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ORIGINAL PAPER

Early outgrowth EPCs generation is reduced in patients with Buerger's disease

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Abstract
Background Buerger's disease often shows poor collateral artery generation (i.e. neovascularization) in the ischemic limbs. However, the etiology has not yet been clarified. Circulating endothelial progenitor cells (EPCs) derived from bone marrow contribute to neovascularization in the multi-step process which includes the following capacities; mobilization, differentiation, adhesion, migration, invasion and secretion.
Materials and methods We assessed EPCs capacities in vitro and ex vivo in age- and sex-matched controls ($n = 12$) and patients with Buerger's disease ($n = 12$), derived from peripheral blood-derived mononuclear cells (PB-MNCs).
Results In the flow cytometry analysis, the numbers of circulating EPC ($CD34^+/KDR^+$ or $CD133^+/KDR^+$ PB-MNC) were similar between controls and patients with Buerger's disease. Next, we cultured PB-MNC to obtain EPCs. The number of early outgrowth EPCs was significantly decreased in patients with Buerger's disease ($p < 0.005$), indicating the reduced generation of early outgrowth EPCs in Buerger's disease. However, adhesion, migration, invasion and secretion capacities were not impaired in patients with Buerger's disease.
Conclusions The early outgrowth EPCs generation is reduced in patients with Buerger's disease.

Keywords Buerger's disease · Thromboangiitis obliterans · Endothelial progenitor cell · Cell function

Abbreviations

EPCs	Endothelial progenitor cells
PB-MNCs	Peripheral blood-derived mononuclear cells
TAO	Thromboangiitis obliterans
ECs	Endothelial cells
KDR	Kinase domain receptor
VEGF	Vascular endothelial growth factor
FACS	Fluorescence-activated cell sorting
HUVEC	Human umbilical vein endothelial cell
Dil-acLDL	1,1′-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein
DAPI	4′,6-diamidino-2-phenylindole
b-FGF	Basic fibroblast growth factor
PDGF-BB	Platelet-derived growth factor BB
IGF	Insulin growth factor
SDF-1α	Stromal cell-derived factor-1α
ELISA	Enzyme-linked immunosorbent assay
IL-1, -6, -8	Interleukin-1, -6, -8
TGF-1α	Tumor growth factor-1α
VCAM-1	Vascular cell adhesion molecule-1
MCP-1	Monocyte chemotactic protein-1
hs-CRP	High-sensitivity C-reactive protein
m-RNA	Messenger ribonucleic acid

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Introduction

Buerger's disease, also known as thromboangiitis obliterans, is a non-atherosclerotic segmental inflammatory

disease that typically occurs in young male smokers [1]. It was first described in 1879 [2], and its detailed pathological findings were initially reported by Leo Buerger in 1908 [3]. Occlusive change of the peripheral arteries in the fore- and hindlimbs induces chill and intermittent claudication as the initial symptoms, and thereafter ischemic rest pain and skin ulcers often additionally occur. Although the generation of collateral arteries (i.e. neovascularization) is a physiological process to salvage critical tissue ischemia, neovascularization in Buerger's disease is generally poor. The progression of Buerger's disease often leads to limb amputation, and therefore the quality of life in the patient remarkably decreases. It is generally known that exposure to tobacco induces the progression of Buerger's disease; however, the pathological etiology has not yet been clarified.

Asahara et al. previously reported that postnatal neovascularization is induced by not only the sprouting from pre-existing vessels (i.e. angiogenesis) [4–6], but also by the generation of new vessels by the recruitment of endothelial progenitor cells (EPCs) that are derived from bone marrow (i.e. vasculogenesis) [7–9]. Circulating EPCs in the peripheral blood accumulate in ischemic tissue, and work in concert with the existing mature endothelial cells (ECs), thereby generating new arteries in ischemic tissues. The neovascularization process is considered to progress by the following steps: (1) mobilization from the bone marrow to the circulation and then to ischemic tissues, (2) chemotaxis and adhesion to mature ECs, (3) (*trans*-) migration and invasion of the intracellular space in adjacent ECs, (4) in situ differentiation to ECs and (5) the secretion of neovascularization-related cytokines to stimulate neovascularization by sprouting new capillaries from pre-existing arteries [10].

The number of circulating EPCs and the function have been reportedly decreased in patients having atherosclerotic risk factors [11–13] and patients with cardiovascular diseases [14–16], suggesting that the malfunction of EPCs contributes to impaired neovascularization in patients with atherosclerotic obliterance. In this study, we assessed the neovascularization-related capacities (mobilization, adhesion, migration, invasion and secretion) of EPCs in patients with Buerger's disease.

Materials and methods

Study population

We collected peripheral blood via venous puncture from age- and sex-matched control subjects ($n = 12$) and patients with Buerger's disease ($n = 12$; 6 in Fontaine class II and 6 in class IV). Buerger's disease was diagnosed by the diagnostic criteria proposed by the committee for Buerger's disease of Japanese Ministry of Health and welfare, which was similar to that of Shionoya [17] and Olin [1]. All non-smokers in healthy controls and in patients with Buerger's disease quitted smoking more than 1 month before the collections of blood samples. This study was approved by the Committees on the Ethics Review Board of the Kurume University School of Medicine, and written informed consent was obtained from all subjects.

Cell culture

Peripheral blood-derived mononuclear cells (PB-MNCs) were isolated from a total of 30 ml peripheral blood by density gradient centrifugation with Ficoll (Ficoll-Paque PLUS; GE Healthcare Bio-Sciences AB, Sweden). PB-MNCs were re-suspended in endothelial basal medium (EBM-2; Clonetics, San Diego, CA, USA) with supplements (0.1% hEGF, 0.4% hFGF-B, 0.1% VEGF, 0.1% ascorbic acid, 0.04% hydrocortisone, 0.1% long R3-IGF-1, 0.1% heparin, 0.1% gentamicin, 0.1% amphotericin and 20% FBS) and seeded onto a six-well tissue plate coated with fibronectin (Sigma, St. Louis, MO, USA) to culture them for the following 4 days at 37°C, 5% CO_2, in a humidified incubator.

Flow cytometric analysis

Because circulating EPCs are mobilized from the bone marrow into the peripheral circulation [8, 9], we calculated the ratio of circulating EPCs in PB-MNCs to evaluate the mobilization capacity of EPC [8, 9]. To identify circulating EPCs in peripheral blood, we adopted the characteristic cell-surface antigens, CD34 (PE-labeled mouse antibody; BD Biosciences Pharmingen, San Jose, CA, USA), CD133 (PE-labeled mouse antibody; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), KDR (VEGFR2) (biotin-conjugated mouse antibody; Sigma, St. Louis, MO, USA, SAv-FITC conjugate; BD Biosciences, San Jose, CA, USA) [8, 10] that are recognized as the specific cell-surface markers of circulating EPC. After the incubation of PB-MNCs with the above antibodies, samples were analyzed by fluorescence-activated cell sorting (FACS) (Becton-Dickinson, San Diego, CA, USA). $CD34^+KDR^+$ or $CD133^+KDR^+$ PB-MNCs were defined as circulating EPCs [8, 10, 18]. Then to identify whether the cultured cells were EPCs, we checked the expressions of endothelial lineage markers on the surface of the cells. We used healthy human umbilical vein ECs as positive controls. After detaching the cells with trypsin (TrypLE^{TM} Express, GIBCO Invitrogen, Carlsbad, CA, USA) from the culture plates, we incubated them with the monoclonal antibodies of CD31 (PE-labeled mouse antibody; BD Biosciences, San Jose, CA, USA) and KDR (biotin-conjugated mouse

antibody; Sigma, St. Louis, MO, USA, SAv-FITC conjugate; BD Biosciences, San Jose, CA, USA) and analyzed the expressions with FACS.

Generation of early outgrowth EPCs

After 4 days of PB-MNCs culture, we washed the adherent cells on the culture plate with PBS and incubated them with Dil-AcLDL (Biomedical Technologies Inc., Stoughton, MA, USA) for 1 h. Then, we fixed them with 2% paraformaldehyde and counterstained with FITC-labeled lectin from Ulex europaeus (VECTOR Laboratories Inc., Burlingame, CA, USA) and DAPI (Dojindo Molecular Technologies Inc., Kumamoto, Japan). The adherent cells that were stained by all dyes of Dil-AcLDL, lectin and DAPI were defined as early outgrowth EPCs [19], which were generated from PB-MNCs. After detaching the adherent cells with trypsin, we counted them and calculated the generation ratio by dividing the number of early outgrowth EPCs with the number of PB-MNCs that were seeded at the first time.

Adhesion assay

We performed cell-matrix adhesion assay [20]. In brief, after washing and detaching of early outgrowth EPCs on the culture plate at day 4 of the culture, the cells were labeled with fluorescent green reagent (CellTracker™ Green CNFDA; Molecular probes Inc., Eugene, Oregon, USA) and plated on a fibronectin (10 μg/ml)-coated dish. After 40 min of the incubation at 37°C, 5% CO_2, in a humidified incubator, we washed out the non-adherent cells and manually counted the adherent cells in three randomly selected high-power fields under a fluorescent microscope.

Migration and invasion assay

We used modified-Boyden chamber assay to evaluate the migration and invasion capacities of early outgrowth EPCs. After washing and detaching of the EPCs at day 4 of the culture, a total of 1×10^5 EPCs were re-suspended in 500 μl EBM-2 medium and placed onto the upper chamber whose bottom was made with 8-μm pore size PET membrane coated with an extracellular matrix, Matrigel (BD BioCoat™ Matrigel™ invasion chamber, BD Biosciences, San Jose, CA, USA) to test the invasion capacity. In migration assay, we used another upper chamber in absence of Matrigel coating. Each upper chamber was placed onto a 24-well culture dish that was filled by 500 μl EBM-2 medium with 20% fatal bovine serum and VEGF (50 ng/ml rhVEGF; R&D Systems Inc., Minneapolis, MN, USA). After 24 h of the incubation at 37°C, the transmigrated cells into the lower chamber via PET membrane of the upper chamber were fixed with 2% paraformaldehyde and counterstained with DAPI. Then, we counted the transmigrated cell number in three randomly selected high-power fields under fluorescent microscope.

Cytokines in culture medium and peripheral blood

To evaluate secretion capacities of early outgrowth EPCs, we measured the concentration of neovascularization-related cytokines [21, 22] that were secreted from cultured EPCs into the culture medium. The concentration of VEGF, b-FGF, PDGF-BB, IGF-1, angiopoietin 1, angiopietin 2 and SDF-1α in the supernatant of culture medium without supplements were measured after 4 days of the cell culture using a commercially available high-sensitivity enzyme-linked immunosorbent assay (ELISA) system (Amersham Pharmacia Biotech, Little Chalfont, UK). In the peripheral blood, serum levels of VEGF, interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), angiopoietin 1, angiopietin 2, SDF-1α, TGF-1α, VCAM-1 and MCP-1 were also measured by ELISA according to the instructions of the manufacturer.

Statistical analysis

Continuous variables are presented as mean ± SEM. Statistical comparisons between control subjects and patients with Buerger's disease were analyzed by *t* test (two-sided). The comparisons of categorical variables were generated by the Pearson χ^2 test. Statistical correlation between the percentage of circulating EPC and the concentration of high-sensitivity C-reactive protein (hs-CRP) or IL-6 in peripheral blood were assessed by univariate analysis. The serum concentrations of hs-CRP and IL-6 were further assessed by a multiple linear and stepwise regression analyses. Statistical significance was assumed at a value of $p < 0.05$. All analyses were performed with SPSS 14.0 software.

Results

The baseline characteristics of control subjects and patients with Buerger's disease are shown in Table 1. The baseline characteristics were similar between control subjects and patients with Buerger's disease except hs-CRP, which was significantly elevated in patients with Buerger's disease ($p < 0.005$). Serum concentrations of neovascularization-related cytokines are shown in Table 2. IL-6 was significantly elevated in patients with Buerger's disease ($p < 0.05$).

Table 1 Baseline characteristics of control subjects and patients with Buerger's disease

	Control ($n = 12$)	Buerger's disease ($n = 12$)	p
Age (years)	44.3 ± 3.3	44.3 ± 3.2	n.s
Gender			
Male	11	11	n.s
Female	1	1	n.s
Hypertension	4	2	n.s
Hyperlipidemia	3	3	n.s
Diabetes mellitus	0	0	n.s
Current smoking	4	4	n.s
Medication			
RASi	2	2	n.s
Statin	3	3	n.s
hs-CRP[a] (ng/ml)	2.4 ± 0.1	3.2 ± 0.3	$p < 0.005$
WBC (×100 μl)	54.5 ± 4.2	64.2 ± 5.9	n.s
PB-MNC ($\times 10^7$)	3.9 ± 0.5	5.0 ± 0.5	n.s

RASi renin–angiotensin system inhibitor, *hs-CRP* high-sensitivity C-reactive protein, *WBC* white blood cell, *PB-MNC* peripheral blood-derived mononuclear cell

[a] Log-transformed values were used in analyses

Number of circulating EPCs and capacities of EPCs

In the flowcytometric analysis, the percentage of $CD133^+/KDR^+$ PB-MNCs was similar between controls and patients with Buerger's disease (Fig. 1a). The percentage of $CD34^+/KDR^+$ PB-MNCs was also similar in the two groups (Fig. 1a). These results indicate the preserved number of circulating EPCs and suggest the preserved mobilization capacity in patients with Buerger's disease.

After 4 days of PB-MNCs culture, all adherent cells on the culture plate were stained by three fluorescent dyes, DiI-acLDL, lectin and DAPI in all fields of view under fluorescence microscopy (Fig. 1b). In flow cytometric analysis, the adherent cells were positively expressed CD31 and KDR as endothelial lineage markers. The expression patterns were similar between controls and patients with Buerger's disease (Fig. 1c). Taken together, we considered that the adherent cells were early outgrowth EPCs [12, 19, 23]. In patients with Buerger's disease, the number of early outgrowth EPCs on each culture plate appeared smaller than that of the controls (Fig. 1d). In fact, the percentage of ex vivo EPC generation was significantly smaller ($p < 0.005$) in patients with Buerger's disease (Fig. 1e). These results suggest that early outgrowth EPCs generation is reduced in Buerger's disease. In the adhesion assay, the number of adherent cells on the fibronectin-coated well was similar between controls and patients with Buerger's disease (Fig. 1f). Likewise, the migration (Fig. 1g) and invasion (Fig. 1h) capacities were similar between the two groups. In the ELISA assay, all the concentrations of VEGF, b-FGF, PDGF-BB, IGF-1, angiopoietin 1, angiopietin 2 and SDF-1α in the culture medium were similar between controls and patients with Buerger's disease (Table 3).

Table 2 Serum concentrations of neovascularization-related cytokines

	Healthy control ($n = 12$)	Buerger's disease ($n = 12$)	
VEGF (pg/ml)	77.0 ± 19.2	101.8 ± 14.8	n.s
MCP-1 (pg/ml)	68.5 ± 2.5	73.3 ± 7.8	n.s
IL-1β (pg/ml)	4.2 ± 0.2	4.0 ± 0.3	n.s
IL-6 (pg/ml)	3.7 ± 0.1	5.4 ± 0.7	$p < 0.05$
IL-8 (pg/ml)	19.7 ± 0.7	19.7 ± 0.8	n.s
VCAM-1 (×10 ng/ml)	26.9 ± 2.0	28.2 ± 0.6	n.s
TGF-1α (×1,000 pg/ml)	28.4 ± 1.9	26.2 ± 3.0	n.s
SDF-1α (×100 pg/ml)	18.4 ± 0.5	20.2 ± 1.0	n.s
Angiopoietin 1 (×100 pg/ml)	22.0 ± 7.0	50.0 ± 12.0	n.s
Angiopoietin 2 (×100 pg/ml)	14.9 ± 1.1	17.5 ± 1.6	n.s

VEGF vascular endothelial growth factor, *IL* interleukin, *SDF-1α* stromal cell-derived factor-1α, *TGF-1β* transforming growth factor-1β, *VCAM-1* vascular cell adhesion molecule-1, *MCP-1* monocyte chemotactic protein-1

Serum concentrations of hs-CRP and IL-6 were significantly increased in patients with Buerger's disease. In a multiple linear regression analysis, the concentration of hs-CRP was significantly correlated with the concentration of IL-6 in serum ($p < 0.05$) and b-FGF in culture medium ($p < 0.05$). The concentration of IL-6 significantly was correlated with hyperlipidemia ($p < 0.001$), hs-CRP ($p < 0.01$) or adhesion capacity of early outgrowth EPCs ($p < 0.05$). By the use of multiple stepwise regression analysis, serum hs-CRP and IL-6 concentration was significantly related to IL-6 ($p < 0.05$) and hyperlipidemia ($p < 0.001$), respectively.

There were six patients in Fontaine class II and six in class IV in this study. The capacities of early outgrowth EPCs, patient characteristics and serum concentrations of neovascularization-related cytokines were not different between the two groups (data not shown). The migration and invasion capacities were significantly impaired in four current smokers of patients with Buerger's disease ($p < 0.05$ vs. 4 current non-smokers in patients with Buerger's disease, respectively), however; we were not able to draw any conclusion from the results because the number of smokers was too small.

Discussion

To assess the neovascularization-related capacities of early outgrowth EPCs in patients with Buerger's disease, we

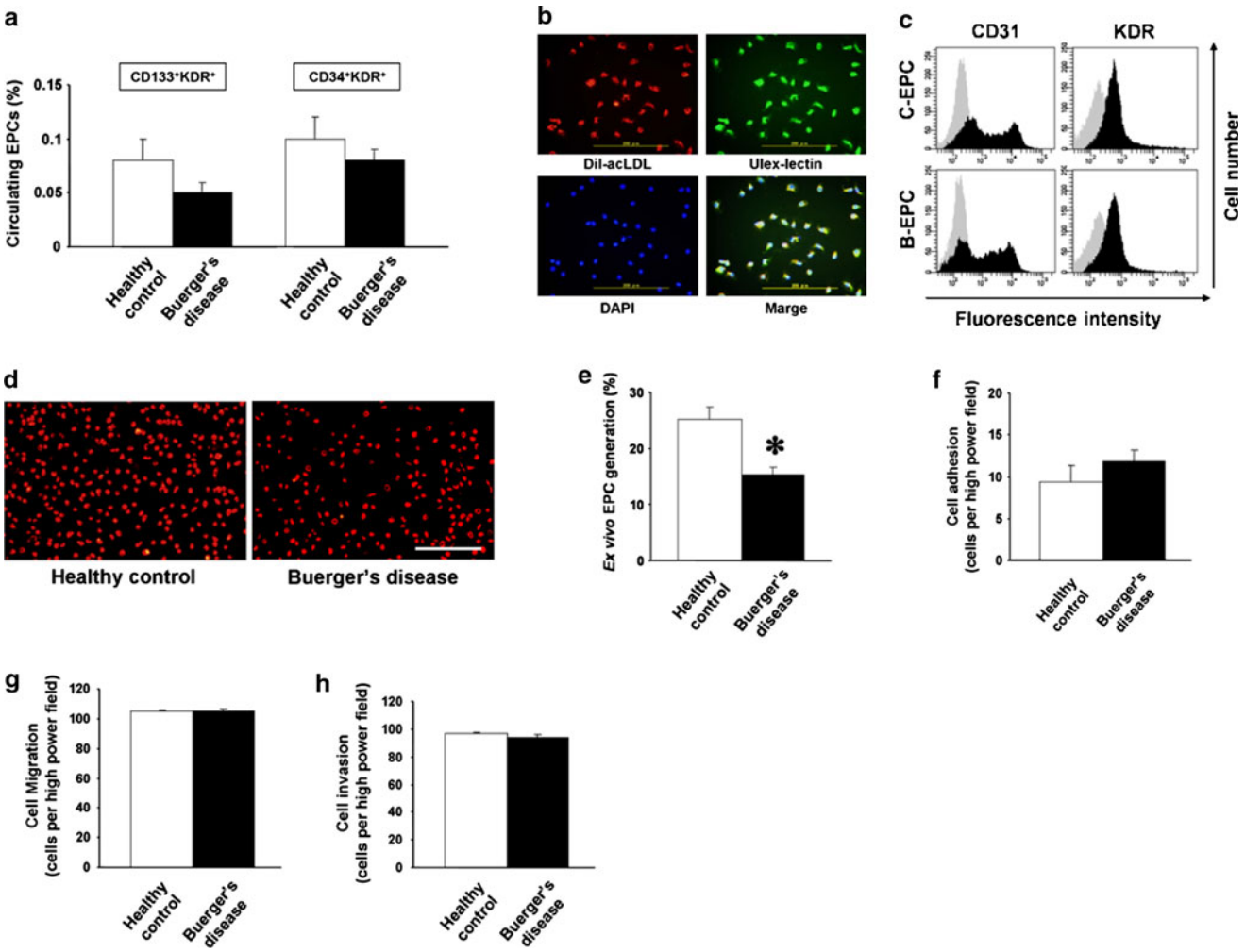

Fig. 1 **a** The percentage of peripheral blood-derived mononuclear cells (PB-MNCs), expressing $CD133^{+}KDR^{+}$ or $CD34^{+}KDR^{+}$. In flow cytometric analysis, the percentages of circulating EPCs in patients with Buerger's disease did not differ from those of age- and sex-matched controls. **b** Microscopic images of EPCs that were differentiated from PB-MNCs and adhered on fibronectin-coated plate after 4 days of a culture. The adherent EPCs were stained by the following three fluorescent colors: *red* Dil-acLDL incorporated into endothelial cell (EC), *green* Ulex-lectin binding to EC, *blue* DAPI staining nuclear. The adherent cell that was stained by all colors was defined as EPC. *Scale bar* 100 μm. **c** FACS analysis for the EPCs cultured from PB-MNCs in controls (C-EPC) and patients with Buerger's disease (B-EPC). *Gray color histograms* indicate isotype-matched IgG controls. Both C-EPC and B-EPC similarly expressed CD31 and KDR, which were surface markers of endothelial cell, on the *black color histograms*. The same expression patterns were observed in three experiments. **d** Representative photos of Dil-acLDL-positive EPCs that were cultured from PB-MNCs in controls and patients with Buerger's disease. The number of EPCs appeared smaller in a patient with Buerger's disease than in a control. **e** The percentage of ex vivo generation of early outgrowth EPCs after 4 days of PB-MNCs culture. The early outgrowth EPCs generation was significantly reduced in Buerger's disease ($*p < 0.005$ vs. controls). **f–h** For the adhesion, migration and invasion capacities of EPCs, no significant differences were observed between controls and patients with Buerger's disease

examined the capacities in the multi-step process. The number of circulating EPCs in peripheral blood was similar between control subjects and patients with Buerger's disease, however; the early outgrowth EPCs generation was reduced in Buerger's disease.

Although it was previously reported in patients with coronary artery disease that the number of circulating EPCs was decreased [15, 16] and migration capacity was impaired [14] reports examining the number of circulating EPCs and ex vivo neovascularization capacities of EPCs in patients with Buerger's disease are very few. In this study, we counted the number of PB-MNCs expressing the specific cell-surface markers of circulating EPCs, $CD34^{+}KDR^{+}$ or $CD133^{+}KDR^{+}$ [10], which were probably mobilized from the bone marrow into the circulation [8, 9]. The number of circulating EPCs was similar between controls and patients with Buerger's disease, indicating the preserved mobilization capacity of EPCs in Buerger's disease. Although the other functions, such as adhesion, migration, invasion and secretion capacities were

Table 3 The concentrations of neovascularization-related cytokines in the culture medium

	Healthy control ($n = 12$)	Buerger's disease ($n = 12$)	
VEGF (pg/ml)	17 ± 0.6	16.1 ± 0.4	n.s
b-FGF (pg/ml)	13.5 ± 0.3	13.2 ± 0.2	n.s
PDGF-BB (pg/ml)	22.7 ± 0.4	21.9 ± 0.5	n.s
IGF-1 (×0.1 ng/ml)	4.5 ± 0.4	4.4 ± 0.4	n.s
SDF-1α (×10 pg/ml)	24.8 ± 1.0	25.2 ± 1.2	n.s
Angiopoietin 1 (×10 pg/ml)	16.5 ± 0.4	17.7 ± 0.6	n.s
Angiopoietin 2 (×10 pg/ml)	87.4 ± 1.7	87.6 ± 6.1	n.s

VEGF vascular endothelial growth factor, *b-FGF* basic-fibroblast growth factor, *PDGF-BB* platelet-derived growth factor-BB, *IGF-1* insulin-like growth factor-1, *SDF-1α* stromal cell-derived factor-1α

similar to those of controls, only the generation of early outgrowth EPCs was reduced in patients with Buerger's disease. Nishioka et al. [24] have previously reported that the number of circulating EPCs and migration capacity were not impaired in patients with Buerger's disease, consisting with our results. It has been previously reported that EPCs number and functions were decreased in inflammatory diseases, such as rheumatoid arthritis [25, 26] and systemic lupus erythematosus [27, 28]. Although the reason is unclear at present, there may be several possibilities. First, hs-CRP was significantly higher than that of controls, but not so high when compared with these inflammatory diseases. Second, the early outgrowth EPCs in patients with Buerger's disease might be less susceptible to inflammation than other inflammatory diseases.

The reason for the reduced generation of early outgrowth EPCs was not clarified in this study. Although it was reported that accumulation of risk factors for atherosclerosis impair functions of EPCs [11–14], the reduced generation of early outgrowth EPCs in Buerger's disease is not likely due to differences in backgrounds because the number of the risk factors were similar between controls and patients with Buerger's disease. Our results may be partially in compatible with those of Yamamoto et al. who have reported the decreased mRNA expressions of EPC-specific molecules in four patients with ischemic limbs, suggesting decreased angiogenic potentials in patients with peripheral artery occlusive disease [29]. Although we measured several kinds of angiogenic cytokines including VEGF, which play an important role in the differentiation of ECs [30–32], the concentrations of the cytokines secreted from EPCs into the culture medium were similar between controls and patients with Buerger's disease. Some reported effects of hs-CRP and IL-6 on functions of EPCs [33, 34]. Accordingly, we examined the associations between EPCs functions and hs-CRP or IL-6. The serum concentration of hs-CRP and IL-6 was significantly elevated in patients with Buerger's disease, however; the elevations did not correlate with the percentage of ex vivo generation of early outgrowth EPCs in a univariate analysis (data not shown).

Because smoking is major risk factor for Buerger's disease [1], we examined the effects of smoking on the number of circulating EPCs and capacities of EPCs in controls and patients with Buerger's disease. The migration and invasion capacities were significantly impaired only in current smokers with Buerger's disease. We checked several neovascularization-related cytokines in the circulation and culture medium to clarify this mechanism, however; there were no relationships between the concentrations of these cytokines and functions of EPCs in patients with Burger's disease (data not shown). Unfortunately, we did not test whether smoking cessation would recover the impaired migration and invasion capacities of the EPCs in patients with Buerger's disease. Anyway, the number of smokers was too small to draw any conclusion from our results.

Limitations

This study had some limitations. First, the number of enrolled subjects was pretty small. Second, we assessed the several capacities of EPCs cultured from PB-MNCs not bone marrow-derived mononuclear cells (BM-MNCs). The results may be different when we used the EPCs cultured from BM-MNCs. Third, we assessed the several capacities of early, not late, outgrowth EPCs. Fourth, we did not determine colony forming units in early and late outgrowth EPCs. Fifth, we did not verify whether the reduced generation of early outgrowth EPCs in Buerger's disease would lead to poor angiogenesis on ex vivo and/or in vivo experiment. In conclusion, the early outgrowth EPCs generation is reduced in patients with Buerger's disease.

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Conflict of interest statement There is no conflict of interest or financial disclosure by the authors.

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