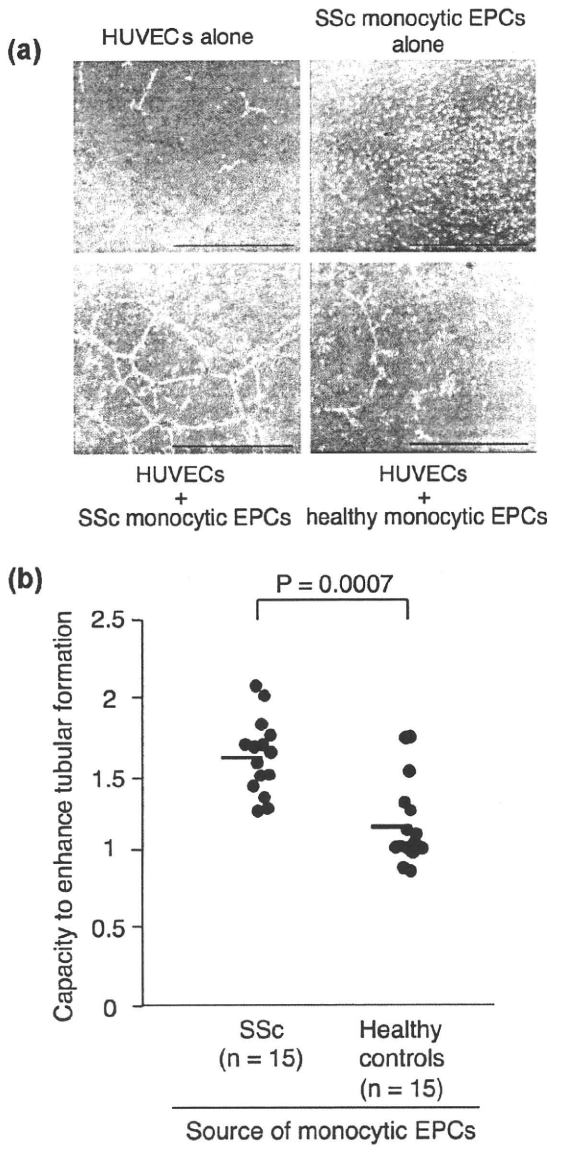

Figure 3 Capacity of monocytic EPCs to promote tubular formations in co-culture with HUVECs in Matrigel®. (a) Representative images of Matrigel® cultures of HUVECs (10^4) alone, monocytic EPCs (10^4) from an SSc patient alone, and HUVECs (10^4) plus monocytic EPCs (10^4) from an SSc patient and a healthy control. Scale bars = 1 mm. **(b)** Capacity of monocytic EPCs to enhance the tubular formation was expressed as the ratio of total tube length in the culture of HUVECs plus monocytic EPCs to the length in the culture of HUVECs alone, and compared between SSc patients and healthy controls. Horizontal bars indicate the mean values.

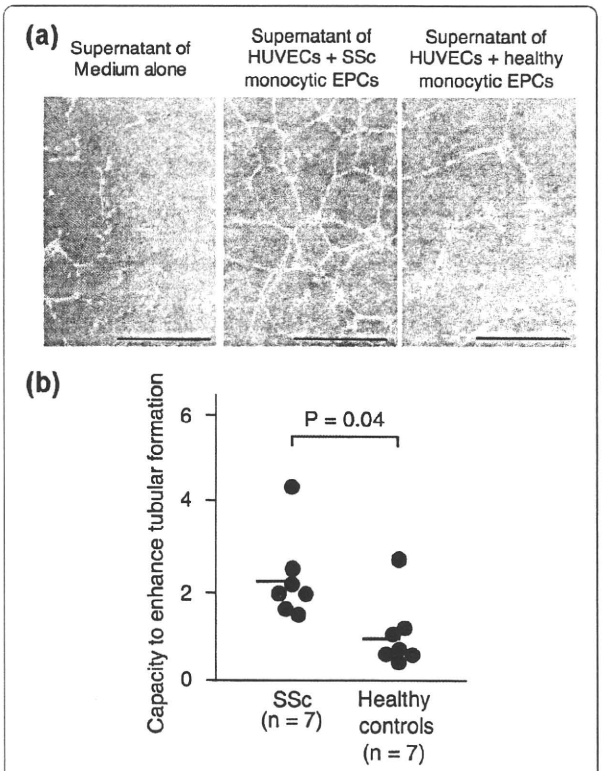

Figure 4 Capacity of monocytic EPC-derived conditioned medium to promote tubular formations in culture of HUVECs in Matrigel®. (a) Representative images of Matrigel® cultures of HUVECs in the presence of culture supernatants of HUVECs (10^4) alone, HUVECs (10^4) plus monocytic EPCs (10^4) from an SSc patient, and HUVECs (10^4) plus monocytic EPCs (10^4) from a healthy control. Scale bars = 500 μm. **(b)** Capacity to enhance tubular formation was expressed as the ratio of total tube length in the culture with conditioned medium of HUVECs plus monocytic EPCs to the length in the culture with conditioned medium of HUVECs alone, and compared between SSc patients and healthy controls. Horizontal bars indicate the mean values.

monocytic EPCs. A comparison of tumor size resulting from the co-transplantation of monocytic EPCs from SSc patients or healthy controls showed that the SSc-derived monocytic EPCs promoted significantly faster tumor growth (P = 0.03).

A histological examination of the tumors showed that the co-transplantation of monocytic EPCs dramatically increased the number of blood vessels carrying erythrocytes compared with the transplantation of CT-26 cells alone, especially when SSc-derived monocytic EPCs were used (Figure 7a). Consecutive sections of the tumors co-transplanted with SSc-derived monocytic EPCs confirmed lateral connection of blood vessels detected as a longitudinal vessel section in the single section (Figure 7b). As shown in Figure 7c, the number of blood vessels in the tumor tissue increased with the number of transplanted monocytic EPCs, and was significantly greater in tumors that arose from the transplantation of CT-26 cells with SSc-derived monocytic EPCs than with control monocytic EPCs (P = 0.03 for the transplantation of 10^4 and 10^5 monocytic EPCs). Thus,

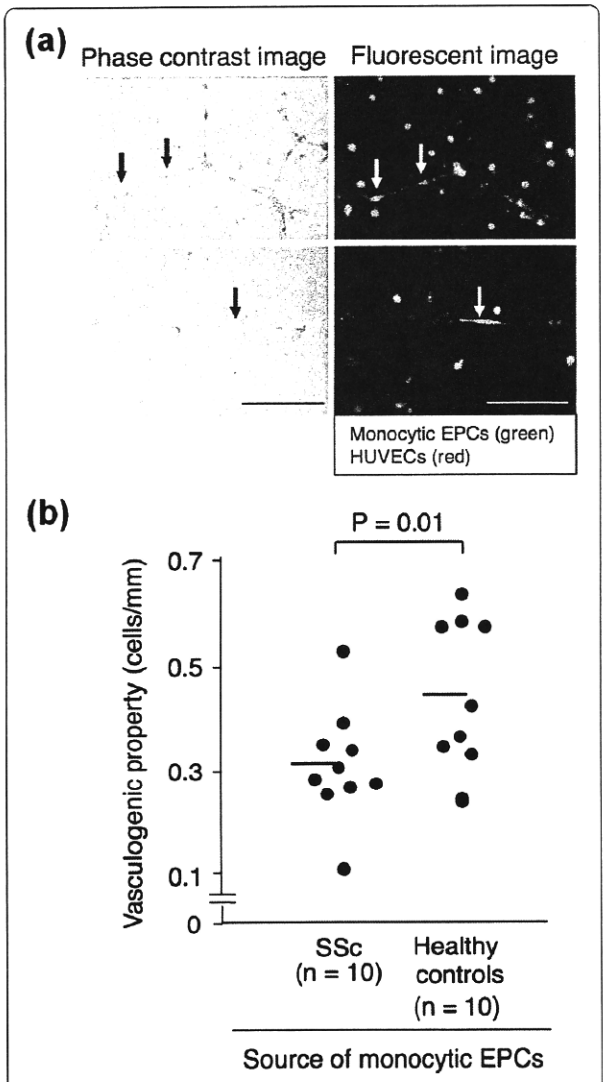

Figure 5 *In vitro* vasculogenic property of monocytic EPCs in Matrigel® culture. Monocytic EPCs labeled with PKH67 (green) and HUVECs labeled with DiI-acetylated LDL (red) were cultured together on Matrigel®. **(a)** Typical phase-contrast (left) and fluorescent (right) images of the same field of monocytic EPCs from an SSc patient (upper) and a healthy control (lower). An arrow indicates a monocytic EPC-derived cell incorporated into the tubular structure. Scale bars = 100 μm. **(b)** The vasculogenic property of monocytic EPCs was calculated as the number of monocytic EPCs within the tubular structure divided by the total tube length (cells/mm), and compared between SSc patients and healthy controls. Horizontal bars indicate the mean values.

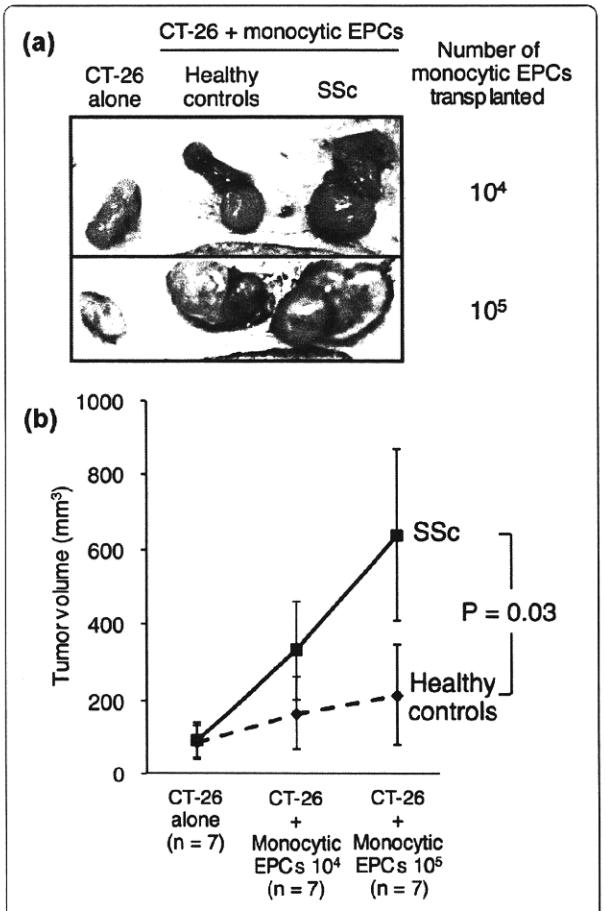

Figure 6 Tumor growth after transplantation of monocytic EPCs in the *in vivo* tumor neovascularization model. Tumors from colon carcinoma CT-26 cells injected subcutaneously into the back of mice alone or in combination with monocytic EPCs (10^4 or 10^5) derived from SSc patients or healthy controls. Tumor growth was assessed 10 days later. **(a)** Representative subcutaneous tumors from mice that received transplanted CT-26 cells alone, or CT-26 cells along with monocytic EPCs (10^4 or 10^5) from a healthy control or an SSc patient. **(b)** Tumor volumes in mice that received transplants of CT-26 cells alone, or CT-26 cells in combination with monocytic EPCs from SSc patients or healthy controls (10^4 or 10^5). Results are shown as the mean and standard deviation.

the increased tumor growth caused by the presence of SSc-derived monocytic EPCs could be explained by the EPCs' enhanced ability to promote blood vessel formation *in vivo*.

To further examine the contribution of vasculogenesis in this process, we evaluated the distribution of the transplanted human monocytic EPCs in the tumors by detecting cells expressing human CD31 (Figure 8a). The majority of transplanted monocytic EPCs expressing human CD31 were detected outside of the vascular lumen, but some blood vessels included cells expressing human CD31 but did not express mouse CD31 (Figure 8b). Similar findings were observed when mAb to HLA class I was used instead of anti-human CD31 mAb. When the proportion of blood vessels carrying endothelial cells expressing human CD31 was evaluated, fewer monocytic EPCs from SSc patients were incorporated into the vascular wall than monocytic EPCs from healthy

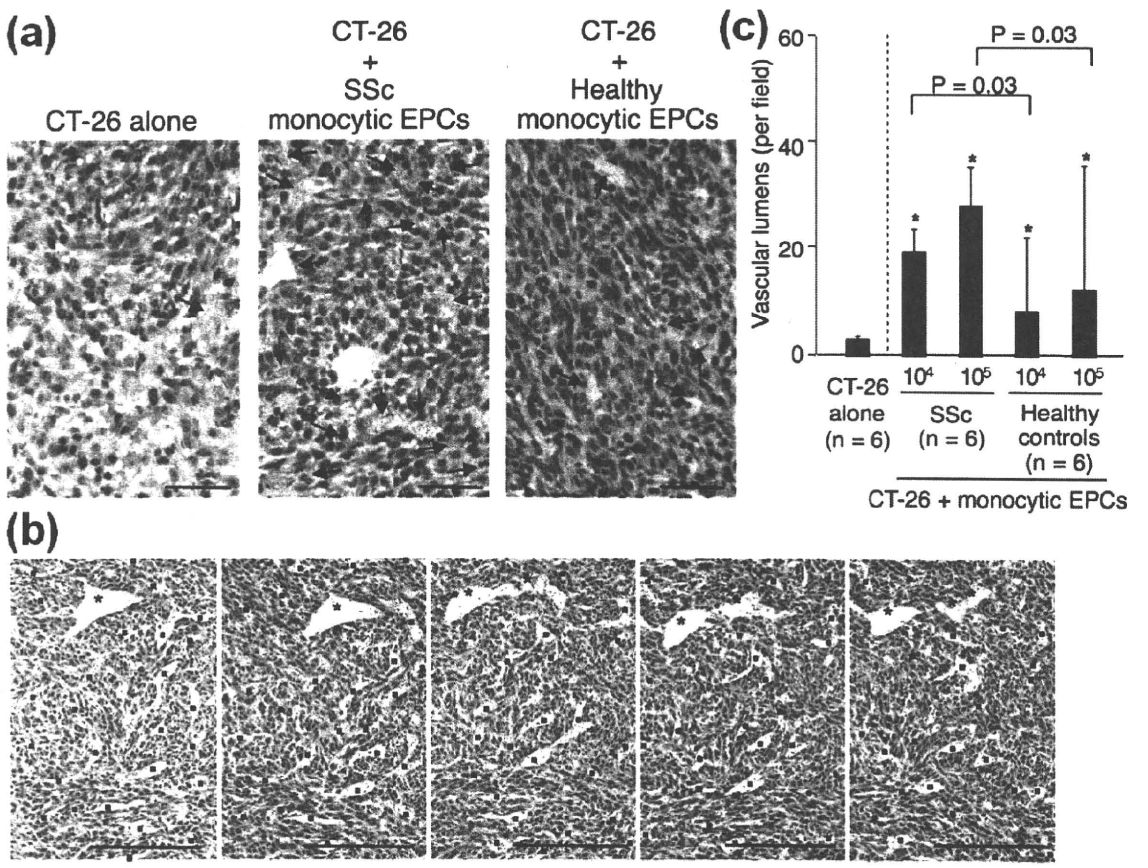

Figure 7 Capacity of monocytic EPCs to promote the formation of blood vessels in a tumor neovascularization model. **(a)** Representative tumor sections stained with hematoxylin and eosin, from mice with transplants of CT-26 cells alone or of CT-26 cells and monocytic EPCs from an SSc patient or a healthy control. Arrows indicate blood vessels carrying erythrocytes. Scale bars = 200 μm. **(b)** Representative consecutive sections of the tumor stained with hematoxylin and eosin, from mice with transplants of CT-26 cells and monocytic EPCs from an SSc patient. Asterisks indicate a relatively large blood vessel found in all consecutive sections. Dots indicate other blood vessels carrying erythrocytes. Scale bars = 500 μm. **(c)** Vascular lumen density in tumors that arose from transplanted CT-26 cells alone or CT-26 cells with monocytic EPCs (10^4 or 10^5) from SSc patients or healthy controls.

controls; this difference was statistically significant when 10^4 and 10^5 monocytic EPCs were used for transplantation ($P = 0.03$ for both comparisons, Figure 8c).

Discussion

In this study, we demonstrated that circulating monocytic EPCs were increased in the peripheral blood of SSc patients. In addition, *in vitro* and *in vivo* functional analyses revealed that monocytic EPCs derived from SSc patients had an enhanced ability to promote blood vessel formation. This characteristic was primarily attributable to an enhanced angiogenic property through production of angiogenic factors. Additional studies to identify monocytic EPC-derived soluble factors responsible for the difference in angiogenic property between SSc patients and healthy individuals are underway. In contrast, the EPCs' ability to be incorporated into vessels and differentiate into mature endothelial cells was rather impaired in SSc patients. This finding may support an early report showing that the angiogenic capacity of PBMCs from SSc patients was inferior to that of healthy controls, but when monocytes were enriched and used in the same assay system, the SSc patients' samples showed an enhanced angiogenic capacity [21].

Monocytic EPCs promote angiogenesis by secreting a variety of angiogenic factors, in a paracrine manner [12,15,22,23], and by differentiating into other elements of the vasculature, such as pericytes and smooth muscle cells, thereby contributing to the outer layers of blood vessels [11,12,24]. Skin biopsies from SSc patients were investigated for their angiogenic activity using the chick embryo chorioallantoic membrane assay [25] and the SCID mouse skin xenograft model [26], and in both

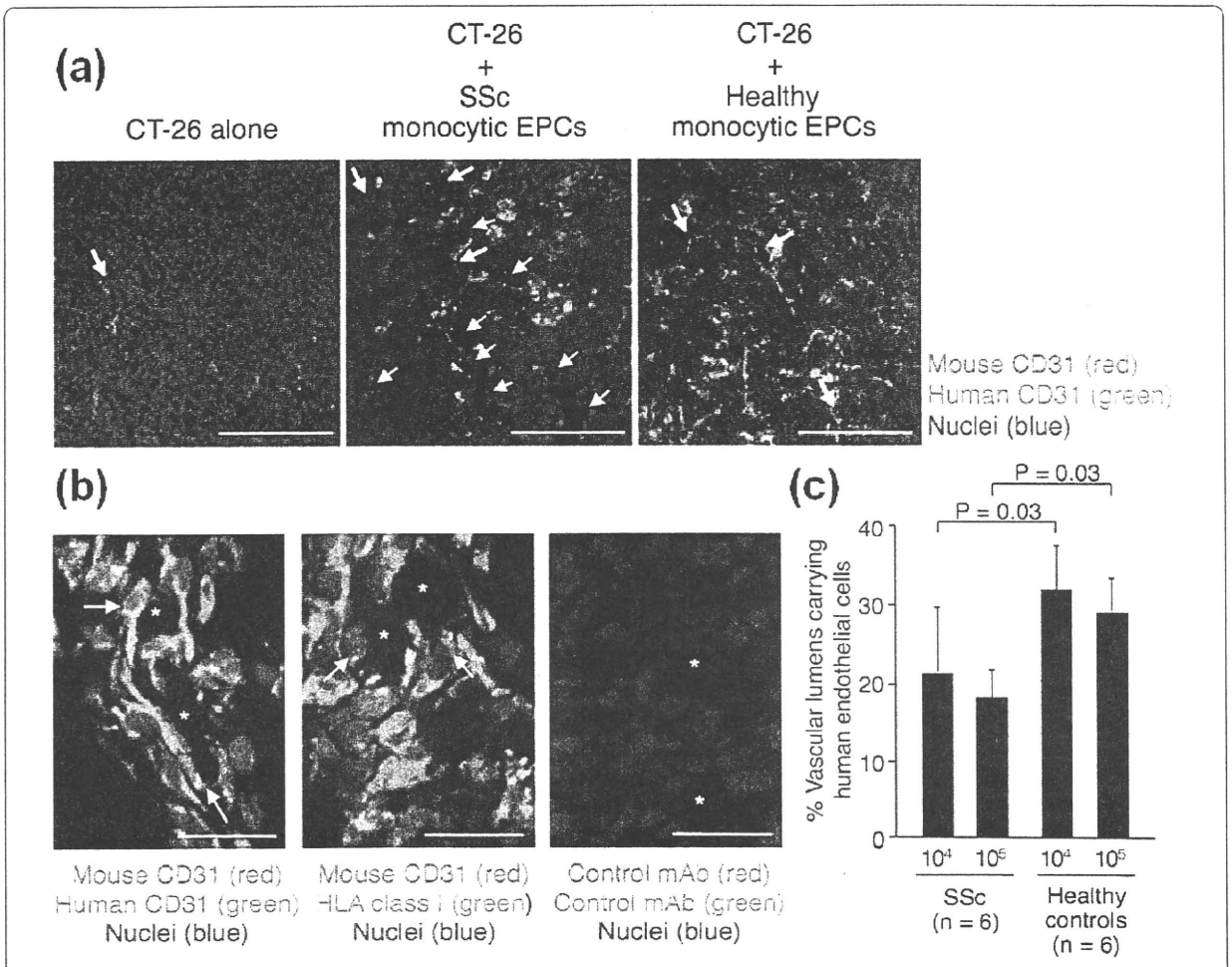

Figure 8 Incorporation of monocytic EPCs into vascular lumen *in vivo* in a tumor neovascularization model. (a) Representative tumor sections stained for mouse CD31 (red) and human CD31 (green), from mice with transplants of CT-26 cells alone or of CT-26 cells and monocytic EPCs from an SSc patient or a healthy control. Nuclei were counterstained with TO-PRO3 (blue). Arrows indicate blood vessels. Scale bars = 200 μm. **(b)** Representative tumor sections stained for mouse CD31 (red) and human CD31 or HLA class I (green) from mice with transplants of CT-26 cells and monocytic EPCs from a healthy control. Negative controls were sections incubated with isotype-matched mouse or rat mAb to an irrelevant antigen, instead of the primary antibody. Nuclei were counterstained with TO-PRO3 (blue). Asterisks indicate blood vessel lumen, while arrows indicate transplanted human monocytic EPCs located at the vascular wall. Scale bars = 50 μm. **(c)** The vasculogenic potency of monocytic EPCs was assessed by determining the proportion of vascular lumens carrying human $CD31^+$ endothelial cells in tumors arising from CT-26 cells co-transplanted with monocytic EPCs (10^4 or 10^5) from SSc patients or healthy controls.

studies the SSc grafts induced a prominent increase in new blood vessel formation in the surrounding normal tissue, compared with grafts from healthy subjects. Since dense mononuclear cell infiltrates were detectable around the newly formed blood vessels in those models, it is likely that SSc skin has a strong intrinsic activity that recruits angiogenic cells, such as monocytic EPCs, from the circulation, probably through chemokine production. Taken together, the robust angiogenic push observed in SSc patients may result, in part, from crosstalk between the affected tissue and circulating monocytic EPCs. In addition, capacity of circulating monocytic EPCs in SSc patients to home to the pathogenic site appears to be intact.

One of the limitations of this study is the method employed to quantify circulating monocytic EPCs, which used the short-term culture, instead of a direct analysis of freshly prepared cells. This is because of a lack of a definitive marker for monocytic EPCs in circulation. Our method is able to enrich monocytic EPCs from PBMCs by utilizing the capacity of monocytic EPCs to bind to fibronectin. Variability in the fibronectin binding capacity may influence the recovery of monocytic EPCs in the culture, but there was no difference in an

expression level of α1β5 integrin, a receptor for fibronectin, between circulating $CD14^+$ monocytes from SSc patients and those from healthy individuals (data not shown). It has also been shown that weak proliferation of adherent $CD14^+$ monocytes occurs during the first 24 hours of the culture [13], and rate of proliferation was similar between SSc patients and healthy controls.

Although SSc patients have high levels of circulating monocytic EPCs with enhanced angiogenic potential, blood vessel formation is apparently insufficient in these patients [1]. This suggests the presence of mechanisms that inhibit angiogenesis at SSc-affected sites. Postnatal angiogenesis governed by endothelial cells requires a series of events, including a response to angiogenic stimuli, proliferation, the coordinated expression of proteolytic enzymes, degeneration of the extracellular matrix, and migration into the matrical space [27,28]. In this regard, microvascular endothelial cells derived from the skin of SSc patients show an overproduction of metalloproteinase-12 and the resultant impairment of urokinase-type plasminogen activator receptor signaling [29] as well as a reduction in tissue kallikreins 9, 11, and 12, which are powerful effectors of angiogenesis [30]. In addition, a recent microarray analysis of microvascular endothelial cells derived from the skin of SSc patients and controls revealed the up-regulation of genes that suppress angiogenesis and the down-regulation of genes critical to cell migration and extracellular matrix-cytoskeleton coupling, which impedes angiogenesis [31]. This anti-angiogenic environment in SSc-affected tissue might interfere with the pro-angiogenic property of monocytic EPCs. Given the defective vasculogenic capacity of monocytic EPCs as well as of "classic" EPCs [7], it seems that the final balance between blood vessel formation and repair favors the suppression of neovascularization in SSc patients.

Monocytic EPCs are recruited into the circulation in response to chemokines, such as monocyte chemoattractant protein-1 (MCP-1) [32], which are up-regulated in the affected skin of SSc patients [33,34]. In addition, endothelial cells are shown to strongly induce circulating monocytes to differentiate into EPCs under hypoxic conditions [35], which is a typical feature of SSc skin [36]. In contrast, Zhu *et al.* reported that SSc serum induces the apoptosis of circulating EPCs through up-regulation of the pro-apoptotic protein Bim, an effect mediated by the inhibition of the activation/phosphorylation of Akt [37]. Therefore, in SSc patients, it is likely that the signals that mobilize monocytic EPCs are so intense that they overcome the mechanisms that reduce the number of circulating monocytic EPCs. The presence of multiple confounding factors that affect the number of circulating monocytic EPCs may explain why their number did not correlate with any clinical characteristics of the SSc patients.

In SSc patients, functionally altered monocytic EPCs would be expected to accumulate at the affected tissue, but it is unclear how they are involved in the pathogenesis of SSc, given the strong anti-angiogenic microenvironment in the affected tissue. We previously reported that monocytic EPCs can differentiate not only into endothelial cells, but also into a variety of mesenchymal-lineage cells, including adipocytes, osteoblasts, chondrocytes, fibroblasts, and skeletal and cardiac myoblasts, when they are exposed to lineage-specific induction stimuli [13,14]. Since it has been reported that monocytes acquire the ability to produce extracellular matrix components, such as collagens, in the presence of MCP-1 [38], fibrogenic environment of the affected tissues in SSc patients may induce the differentiation of monocytic EPCs into fibroblast-like cells. Alternatively, monocytic EPCs recruited to the SSc-affected sites could be a source of soluble factors, such as MCP-1, platelet-derived growth factor, and interleukin-6, all of which accelerate fibrosis.

Conclusions

In summary, despite insufficient vascular formation and repair in SSc patients, monocytic EPCs are paradoxically increased in the circulation and possess a prominent angiogenic potential. These functionally altered monocytic EPCs apparently cannot overcome the anti-angiogenic environment at SSc-affected sites, and may eventually be involved in other aspects of SSc pathogenesis, such as the promotion of excessive fibrosis. Further studies investigating the role of monocytic EPCs in the tissue fibrosis in SSc patients are underway.

Abbreviations

acLDL: acetylated low-density lipoprotein; ACR: American College of Rheumatology; DiI: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine; EPCs: endothelial progenitor cells; FITC: fluorescein isothiocyanate; HLA: human leukocyte antigen; HUVEC: human umbilical vein endothelial cell; mAb: monoclonal antibody; MCP-1: monocyte chemoattractant protein-1; PBMC: peripheral blood mononuclear cells; RA: rheumatoid arthritis; SSc: systemic sclerosis; SCID: severe combined immunodeficient; VEGF: vascular endothelial growth factor; vWF: von Willebrand factor.

Acknowledgements

This work was supported by a research grant on intractable diseases from the Japanese Ministry of Health, Labour and Welfare, and a grant from the Japanese Ministry of Education, Science, Sports and Culture (to MK).

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Authors' contributions

YY performed the acquisition of data, and analysis and interpretation of data, and wrote the manuscript. YO, NS, and TS performed the acquisition of data. KT and ZI provided peripheral blood samples and clinical information, and performed analysis of data. MK designed the experiments, performed data analysis and interpretation, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 11 June 2010 **Revised:** 9 October 2010
Accepted: 4 November 2010 **Published:** 4 November 2010

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doi:10.1186/ar3180
Cite this article as: Yamaguchi *et al.*: Enhanced angiogenic potency of monocytic endothelial progenitor cells in patients with systemic sclerosis. *Arthritis Research & Therapy* 2010 **12**:R205.

Clinical and Applied
Thrombosis/Hemostasis
16(6) 622-627

DOI: 10.1177/1076029609350889
http://cath.sagepub.com
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Effects of Bisphosphonate Administration on the Bone Mass in Immune Thrombocytopenic Purpura Patients Under Treatment With Steroids

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Abstract

Immune thrombocytopenic purpura (ITP) is an acquired hemorrhage condition involving accelerated platelet consumption caused by antiplatelet autoantibodies. Although various therapeutic strategies are used to treat patients with ITP, the standard treatment method is steroid therapy. The most important problem with steroid administration may be a prolonged use tendency in many cases, because there are many refractory chronic patients. To elucidate the effects of glucocorticoid on bone mineral density (BMD) in patients with ITP, we retrospectively evaluated the relationship between BMD and the total dose of glucocorticoid or the mean daily dose given. We observed decreased BMD in 66.7% of the patients with ITP to whom glucocorticoid was given, although normal bone BMD was observed in 28.6% of patients with ITP treated without steroids. The mean level of BMD was markedly decreased in steroid-treated patients compared with nonsteroid-treated patients ($P < .01$). The relationship between BMD and the total dose of glucocorticoid ($P = .023$) or the mean daily dose revealed a negative correlation ($P = .022$). Administration of bisphosphonate revealed a significant increase in bone mass in patients at 6 and 12 months after the start of bisphosphonate treatment, despite the aggravation of thrombocytopenia. In conclusion, glucocorticoid-induced osteoporosis was observed in patients with ITP, similar to situation seen in patients with other diseases. Bisphosphonate may be an effective agent for the prevention and treatment of glucocorticoid-induced osteoporosis in patients with ITP scheduled to receive long-term steroid treatment.

Keywords

immune thrombocytopenic purpura, bisphosphonate, steroid, bone mass, quality of life

Introduction

Immune thrombocytopenic purpura (ITP) is an acquired hemorrhage condition of accelerated platelet consumption caused by antiplatelet autoantibodies.[1] In addition to markedly shortened platelet survival, impaired platelet production is also responsible for thrombocytopenia in ITP.[2] Recently, various new therapeutic strategies such as rituximab and thrombopoietin-like agents are used for the treatment of patients with ITP.[2-4] However, the standard treatment method is a steroid therapy.[5] The most important problem about steroid administration may be a prolong-used tendency in many cases because there are many refractory chronic patients. However, there is a report that the incidence of patients with ITP aged 60 years or older has been increasing in recent years.[6] Given the fact that ITP is a disease common among women and increasing in the elderly population, glucocorticoid-induced osteoporosis in patients with ITP is important in considering the patient's prognosis. Therefore, in recent studies, high doses of steroids for short periods of time is advocated in patients with ITP.[2,5]

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As tests for the assessment of osteoporosis have become widely available in recent years, osteoporosis associated with the administration of corticosteroids (hereinafter, steroids) has become a focus of attention. Because osteoporosis is common in the elderly population and vertebral compression fracture and femoral neck fracture lead to a decline in the quality of life (QOL), its prevention is important. It is widely known that osteoporosis can be caused by steroids. The fracture risk in patients with glucocorticoid-induced osteoporosis increases with escalation of steroid dose and is likely to occur especially in vertebral bones.[7] It has been reported that the bone mineral density (BMD) in fractured bones in patients with glucocorticoid-induced osteoporosis is higher than that in fractured in patients with primary osteoporosis, and even if the loss of BMD is slower, the fracture risk of patients with glucocorticoid-induced osteoporosis is higher than that of patients with primary osteoporosis.[8]

Based on these backgrounds, we examined the relationship between steroids and BMD in patients with ITP and fluctuations of bone metabolism markers by administering bisphosphonate in patients under long-term treatment with steroids. We report the results of our examination.

Materials and Methods

Patients

In 25 patients with ITP (6 men, 19 women) aged 19 to 75 years old in whom the BMD was measured, the history of steroid administration (presence or absence of steroid administration, total dose, and average daily dose) from the time of onset of ITP to the time of BMD measurement was investigated retrospectively, and its relationship with the BMD was examined. Of these, 6 were treated with Kami-kihi-to, 4 with danazol, and 5 with Helicobactor pylori eradication therapy. However, there were no long-term drugs other than steroids. Concerning the steroid treatment, the steroid doses were calculated as prednisolone-equivalent doses. The usual dosages of steroids used for chronic ITP were 5 to 30 mg/d. The duration of treatment was calculated from the start of steroid administration to the time of the BMD measurement, and the average daily dose was calculated by dividing the total steroid dose during this period by the duration.

Measurements of BMD

As for BMD measurement, the BMD from the second to fourth lumbar vertebrae (L2-4) was measured by dual-energy x-ray absorptiometry (DXA).[9] For comparison of the BMD between the groups treated and groups not treated with steroids, the measured values were classified into normal, osteopenia, and osteoporosis levels according to the Diagnostic Criteria for Primary Osteoporosis (year 2000 version) laid down by the Japanese Society for Bone and Mineral Metabolism. The Mann-Whitney *U* test was used to determine the significance of differences between the 2 groups.

Because men and postmenopausal women are also subject to measurements of the BMD, BMD is expressed as the deviation score (*z* score) from the mean values in a sex- and age-matched healthy control group.

$$z \text{ score} = (\text{Measured values} - \text{Mean value})/\text{SD}$$

(SD : standard deviation)

Effects of Bisphosphonate

In the next step, among the 32 patients with ITP, 27 patients (4 men, 23 women) aged 19 to 75 years old in whom the BMD was measured after obtaining informed consent were treated with alendronate to examine the clinical effects of bisphosphonates in ITP patients with glucocorticoid-induced osteoporosis.[10-12] The BMD and bone metabolism markers in these patients were then examined prospectively. The markers of bone metabolism were examined at the start of the drug (bisphosphonate) administration and at 6 and 12 months after the start of the drug administration. The markers of bone metabolism measured were the serum level of bone-specific alkaline phosphatase (hereinafter, BAP) and urinary level of collagen type 1 cross-linked *N*-telopeptides (hereinafter, NTX). Mann-Whitney *U* test was used to test the significance of the differences between the 2 groups. All study protocols were approved by the Institutional Review Board (IRB) of our institutions, and written informed consent was obtained from each patient prior to the start of the trial.

Results

Bone mass was examined according to the Diagnostic Criteria for Primary Osteoporosis (year 200 revision) laid down by the Japanese Society for Bone and Mineral Metabolism. There were 18 patients in the steroid administration group and 7 patients who were not on steroid treatment; the mean ages of the patients in the 2 groups were 57.6 $\pm$ 16.0 and 57.9 $\pm$ 19.8 years, respectively, with no significant difference in age between the 2 groups. The average total dose and average daily dose (as predonisolone-equivalent dose) in the steroid treatment group were 5233.1 $\pm$ 3540.8 and 9.3 $\pm$ 5.2 mg, respectively. The platelet count was 118 000/μL in the steroid treatment group, which was higher than 68 000/μL in the group not under steroid treatment. Among the patients in the steroid treatment group, 8 had taken either active vitamin D or vitamin K2 (Table 1).

In relation to the distribution of the bone mass in the 2 groups, the frequencies of both osteopenia and osteoporosis were higher in the steroid treatment group than in the group not treated with steroids, although no significant difference in the distribution was observed between the 2 groups (Table 2).

Then, the changes in the bone mass (*z* score) were examined as a function of the steroid dose. Comparison of the average BMD between the steroid treatment group and the group not treated with steroids revealed that the average BMD in the former was significantly lower (-0.46 ± 1.09) than that in the