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Vitamin C Reversed Malfunction of Peripheral Blood-Derived Mononuclear Cells in Smokers Through Antioxidant Properties

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Background Smoking impairs neovascularization, possibly, through the impaired function of peripheral blood-derived mononuclear cells (PB-MNCs). Thus, the mechanism of impaired function of PB-MNCs caused by chronic smoking was examined, and whether vitamin C reversed the malfunction of PB-MNCs in smokers was investigated.

Methods and Results The cohort comprised 27 healthy male volunteers (16 smokers and 11 age-matched non-smokers). For evaluation of the colony-forming activity of PB-MNCs, the number of endothelial colony-forming units (e-CFUs) was counted in a culture assay. Migration activity of PB-MNCs was evaluated by the modified Boyden chamber method. In smokers, the number of e-CFUs was reduced to 56% and migratory activity of PB-MNCs to 40% compared with non-smokers ($p<0.01$). The urinary level of 8-isoprostane, an oxidative stress marker, was greater in smokers than in non-smokers ($p<0.05$). There was an inverse correlation between migratory activity of PB-MNCs but not between the number of e-CFUs and urinary level of 8-isoprostane. Furthermore, oral administration of vitamin C for 2 weeks ameliorated the impaired migratory activity of PB-MNCs in smokers.

Conclusion Chronic smoking impairs the function of PB-MNCs. Smoking-induced oxidative stress may be involved in impaired migratory activity of PB-MNCs. (*Circ J* 2008; **72:** 654–659)

Key Words: Cells; Oxidative stress; Smoking

Neovascularization in adults results from not only angiogenesis but also from vasculogenesis in which endothelial progenitor cells (EPCs) are mobilized from the bone marrow[1,2] Recently, we and others have reported that cell transplantation with peripheral or bone marrow mononuclear cells (MNCs), being the major source of EPCs, is a promising new strategy to improve peripheral tissue ischemia[3–5] In those studies, the injection of MNCs enhanced neovascularization and accelerated the restoration of blood flow of ischemic limbs in animals and humans, indicating the significant contribution of MNCs to adult neovascularization. Accordingly, the decreased activity of MNCs may contribute to the pathogenesis of peripheral artery occlusive diseases or reduced therapeutic potential. Indeed, MNCs isolated from patients with ischemic heart disease had reduced migratory and colony-forming activities in vitro[6] and had impaired neovascularization capacity in animal ischemic models[6]

Smoking is a major risk factor for arteriosclerosis obliterance (ASO), suggesting the role of smoking in impaired neovascularization in this disorder. Recently, several studies have shown that the number of coronary risk factors is inversely correlated with the colony-forming activity of peripheral blood-derived MNCs (PB-MNCs)[7,8] However, these studies were done with middle-aged to elderly subjects, of heterogeneous backgrounds and multiple risk factors. Furthermore, no mechanistic data were provided. To clarify the effect of smoking on PB-MNCs more simply, we enrolled only young healthy subjects with no risk factors other than smoking, and evaluated the function of PB-MNCs, colony-forming activity and migration activity. We measured the plasma levels of several angiogenic cytokines and an oxidative stress marker to elucidate the mechanisms for impaired function of PB-MNCs in chronic smokers. In addition, we examined the effects of orally administered vitamin C, an antioxidative agent, on smoking-induced malfunction of PB-MNCs.

Methods

Study Participants

The protocol was confirmed with the principles outlined in the Declaration of Helsinki and approved by the Institutional Ethic Committee of the Kurume University. Written informed consent was obtained from all participants. We

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Table 1 Background Characteristics of Smokers and Non-Smokers

	Smokers (n=11)	*Non-smokers (n=11)*	*p value*
Age (years)	*24.2±2.2*	*24.9±3.6*	*NS*
Gender (M/F)	*11/0*	*11/0*	*NS*
BMI (kg/m²)	*22.9±3.6*	*22.2±1.6*	*NS*
SBP (mmHg)	*121.5±11.1*	*122.4±8.6*	*NS*
DBP (mmHg)	*73.3±9.9*	*75.6±5.3*	*NS*
Total cholesterol (mg/dl)	*168.3±21.8*	*158.3±17.7*	*NS*
LDL-cholesterol (mg/dl)	*91.0±15.0*	*78.1±16.0*	*NS*
HDL-cholesterol (mg/dl)	*54.9±7.6*	*62.8±12.3*	*NS*
Triglyceride (mg/dl)	*85.8±32.4*	*98.8±46.4*	*NS*
FBS (g/dl)	*97.6±16.7*	*102.8±9.7*	*NS*
Homocystine (nmol/ml)	*10.2±2.7*	*12.2±3.6*	*NS*

Data are presented as the mean±SD.
NS, not significant; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBS, fasting blood sugar.

enrolled healthy male volunteers (smokers or non-smokers), who were taking no medication. All smokers smoked >10 cigarettes/day every day over 3 years (mean no. cigarettes, 16.4±3.9 per day). Non-smokers never smoked, and were instructed to keep away from second-hand smoke. Abstinence from smoking was confirmed by no detection of urinary cotinine, the end metabolite of nicotine. Smoking habit in chronic smokers was confirmed by the pesence of urinary cotinine. Other than smoking, no participants had coronary risk factors, including diabetes, hypertension and hyperlipidemia (Table 1).

Protocol 1 To clarify the effects of smoking on the colony-forming activity and migratory function of PB-MNCs, we enrolled 11 smokers and 11 age-matched non-smokers. The mean age was 24.2±2.2 for smokers and 24.9±3.6 years for non-smokers (not significant). Peripheral blood was drawn from the antecubital vein for PB-MNC preparation and for the measurements of several growth factors, and 24-h urine was collected for measurement of 8-isoprostane, which is a marker of oxidative stress.

Protocol 2 To examine the effects of an antioxidant on smoking-induced PB-MNC malfunction, we enrolled 5 additional smokers (mean age, 25.4±4.0 years). Vitamin C (3 g/day) was given orally to the smokers every day for 2 weeks. Peripheral blood was drawn from the antecubital vein before and 2 weeks after administration (ie, 3 g/day× 2 weeks) and 2 weeks after cessation of vitamin C administration.

Mononuclear Cell Isolation and Culture

The PB-MNCs were isolated by a density gradient centrifugation method from 20 ml of peripheral blood, as previously described[5] The PB-MNCs (1×10^6 cells/well) were then cultured on gelatin-coated, 6-well plates in Medium-199 (Gibco, Carlsbad, CA, USA) containing 20% fetal bovine serum (FBS), bovine pituitary extract (as endothelial cell growth supplement) (Invitrogen, Carlsbad, CA, USA), antibiotics (Gibco) and heparin (10 U/ml). On Day 7, adherent cells were characterized by immunocytochemistry using antibodies against kinase insert domain receptor (KDR) (Sigma, St Louis, MO, USA), CD31 (DakoCytomation, Glostrup, Denmark) and CD45 (DakoCytomation), and by staining for fluorescein isothiocyanate-labeled Ulex europaus agglutinin 1 (lectin, 10 μg/ml; Sigma-Aldrich, St Louis, MO, USA) and Dil-acetylated low-density lipoprotein (Dil-acLDL; Biomedical Technologies, Stoughton, MA, USA)[1,5,7] $KDR^+CD31^+CD45^-$ adherent cells that were double-positive for Dil-acLDL incorporation and lectin binding were defined as EPC-like cells[9] The number of endothelial colony-forming units (e-CFUs)[10] was counted under light microscopy at Day 7 by 3 investigators blinded to the study's protocol. Five randomly selected microscopic fields of 6 culture wells were counted in each subject (ie, 30 fields/subject), and the average number of e-CFUs (per field) was calculated. The intra- and inter-observer variabilities were less than 5%. We evaluated the number of e-CFUs as colony-forming activity of PB-MNCs.

Migration Assay

In the migration assay, vascular endothelial growth factor (VEGF)-induced migratory activity of MNCs was tested. The MNCs from peripheral blood were harvested by centrifugation, and subjected to migration assay using a modified Boyden chamber apparatus (Neuroprobe, Gaithersburg, MD, USA), as previously described[9,11] Briefly, polyvinylpyrrolidone-free polycarbonate filters with multiple pores (8 μm in diameter) were coated with 0.1% gelatin and 50 μg/ml fibronectin, and left exposed to the air for 6 h. After a coated filter was placed between the lower and upper chambers, 5×10^5 MNCs, which were suspended in 50 μl of Medium-199 containing 1% FBS without VEGF, were then seeded in the upper chamber. Medium-199 (25 μl) supplemented with 1% FBS and 10 ng/ml recombinant human VEGF was placed in the lower chamber of the apparatus. The whole apparatus was then incubated for 5 h at 37°C in a humidified incubator to render any cells that were able to migrate through the membrane. After the incubation period, the filter was removed and any non-migrated cells on the topside of the filter were scraped off with a rubber. The filters were then fixed with methanol and stained with Giemsa solution. Three investigators blinded to the study's protocol then counted the number of migrated cells attached to the underside of the filter, in 3 randomly selected microscopic fields in 10 chambers for each subject (ie, 30 fields/subject), and the migrated cell counts were expressed as the number of cells per high-power fields (×100) and then averaged. Data were expressed as the average number of migrated cells per field.

Biochemical Measurements

Plasma levels of VEGF and hepatocyte growth factor (HGF) were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA). Urinary 8-isoprostane was measured using commercially available

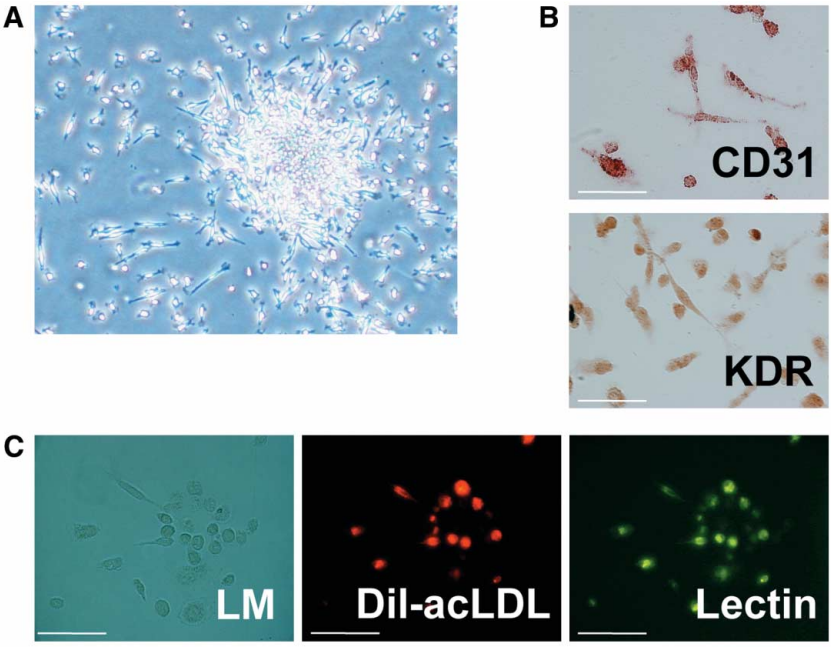

Fig 1. Enumeration of endothelial colony-forming units (e-CFUs). (A) A representative photomicrograph showing e-CFUs. (B) e-CFUs express the endothelial cell lineage markers (CD31 and kinase insert domain receptor (KDR)). (C) e-CFUs incorporate Dil-labeled acetylated low-density lipoprotein (Dil-acLDL), and bind to Ulex europaus agglutinin 1 lectin. LM, light microscope. Bars = 20 μm.

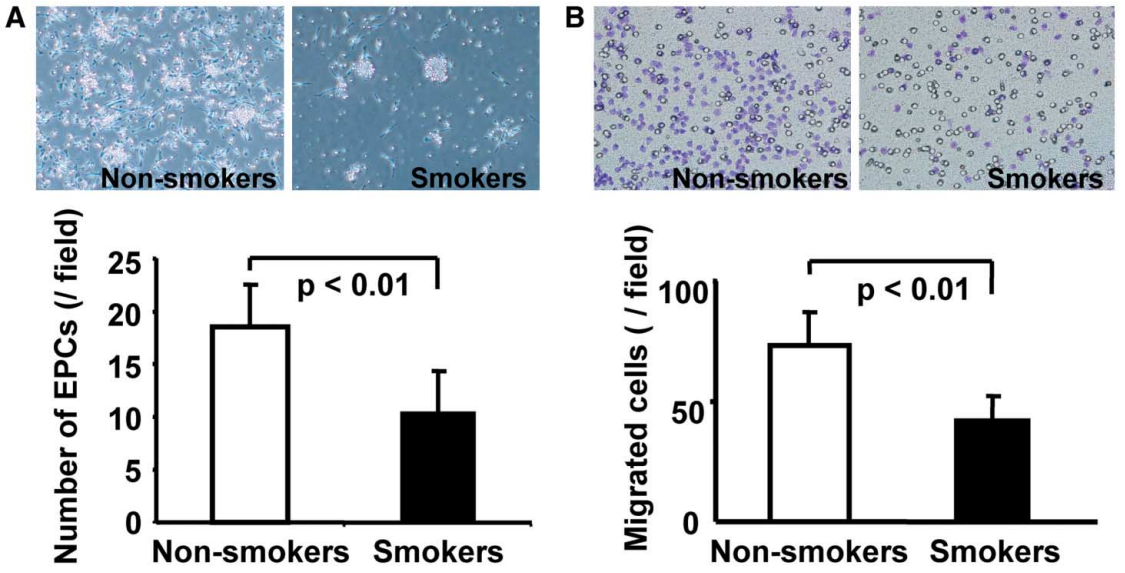

Fig 2. Effects of chronic smoking on the number of endothelial colony-forming units (e-CFUs) and migration activity of peripheral blood-derived mononuclear cells (PB-MNCs). (A) Top: Representative photographs of in vitro culture assay demonstrating the reduced number of e-CFUs in smokers. Photomicrographs were taken at Day 7 of culture. Bottom: Pooled data on the number of e-CFUs at Day 7. The number of CFUs was significantly greater in non-smokers than in smokers. Bar = 1 SD (n=11). (B) Top: Representative photographs of modified Boyden chamber experiments showing the reduced number of migrated PB-MNCs in smokers. Migrated cells onto the underside of the filter are violet in color. Bottom: Pooled data on the number of PB-MNCs that migrated in response to vascular endothelial growth factor. Bar = 1 SD (n=11). EPCs, endothelial progenitor cells.

ELISA kits (Cayman, Ann Arbor, MI, USA).[12] Urinary cotinine was measured at a commercially available laboratory (SRL, Fukuoka, Japan).

Statistical Analysis

The migrated cell counts were not normally distributed. Thus, the counts were logarithmically transformed, and statistical analysis was then performed. Data were expressed as the mean ± SD. Differences between the 2 groups were analyzed by unpaired Student's t-test. Univariate correlations were analyzed by Pearson's correlation. Differences were considered statistically significant when $p<0.05$.

Results

When PB-MNCs isolated from peripheral blood were cultured on gelatin-coated plates, e-CFUs developed from which spindle-shaped attaching cells sprouted on Day 7 (Fig 1A). Morphologically, these adherent cells resembled EPCs that were similar to those of previous reports.[1,5] Most adherent cells were labeled with endothelial cell lineage markers, such as KDR and CD31 (Fig 1B), but not with

Table 2 Number of PB-MNCs, Plasma Levels of Angiogenic Cytokines and Urinary Levels of Oxidative Stress Markers and Cotinine

	Smokers (n=11)	*Non-smokers (n=11)*	*p value*
PB-MNCs (×10⁶/ml)	*1.72±0.52*	*1.77±0.39*	*NS*
VEGF (pg/ml)	*39.83±16.63*	*35.25±31.83*	*NS*
HGF (pg/ml)	*587.70±92.41*	*594.58±85.47*	*NS*
8-Isoprostane (pg/mg creatinine)	*231.9±63.2*	*173.8±39.8*	*p<0.05*
Cotinine (ng/ml)	*1,247.3±677.8*	*ND*	

Data are presented as the mean±SD.
PB-MNCs, peripheral blood-derived mononuclear cells; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; ND, not detected. Other abbreviation see in Table 1.

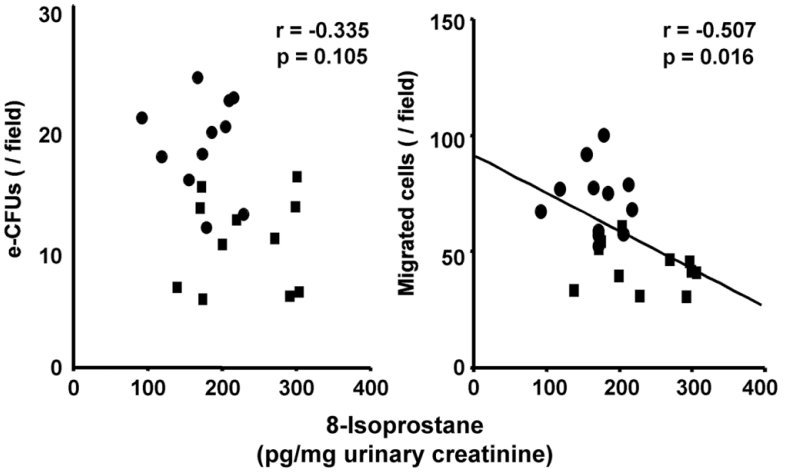

Fig 3. Correlation between 8-isoprostane and number of endothelial colony-forming units (e-CFUs) or the number of vascular endothelial growth factor (VEGF)-induced migrated cells. Left: Number of e-CFUs had no correlation with urinary levels of 8-isoprostane. Right: The VEGF-induced migrated cells inversely correlated with urinary levels of 8-isoprostane, suggesting that migratory activity is impaired by oxidative stress. (●), Non-smokers; (■), smokers.

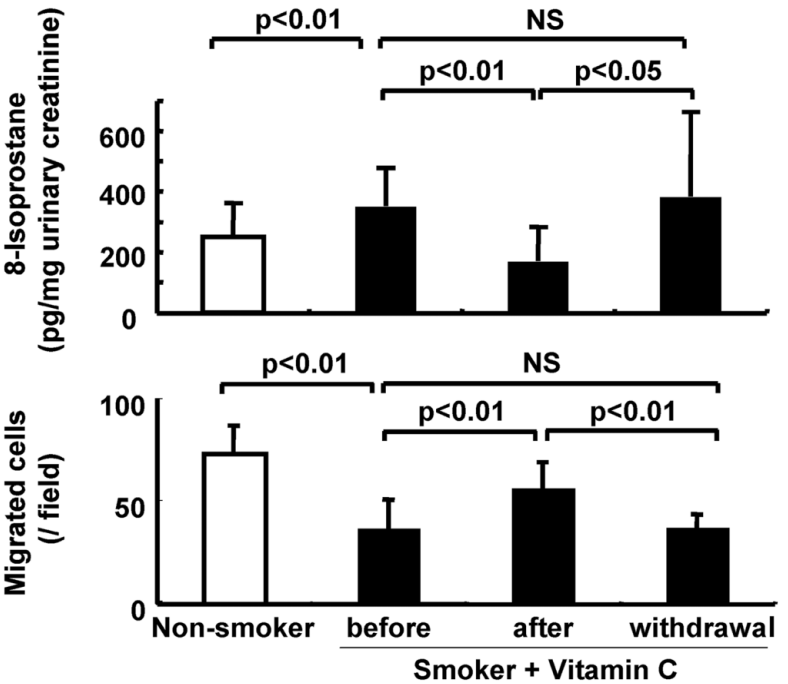

Fig 4. Effects of vitamin C. Top: The level of 8-isoprostane was higher in chronic smokers than in non-smokers. The level in smokers decreased to that of non-smokers with vitamin C, and became higher again after the administration of vitamin C was withdrawal. Bottom: Vitamin C ameliorated the impaired vascular endothelial growth factor-induced migratory activity in smokers. At baseline, the number of migrated cells (migratory activity) was fewer in smokers than in non-smokers. After withdrawal of vitamin C, migratory activity was again impaired. NS, not significant.

CD45, a common leukocyte antigen (data not shown). In addition, these cells showed lectin binding and Dil-acLDL incorporation (Fig 1C). Thus, the major populations of the adherent cells were defined as EPC-like cells[9].

Protocol 1

Colony-Forming Assays To determine the effects of chronic smoking on the colony-forming activity of PB-MNCs, culture assay was performed for both groups (ie, smokers and non-smokers). As shown in Fig 2A, the number of adherent e-CFUs was remarkably less in smokers than in non-smokers. In smokers, the number of e-CFUs was decreased to 56% of that of non-smokers (Fig 2A).

Migration Assay The migratory activity of PB-MNCs

in response to VEGF was evaluated using the modified Boyden chamber. The VEGF-induced migratory activity of PB-MNCs was remarkably impaired in smokers compared with non-smokers (Fig 2B). In smokers, the number of VEGF-induced, migrated PB-MNCs was 41% of that of non-smokers (Fig 2B).

Biochemical Markers There were no significant differences in the mean levels of plasma VEGF and HGF between smokers and non-smokers (Table 2). Urinary 8-isoprostane was significantly greater in smokers. Urinary cotinine, the end metabolic product of nicotine, was detected exclusively in smokers but not in non-smokers.

Relationship Between Oxidative Stress and Function of MNCs

The migratory activity of MNCs correlated inversely with the urinary level of 8-isoprostane ($r=-0.507$, $p=0.0159$; Fig 3, Right). There was no such relationship for the number of e-CFUs (Fig 3, Left).

Effects of Vitamin C (Protocol 2)

Administration of vitamin C for 2 weeks decreased the levels of 8-isoprostane in smokers to that of non-smokers. After withdrawal of vitamin C, levels of 8-isoprostane again became elevated (Fig 4, Top). Vitamin C administration had no effect on the number of e-CFUs in chronic smokers (data not shown). By contrast, the impaired VEGF-induced migratory activity of PB-MNCs was significantly ameliorated ($p<0.05$). The beneficial effect of vitamin C disappeared 2 weeks after administration was ceased (Fig 4, Bottom).

Discussion

There are 3 major findings of the present study. First, chronic smoking impaired colony-forming and migratory activities of PB-MNCs in healthy young men. Second, migratory activity correlated inversely with the level of urinary 8-isoprostane. Third, 2 weeks administration of vitamin C significantly improved migratory activity of PB-MNCs in smokers, suggesting that smoking-induced oxidative stress may impair MNC migratory activity.

Study Population

Two previous reports[7,8] demonstrated that the number of e-CFUs and the colony-forming activity of PB-MNCs were both reduced as the number of risk factors increased in middle-aged subjects. It was concluded from those studies that risk factors impair the function of PB-MNCs. However, the participants in those studies had multiple risk factors and heterogeneous backgrounds and, therefore, the specific effects of smoking on function of PB-MNCs and the mechanisms are unknown. By contrast, the cohort of the present study was homogenous, young and had only smoking as a risk factor. Thus, in the present study we were able to exclude other risk factors (except smoking) as contributing to impaired function of PB-MNCs.

During the study, we monitored the smoking status of participants very carefully. As shown in Table 2, urinary cotinine, the end metabolite of nicotine, was high in chronic smokers but not detectable in non-smokers. Thus, it was considered that the observed findings were attributable exclusively to chronic smoking. Also, we measured a marker of oxidative stress, 8-isoprostane levels, in urine. The chronic smokers had elevated levels of 8-isoprostane, results similar to those of many previous studies[12] Thus, not only was smoking status confirmed in the present study, but also increased oxidative stress was demonstrated in chronic smokers.

Malfunction of PB-MNCs in Chronic Smokers

Causes of impaired neovascularization in chronic smokers are multifactorial. Because plasma levels of VEGF and HGF were similar between smokers and non-smokers, it is unlikely that chronic smoking impairs neovascularization by reducing the secretions of angiogenic cytokines. Besides, the young participants in our study did not show any evidence of impaired neovascularization. Among the multiple mechanisms possible, malfunction of EPCs may be an attractive option. Accordingly, in the present study, we examined the function of PB-MNCs. Acknowledging that PB-MNCs may not be exactly the same as EPCs, it must be considered that the exact cell surface markers of EPCs remain incompletely determined. It has been reported that the percentage of EPCs in peripheral blood is 0.01–0.03% of PB-MNCs. Furthermore, PB-MNCs have many features similar to endothelial cells, as shown in Fig 1. Thus, PB-MNCs were considered endothelial progenitor-like cells, as also considered by other studies!

PB-MNCs possess several functions, which may contribute to neovascularization in adults. They include mobilization from the bone marrow, chemoattractant, adhesion, migration, invasion and colony-forming activity. The present study demonstrated that the numbers of e-CFUs and migrated cells were significantly reduced in healthy male chronic smokers, indicating impaired colony-forming and migratory activities in chronic smokers. In order to elucidate the mechanism by which chronic smoking impairs the function of PB-MNCs, we investigated the relationship between 8-isoprostane (a marker of systemic oxidative stress) and numbers of e-CFUs or migrated cells.

As shown in Fig 3, there was an inverse relationship for migrated cells but not for e-CFU, suggesting the role of smoking-induced oxidative stress for impaired migratory activity but not for colony-forming activity. This notion may be supported by recent studies showing that the reduction in circulating EPCs in healthy smokers was reversed by smoking cessation[13] or green tea consumption[14] because these behaviours attenuate oxidative stress in smokers.

In order to further elucidate the contribution of oxidative stress, we gave an antioxidant (ie, vitamin C, 3 g/day) to smokers for 2 weeks. In our previous study, the dose and duration was enough to reverse oxidative stress in smokers![15] As expected, vitamin C reduced the elevated 8-isoprostane levels and recovered impaired migratory activity of PB-MNCs in smokers. Moreover, 2 weeks after the cessation of vitamin C, migratory activity returned to levels similar to those before vitamin C treatment.

These findings clearly suggest that oxidative stress may be one of the mechanisms of impaired PB-MNC migratory activity in chronic smokers. Because there was no relationship between oxidative stress and colony-forming activity in smokers, we did not examine the change in e-CFUs after vitamin C.

It is not clear from the present study why oxidative stress was responsible only for impaired migratory activity and not for colony-forming activity. We speculated that this different effect of oxidative stress might have resulted from the different sensitivity to VEGF. In fact, it is reported that the decreased sensitivity of EPCs to VEGF is the major mechanism of decreased migratory function of EPCs in

patients with atherosclerotic risk factors.[7] In the present study, migration activity was evaluated in the presence of VEGF. Thus, impaired migratory activity of PB-MNCs in this study may have just reflected the reduced sensitivity of PB-MNCs to VEGF. On the other hand, it is unclear whether VEGF has an effect on the colony-forming activity of PB-MNCs. Further studies are needed to clarify the mechanisms of the different effects of oxidative stress on the function of PB-MNCs.

Clinical Implications

Given that migration of PB-MNCs is an early key event in vasculogenesis and probably in the maintenance of endothelial integrity,[1,2] the functional impairment of PB-MNCs may account for aggravated atherosclerosis and impaired neovascularization in chronic smokers. Furthermore, antioxidant agents, such as vitamin C, might improve the efficacy of therapeutic angiogenesis by cell transplantation. Consistent with this notion, we demonstrated previously in rats that statins improved limb ischemia by recruiting EPCs, possibly through the antioxidant activity.[16]

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Disclosure

The authors have no conflict of interest.

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