Vascular Function and Circulating Progenitor Cells in Thromboangitis Obliterans (Buerger’s Disease) and Atherosclerosis Obliterans

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Abstract—Thromboangitis obliterans (TAO; Buerger’s disease) and atherosclerosis obliterans (ASO) are associated with endothelial dysfunction. The purpose of this study was to evaluate the role of circulating progenitor cells (CPCs) in endothelial function in patients with TAO and ASO. We measured flow-mediated vasodilation (FMD), nitroglycerine-induced vasodilation, and circulating CPCs in 30 patients with TAO and 30 age- and sex-matched healthy subjects and in 40 patients with ASO. FMD was smaller in both the TAO group and ASO group than in the control group (6.6±2.7%, 5.7±3.3% versus 9.5±3.1%, P<0.0001, respectively). There was no significant difference in FMD between the TAO group and ASO group. Nitroglycerine-induced vasodilation was similar in the 3 groups. The number of and migration of circulating CPCs were similar in the TAO group and control group, whereas the number of and migration of circulating CPCs were significantly lower in the ASO group than in other groups (ASO 553±297/mL versus TAO 963±543/mL; control 1063±426/mL and ASO 36±18/hpf versus TAO 62±23/hpf; control 68±18/hpf, P<0.0001, respectively). There was a significant relationship between the number of and migration of CPCs and FMD (r=0.43 and r=0.40, P<0.0001, respectively). FMD was impaired in patients with TAO as well as in patients with ASO compared to that in normal control subjects, and the number of and function of circulating CPCs were not decreased in patients with TAO. These findings may partially explain why there are differences in cardiovascular morbidity and mortality rates between patients with TAO and patients with ASO. (Hypertension. 2011;57:70-78.) ● Online Data Supplement

Key Words: endothelial function ■ circulating progenitor cells ■ oxidative stress ■ Buerger’s disease ■ atherosclerosis obliterans

The vascular endothelium is involved in the release of various vasodilators, including nitric oxide (NO), prostaglandins, and endothelium-derived hyperpolarizing factor as well as vasoconstrictors.1,2 A healthy endothelium, mainly NO, maintains vascular tone and structure by regulating the balance between vasodilation and vasoconstriction, growth inhibition and growth promotion, antithrombosis and prothrombosis, anti-inflammation and proinflammation, and also antioxidation and pro-oxidation.3,4 Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis, leading to cardiovascular outcomes.5 It has been shown that thromboangitis obliterans (TAO; Buerger’s disease) and atherosclerosis obliterans (ASO) are associated with endothelial dysfunction.6-9 Interestingly, mortality and morbidity rates of cardiovascular disease are significantly higher in patients with ASO than in patients with TAO, and the mortality rate is not higher in patients with TAO than in age-matched populations.10-12

In 1997, Asahara et al13 demonstrated that circulating progenitor cells (CPCs) contribute to reendothelialization of the injured endothelium and neovascularization at sites of ischemia. Hill et al14 found by measurements of flow-mediated vasodilation (FMD) in healthy men that the number of CPCs is correlated with endothelial function. Endothelial function was found to be associated with the number of CPCs. In addition, Werner et al15 demonstrated the cumulative event-free survival in analysis of a first major cardiovascular event, according to levels of CPCs at the time of enrollment of patients with cardiovascular diseases. The cumulative event-free survival rate increased stepwise across 3 increasing baseline levels of endothelial progenitor cells in an analysis of death from cardiovascular causes. These findings suggest that the number of CPCs may be a predictor of cardiovascular events. However, there is no information concerning the relationship between endothelial function and CPCs in TAO and ASO.
We therefore evaluated vascular function, FMD, and nitroglycerine-induced vasodilation; the circulating number of and function of CPCs; and circulating levels of vascular endothelial growth factor (VEGF) and C-reactive protein (CRP), oxidative stress markers, circulating levels of malondialdehyde-modified low-density lipoprotein (MDA-LDL), and urinary excretion of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in patients with TAO and patients with ASO.

Methods

Subjects
We studied 30 patients with TAO; 30 age- and sex-matched healthy subjects who had no history of cardiovascular or cerebrovascular disease, hypertension, dyslipidemia, diabetes mellitus, liver disease, renal disease, current smoking habit, or other diseases; and 40 patients with ASO. Thirty patients with TAO and 40 patients with ASO were enrolled from the Hiroshima University peripheral arterial disease (PAD) database. TAO was diagnosed by the previous criteria, including results of physical examinations, clinical symptoms, and angiographic findings: smoking history, onset before the age of 50 years, infrapopliteal arterial occlusive disease, either upper limb involvement or phlebitis migrans, and absence of atherosclerotic risk factors other than smoking. To rule out other vasculitides and hypercoagulable states, rheumatoid factor, lupus anticoagulants, and serological investigations were evaluated. Patients who had received surgical interventions such as bypass grafting, skin grafting, and surgical interventions such as bypass grafting, skin grafting, and sympathectomy and patients who had undergone exercise training were excluded. Patients with ankle brachial pressure index <0.90 and with a history of TAO or ASO, including patients who had received peripheral bypass surgery and radiological intervention, were defined as patients having TAO or ASO. All patients were angiographically proven as having stenosis lesions in arteries of the lower extremities. The Ethical Committee of Hiroshima University Graduate School of Biomedical Sciences approved the study protocol. Written informed consent for participation in the study was obtained from all subjects.

Study Protocol
We measured vascular responses to reactive hyperemia and sublingual nitroglycerine, VEGF, CRP, MDA-LDL, 8-OHdG, and CPCs in all subjects. Subjects fasted the previous night for at least 12 hours. The study began at 8:30 AM. The subjects were kept in the supine position in a quiet, dark, air-conditioned room (constant temperature of 22°C to 25°C) throughout the study. A 23-gauge polyethylene catheter was inserted into the left deep antecubital vein to obtain blood samples. Thirty minutes after maintaining the supine position, the basal brachial artery diameter was measured. Then FMD and nitroglycerine-induced vasodilation were measured. These studies were performed in a randomized fashion. Each study proceeded after brachial artery diameter had returned to baseline.

Baseline fasting serum concentrations of total cholesterol, high-density lipoprotein cholesterol, LDL cholesterol, triglycerides, MDA-LDL, glucose, insulin, electrolytes, and CRP and plasma concentrations of VEGF were measured after a 30-minute rest period before the study. Twenty-four-hour urinary excretion of 8-OHdG was determined.

Measurement of Vascular Function
The subjects underwent a study of vascular function using ultrasound equipment and a high-resolution linear artery transducer (13 MHz) coupled to computer-assisted analysis software (e-TRACKING system; ALOKA Co.) that used an automated edge-detection system for measurement of brachial artery diameter. Please see the online Data Supplement for additional details.

Measurement of CPC
The number of CPCs was analyzed by flow cytometry as described previously. Please see the online Data Supplement for additional details.

Characterization of Progenitor Cells
Mononuclear cells were isolated by Ficoll density-gradient centrifugation of human blood buffy coats from 50 mL of peripheral blood. Please see the online Data Supplement for additional details.

Migration Assay
Cell migration was evaluated using a modified Boyden chamber assay as described previously. Please see the online Data Supplement for additional details.

Analytical Methods
Samples of venous blood were placed in tubes containing sodium EDTA (1 mg/mL) and in polystyrene tubes. Please see the online Data Supplement for additional details.

Statistical Analysis
Values are expressed as the mean±SD. All reported probability values were 2-tailed. Results were considered significant at P<0.05. Multigroup comparison of variables was performed by 1-way ANOVA followed by the Bonferroni correction. Relationships between variables were determined by linear regression analysis. The data were processed using the software package StatView IV (SAS Institute Inc.) or by super ANOVA (Abacus Concepts).

Results

Clinical Characteristics
The baseline clinical characteristics of the 30 normal control subjects, the 30 patients with TAO, and the 40 patients with ASO are summarized in Table 1. Age, systolic blood pressure, diastolic blood pressure, and serum concentrations of triglycerides and glucose were significantly higher in patients with ASO than in normal control subjects and patients with TAO (P<0.001, respectively). The serum concentration of CRP was higher in patients with TAO and patients with ASO than in normal control subjects (P<0.0001). There was no significant difference between the serum concentrations of CRP in patients with TAO and patients with ASO. The serum concentration of MDA-LDL and urinary excretion of 8-OHdG were higher in patients with ASO than in patients with TAO and normal control subjects (P=0.0003). There was no significant difference between the serum concentration of MDA-LDL and urinary excretion of 8-OHdG in patients with TAO and those in controls. The serum concentration of VEGF was higher in patients with TAO and ASO than in normal control subjects (P<0.0001). There was no significant difference between the serum concentrations of VEGF in patients with TAO and patients with ASO. Ankle-brachial pressure index was significantly lower in patients with TAO and ASO than in normal controls and was similar in patients with TAO and ASO. There was no significant difference in other parameters between the normal control group and the TAO group.

FMD and Vascular Response to Nitroglycerine
FMD was lower in patients with TAO and ASO than in normal control subjects (6.6±2.7%, 5.7±3.3% versus 9.5±3.1%, P<0.0001, respectively). There was no signifi-
cant difference between FMD in the TAO group and that in the ASO group (Figure 1). Brachial artery diameter at baseline and increase in hyperemic blood flow were similar among the 3 groups (Table 2). Vascular responses to nitroglycerine were not significantly different among the normal control subjects, patients with TAO, and patients with ASO (Table 2).

**Number of CPCs**
The number of CPCs was smaller in patients with ASO than in patients with TAO and normal control subjects (553±297/mL versus 963±543/mL and 1063±426/mL, *P*<0.0001, respectively), whereas there was no significant difference between the

| Table 1. Clinical Characteristics of the Normal Control, TAO, and ASO Groups |
|------------------|-------------------|------------------|
| Variables        | Normal Control    | TAO              | ASO              |
| Age, year        | 45±14             | 43±16            | 65±5†            |
| Gender, man/woman| 26/4              | 26/4             | 31/9             |
| Body mass index, kg/m² | 22.1±3.5     | 22.5±3.7         | 24.7±4.2         |
| Systolic blood pressure, mm Hg | 114.8±10.8 | 118.2±10.7 | 142.0±13.2† |
| Diastolic blood pressure, mm Hg | 63.2±6.4     | 64.2±6.1         | 82.5±10.4†      |
| Heart rate, bpm  | 64.2±12.1         | 69.1±12.9        | 70.2±10.2        |
| Total cholesterol, mmol/L | 4.76±0.61     | 4.92±0.45        | 5.12±0.72        |
| Triglycerides, mmol/L | 1.13±0.65     | 1.16±0.76        | 1.96±0.92†      |
| High-density lipoprotein cholesterol, mmol/L | 1.35±0.12 | 1.29±0.14 | 1.16±0.23†      |
| LDL cholesterol, mmol/L | 2.86±0.24     | 2.75±0.42        | 2.92±0.52        |
| Glucose, nmol/dL  | 4.8±0.1           | 4.7±0.2          | 5.4±0.4†         |
| Insulin, pmol/L  | 54.2±10.7         | 55.8±11.8        | 62.8±17.5        |
| VEGF, pg/mL      | 87.5±10.2         | 388.6±187.3*     | 421.5±307.4*     |
| CRP, mg/dL       | 0.08±0.09         | 2.31±2.14*       | 2.45±2.26*       |
| MDA-LDL, U/L     | 56.1±17.8         | 59.6±22.4        | 89.4±30.2†       |
| Urinary 8-OHdG, ng/mg Cr | 9.6±3.2        | 10.1±2.7         | 16.5±4.1†       |
| Ankle-brachial pressure index | 1.11±0.10 | 0.74±0.15* | 0.71±0.16*      |
| Complication     |                  |                  |                  |
| Hypertension, n (%) | 0 (0)            | 0 (0)            | 13 (33)          |
| Diabetes mellitus, n (%) | 0 (0)         | 0 (0)            | 11 (28)          |
| Hyperlipidemia, n (%) | 0 (0)            | 0 (0)            | 8 (20)           |
| Medication       |                  |                  |                  |
| Anti-platelet agent, n (%) | 0 (0)           | 24 (80)          | 40 (100)         |
| Calcium channel blocker, n (%) | 0 (0)           | 0 (0)            | 8 (20)           |
| ACE inhibitor, n (%) | 0 (0)            | 0 (0)            | 10 (25)          |
| ARB, n (%)       | 0 (0)             | 0 (0)            | 6 (15)           |
| Statin, n (%)    | 0 (0)             | 0 (0)            | 5 (13)           |
| Sulfonurylurea and/or metformin, n (%) | 0 (0)           | 0 (0)            | 11 (28)          |

ACE indicates angiotensin-converting enzyme; ARB, angiotensin type I receptor blocker.

*P*<0.05 vs normal.
P<0.05 vs TAO.
All results are presented as mean±SD.

**Figure 1.** Bar graph shows FMD in the normal control subjects, patients with TAO, and patients with ASO.

**Table 2. Vascular Response of the Normal Control, TAO, and ASO Groups**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal Control</th>
<th>TAO</th>
<th>ASO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachial artery diameter, mm</td>
<td>4.12±0.65</td>
<td>4.08±0.72</td>
<td>4.37±0.61</td>
</tr>
<tr>
<td>Increased hyperemic blood flow, %</td>
<td>412±128</td>
<td>398±146</td>
<td>404±216</td>
</tr>
<tr>
<td>Nitroglycerine-induced vasodilation, %</td>
<td>10.5±3.8</td>
<td>10.4±2.8</td>
<td>10.1±2.9</td>
</tr>
</tbody>
</table>

All results are presented as mean±SD.
number of CPCs in the control group and that in the TAO group (Figure 2).

Cell Migration Response to VEGF

Cell migration response to VEGF was smaller in patients with ASO than in patients with TAO and normal control subjects (36±18/hpf versus 62±23/hpf and 68±18/hpf, \(P<0.0001\), respectively) (Figure 3), whereas there was no significant difference between the cell migration response to VEGF in the control group and that in the TAO group (Figure 3).

Relationships Between FMD, Number of CPCs, and Cell Migration Response to VEGF, Oxidative Stress Markers, and Inflammation Marker

FMD was correlated with the number of CPCs (\(r=0.43, P<0.0001\)), cell migration response to VEGF (\(r=0.40, P<0.0001\)), MDA-LDL (\(r=-0.22, P=0.03\)), urinary excretion of 8-OHdG (\(r=-0.32, P=0.0009\)), and CRP (\(r=-0.33, P=0.0008\)) in all subjects (Figure 4). There was a significant relationship between the number of CPCs and cell migration response to VEGF (\(r=0.33, P=0.0008\)). In patients with
Figure 5. The number of CPCs significantly correlated with serum concentrations of MDA-LDL (r = -0.37, P = 0.0001) and urinary excretion of 8-OHdG (r = -0.41, P = 0.003) but not with CRP (r = -0.13, P = 0.19) in all subjects (Figure 5, left). In patients with ASO, the number of CPCs showed significant correlations with MDA-LDL and urinary excretion of 8-OHdG (r = -0.41, P = 0.003 and r = -0.39, P = 0.01, respectively) but not with CRP (r = 0.07, P = 0.69). In patients with TAO and healthy controls, there were no significant correlations between the number of CPCs and MDA-LDL, urinary excretion of 8-OHdG, and CRP.

Cell migration response to VEGF significantly correlated with serum concentrations of MDA-LDL (r = -0.37, P = 0.0001), urinary excretion of 8-OHdG, and CRP in all subjects.
urinary excretion of 8-OHdG ($r = -0.36$, $P = 0.0002$), and CRP ($r = -0.24$, $P = 0.02$) in all subjects (Figure 5, right). In patients with ASO, the cell migration response to VEGF showed a significant correlation with urinary excretion of 8-OHdG ($r = -0.31$, $P = 0.04$) but not with MDA-LDL or CRP ($r = -0.24$, $P = 0.11$ and $r = -0.07$, $P = 0.62$, respectively). In patients with TAO and healthy controls, there were no significant correlations between cell migration response to VEGF and MDA-LDL, urinary excretion of 8-OHdG, and CRP.

Serum concentrations of VEGF did not correlate with FMD, vascular response to nitroglycerine, number of CPCs, cell migration response to VEGF, MDA-LDL, urinary excretion of 8-OHdG, and CRP in all subjects or in each group.

**Discussion**

The number and function of CPCs were restored in patients with TAO, while endothelial function was impaired in these patients. In patients with ASO, the number and function of CPCs were decreased, and endothelial function was impaired. Circulating levels of MDA-LDL and urinary excretion of 8-OHdG were significantly higher in patients with ASO than in patients with TAO and normal controls. These findings suggest that oxidative stress plays a critical role in endothelial dysfunction and CPC dysfunction, especially in patients with ASO, through inactivation of the VEGF-CPC-NO pathway, leading to different rates of cardiovascular morbidity and mortality between patients with TAO and patients with ASO.

In the present study, FMD was smaller in both patients with TAO and patients with ASO than in the healthy subjects, whereas nitroglycerine-induced vasodilation was similar in the 3 groups. These findings suggest that the vascular endothelium, but not smooth muscle cells, was selectively impaired in patients TAO and patients with ASO.

The precise mechanism of endothelial dysfunction in patients with TAO is unclear. Patients with TAO had no classic cardiovascular risk factors, which influence endothelial function. In addition, confounding factors for endothelial function, such as oxidative stress markers, number of CPCs, and cell migration response to VEGF, other than an inflammation marker, were similar in patients with TAO and
ASO.27–29 Indeed, in the present study, serum concentration decreased in patients with ASO have been postulated. Healthy controls. Some possible mechanisms by which the response to VEGF were significantly decreased in patients groups. However, the number of CPCs and cell migration of CPCs also were similar in the 2 ASO. Therefore, it is postulated that the circulating number of and cell migration of CPCs in patients with TAO and normal populations. Although patients with TAO had increased levels of inflammation marker CRP, there was no association between FMD and circulating levels of CRP, suggesting that systemic inflammation might not directly affect endothelial function in patients with TAO. However, we cannot rule out the possibility that decreased NO bioavailability under the condition of increase in systemic inflammation contributes to endothelial dysfunction in these patients.

It is well known that VEGF gene expression is upregulated by hypoxia-induced factor-1 (HIF-1) under the condition of hypoxia. HIF-1 is a heterodimer composed of 2 subunits, HIF-1α and HIF-1β, and promotes transcription by combining with hypoxia response element in its target gene. Several investigators have recently shown that circulating levels of VEGF are increased by tissue ischemia in animal models of ischemic limb and patients with PAD, including TAO. In the present study also, circulating levels of VEGF were markedly increased in patients with TAO and ASO compared to the levels in healthy subjects. The levels of circulating VEGF were similar in patients with TAO and ASO. Therefore, it is postulated that the circulating number of and cell migration of CPCs also were similar in the 2 groups. However, the number of CPCs and cell migration response to VEGF were significantly decreased in patients with ASO compared to those in patients with TAO and healthy controls. Some possible mechanisms by which the number of CPCs and cell migration response to VEGF were decreased in patients with ASO have been postulated.

Oxidative stress plays an important role in the pathogenesis and development of cardiovascular diseases, including ASO. Indeed, in the present study, serum concentration of MDA-LDL and urinary excretion of 8-OHdG, oxidative stress markers, were significantly higher in patients with ASO than in patients with TAO and healthy subjects. Experimental and clinical studies have clearly shown that excessive oxidative stress decreases the number of CPCs and impairs CPC function. Under the condition of excessive oxidative stress, tissue ischemia failed to increase the number of CPCs and enhance CPC function in patients with ASO, leading to, at least in part, endothelial dysfunction. In addition, it is well known that a balance between ambient levels of superoxide and NO release plays a critical role in the maintenance of normal endothelial function. Reactive oxygen species (ROS), including hydroxy radicals, directly scavenge NO and produce toxic peroxynitrite. Recently, we have shown that inactivation of the renin-angiotensin system, particularly angiotensin II, by successful renal angioplasty decreases oxidative stress, resulting in improved endothelium-dependent vasodilation in patients with renovascular hypertension. These findings suggest that oxidative stress may be involved in impaired NO-mediated vasodilation in humans. Therefore, enhanced production of ROS and an attenuated antioxidant system may contribute to endothelial dysfunction in patients with ASO through a decrease in NO bioavailability. Under the condition of excess oxidative stress, depletion of VEGF-induced mobilization of CPCs and decrease in NO bioavailability may form a vicious circle, leading to endothelial dysfunction in patients with ASO.

Several lines of evidence have shown that PAD is associated with systemic inflammation. In the present study also, levels of the inflammation marker CRP were significantly higher in patients with ASO and patients with TAO than in healthy subjects. Experimental and clinical studies have shown that inflammation generally induces CPC dysfunction. Inflammation has a dual-sword role in CPC function. Although a low grade of inflammation, which probably has a favorable effect on CPCs, augments CPC functions such as mobilization, proliferation, and colony formation, a high grade of inflammation inhibits CPC functions. Verma et al reported that CRP per se directly inhibits CPC differentiation, survival, and function. However, although an increased level of CRP was similar in patients with ASO and patients with TAO, the number and function of CPCs were decreased in patients with ASO compared to those in patients with TAO. It is unlikely that inflammation directly influences the number and function of CPCs.

The number of and function of CPCs play an important role in not only endothelial function per se but also the prevalence of cardiovascular morbidity and mortality. Interestingly, it is postulated that development of ischemia is arrested in TAO patients older than 60 years of age. These is no significant difference in the rate of mortality between patients with TAO and normal populations. Although some investigators have reported that the survival rate of patients with TAO was significantly lower than that in the general population, the cumulative survival rate was significantly higher in patients with TAO than in patients with ASO. In contrast, atherosclerosis progressively develops with aging in patients with ASO. The rate of mortality is markedly worse in these patients. In this cross-sectional study, patients with TAO had endothelial dysfunction but had normal levels of CPCs and normal ability of cell migration. Although the cause of TAO remains unclear, if the condition of TAO stabilizes or reaches remission, the normal number of and function of CPCs may contribute to the restoration of endothelial function, resulting in inhibition or reduction in cardiovascular outcomes. Differences in the number of and function of CPCs and the grade of oxidative stress may lead to differences in the prevalence of
cardiovascular morbidity and mortality between patients with TAO and patients with ASO. Future studies are needed to determine whether FMD is restored in patients with TAO after the condition of remission.

Perspectives
Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis, resulting in cardiovascular complications. In addition, the number and function of CPCs relate to endothelial function. In the present study, the number of CPCs and cell migration response to VEGF were significantly decreased in patients with ASO compared to those in patients with TAO, although circulating levels of VEGF were similar in the 2 groups. The number of CPCs and cell migration response to VEGF were similar in patients with TAO and control subjects. Our results may partially explain why there are differences in the rates of morbidity and mortality of cardiovascular diseases between patients with TAO and patients with ASO. Additional studies are needed to evaluate endothelial function, the number and function of CPCs, and outcomes during a long-term follow-up period in patients with TAO and patients with ASO.

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Disclosures
None.

References
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Vascular Function and Circulating Progenitor Cells in Thromboangitis Obliterans (Buerger’s Disease) and Atherosclerosis Obliterans

Brief title: Buerger’s disease, endothelial function, and CPC

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Methods

Measurement of vascular function
The subjects rested and the right arm was fixed by a special arm-holding device (MIST-100, Saraya Co., Osaka Japan). A blood pressure cuff was first placed around the forearm. The brachial artery was scanned longitudinally 5-10 cm above the elbow. When the clearest B-mode image of the anterior and posterior intimal interfaces between the lumen and vessel wall was obtained, the transducer was held at the same point throughout the scan by a special probe holder (MP-PH0001, ALOKA Co.) to ensure consistency of the image. Pulsed Doppler flow was assessed at baseline and during peak hyperemic flow, which was confirmed to occur within 15 seconds after cuff deflation. The Doppler flow signals were captured with customized equipment. Blood flow velocity was calculated from color Doppler data and was displayed as a waveform in real time. The baseline longitudinal image of the artery was acquired for 30 seconds, and then a blood pressure cuff was inflated to 50 mm Hg above systolic pressure for 5 minutes. The longitudinal image of the artery was recorded continuously until 5 minutes after cuff deflation. Pulsed Doppler velocity signal obtained for 20 seconds at baseline and for 10 seconds immediately after cuff deflation. The response to nitroglycerine was used for assessment of endothelium-independent vasodilation. After acquiring baseline rest image for 30 seconds, a sublingual tablet (nitroglycerine 75μg, Nihonkayaku Co., Tokyo, Japan) was given, and image of the artery was recorded continuously for 5 minutes. The coefficient of variation for the baseline diameter was 2.8% in our laboratory.

Measurement of CPC
Briefly, samples of venous blood were placed in tubes containing sodium EDTA (7 mg/mL) and in polystyrene tubes. The EDTA-containing tubes were chilled promptly in an ice bath. Peripheral blood mononuclear cells were immediately isolated by Ficoll density gradient centrifugation (AXIS-SHIELD, Dundee, Scotland). After thawing, $1 \times 10^6$ peripheral blood mononuclear cells were incubated for 10 minutes with monoclonal antibodies against human FITC-conjugated anti-CD45 (Miltenyi Biotec, Bergisch Gladbach, Germany), PE-conjugated anti-AC133 (Miltenyi Biotec), and APC-conjugated anti-CD34 monoclonal antibody (Becton Dickinson Biosciences, Franklin Lakes, New Jersey). To assess background, isotype controls were used as negative controls based on the species and IgG subclass of each antibody. After incubation, erythrocytes were lysed, and the remaining cells were washed with phosphate-buffered saline, fixed in 2% paraformaldehyde, and analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Biosciences). Each analysis consisted of
500,000 events. To quantify the amount of CD34⁺ACC133⁻CD45low cells, the mononuclear cell fraction was gated and analyzed for the expression of AC133 and CD45. Only the AC133⁻CD45low cells finally investigated for the count of CD34⁺ cells.

**Characterization of progenitor cells**
The 1 × 10⁴ mononuclear cells were plated on 6-well culture dishes coated with human fibronectin and gelatin and maintained in endothelial cell basal medium-2 (EBM-2, CellSystem Co.) supplemented with EGM-2 microvascular single aliquots and 5% fetal bovine serum. After 3 days of culture, nonadherent cells were removed. Cytochemical analysis of adherent cells was performed on day 4. To detect the uptake of 1,1'-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated LDL (Di-AcLDL; Molecular Probes, Carlsbad, CA), cultivated cells were incubated with Di-AcLDL (10 μg/mL) at 37°C for 1 hour. Thereafter, cells were fixed with 2% paraformaldehyde for 10 minutes and incubated with fluorescein isothiocyanate (FITC)-labeled *Ulex europaeus* agglutinin I (lectin, 10 μg/mL; Sigma) for 1 hour. Cells demonstrating double-positive staining lectin and Di-AcLDL were identified to be progenitor cells. They counted 3 randomly selected high-power fields per well.

**Migration assay**
Briefly, isolated progenitor cells were detached mechanically by using a cell scraper, harvested by means of centrifugation, resuspended in 300 μL of EBM, and counted. The 2 × 10⁴ progenitor cells were placed in the upper chamber of a modified Boyden chamber (FluroBlock, Becton Dickinson Biosciences). The chamber was placed in a 24-well culture dish containing EBM and culture medium for control and human recombinant VEGF (50 ng/mL; Sigma). After 24 hours of incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification of cells that had migrated, cell nuclei were stained with 4’,6-diamino-phenylidole (DAPI, Sigma). Migrated cells in the lower chamber were counted manually in the 3 random high-power fields. Each experiment was performed in triplicate.

**Analytical methods**
The EDTA-containing tubes were chilled promptly in an ice bath. Plasma was immediately separated by centrifugation at 3100g for 10 min at 4°C, and serum was separated by centrifugation at 1000g for 10 min at room temperature. Samples were stored at -80°C until the time of assay. Serum concentrations of total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, glucose, and electrolytes were determined by routine chemical methods. Plasma concentrations of VEGF were
measured using ELISA kits (R&D Systems, Minneapolis, Minnesota). Serum concentration of CRP was measured by a CRP kit (Dade Behring, Deerfield, Illinois). The serum concentration of MDA-LDL was assayed by ELISA (anti-MDA-modified LDL antibody, SRL, Atsugi, Japan). The urinary excretion of 8-OHdG also was assayed by ELISA using 8-OHdG kits (Nihon Yushi, Fukuroi, Japan).