Smoking Cessation Rapidly Increases Circulating Progenitor Cells in Peripheral Blood in Chronic Smokers

Takahisa Kondo, Mutsuharu Hayashi, Kyosuke Takeshita, Yasushi Numaguchi, Koichi Kobayashi, Shigeo Iino, Yasuya Inden, Toyoaki Murohara

Objective—Circulating endothelial progenitor cells (EPCs) contribute to postnatal angiogenesis. The number of circulating EPCs has an inverse correlation with coronary risk scores. However, the effect of smoking on the number of circulating EPCs is not well-known.

Methods and Results—We examined the effects of chronic smoking and of smoking cessation on EPC levels. Circulating EPCs were quantified by flow cytometry as CD45lowCD34+CD133+ (progenitor cells [PCs]) or CD45lowCD34+CD133+VEGFR2+ (EPCs) in 14 nonsmokers and in 15 smokers. All smokers quit smoking. Eight quit smoking with nicotine patch and 7 without nicotine patch. PC/EPC levels were inversely correlated with the number of cigarettes smoked. Circulating PCs/EPCs increased rapidly after cessation (P<0.0001) and decreased again after resumption of smoking to the level similar to that before cessation (P=0.0031). The magnitude of increase in EPCs was greater in light smokers than in heavy smokers.

Conclusions—The number of circulating PCs/EPCs was reduced in chronic smokers. Smoking cessation led to a rapid restoration of PC/EPC levels. The recovery of EPC levels was greater in light smokers than in heavy smokers. The decreased number of circulating EPCs would make smokers susceptible to cardiovascular disease, and even short-time cessation of smoking may be an effective means to reduce cardiovascular risk. (Arterioscler Thromb Vasc Biol. 2004; 24:1442-1447.)

Key Words: smoking cessation ■ endothelial progenitor cells ■ CD34 ■ CD133 ■ VEGFR2

Endothelial progenitor cells (EPCs) have been discovered in peripheral blood, bone marrow, and cord blood mononuclear cells.1–3 Experimental and clinical studies showed that EPCs were mobilized in response to tissue ischemia and vascular injury from bone marrow.4–6 Mobilized EPCs thus contribute to not only microvascular angiogenesis but also endothelial repair of large vessels.2–7–9 Recently, Vasa et al reported that the number and functions of circulating EPCs were inversely correlated with the risk of cardiovascular diseases.10 Furthermore, Hill et al showed that an inverse correlation exists between the number of circulating EPCs and Framingham risk scores.11 They also showed that the number of EPCs correlated with endothelial function as measured by flow-mediated forearm vessel dilatation.11 Several studies have demonstrated that the deterioration of flow-mediated forearm vessel dilatation is a good marker for major cardiovascular events.12,13 Furthermore, bone-marrow derived EPCs are reported to be used in the repair process of endothelial injury.9,14 Considering these findings, measuring circulating EPCs in peripheral blood might have potential diagnostic and/or prognostic advantages in evaluating endothelial function and future cardiovascular events. Furthermore, the measurement of circulating EPCs in peripheral blood may also be useful in assessing cardiovascular burden in subjects.

Currently, circulating EPCs in peripheral blood are mainly determined by attaching cells in a culture method, as previously reported.3,10,11 However, this method is time-consuming, and the values vary with the growth factors or the type of fetal bovine serum used. Recently, Peichev et al showed that CD34+CD133+VEGFR2+ expressing progenitor cells (PCs) give rise to endothelial cells.15 Hence, measurement of cells that express CD34+CD133+VEGFR2 would be an ideal marker for assessing the amount of circulating EPCs, especially when examining direct environmental effects such as that of smoking on EPC kinetics. In this study, we define circulating CD45lowCD34+CD133+ cells as PCs and circulating CD45lowCD34+CD133+VEGFR2+ cells as EPCs, and we use these fractions of cells for the measurement of circulating PCs/EPCs.

Smoking is a preventable behavior that is a leading risk factor for cardiovascular diseases. Cigarette smoking accounts for almost 50% of coronary events, and the risk of myocardial infarction or stroke decreases by 50% within the first 2 years after smoking cessation.16,17 Chronic smokers have endothelial dysfunction, and endothelial reactivity is rapidly
restored after smoking cessation. As mentioned, endothelial reactivity clearly predicts future cardiac events, and the restoration of endothelial function by smoking cessation might imply a reduction of future cardiovascular events in chronic smokers. Hill et al demonstrated that endothelial reactivity and circulating EPCs were correlated, so that the measurement of circulating EPCs may predict future cardiac events.

Despite such background, little is known regarding the effects of smoking on the levels of circulating PCs/EPCs. Only Vasa et al have shown that EPC levels decreased in smokers. Accordingly, we first examined the number of circulating PCs/EPCs in chronic smokers and nonsmoking subjects. We then examined whether smoking cessation influenced the levels of circulating PCs/EPCs.

Methods

Study Subjects

We studied 14 nonsmokers and 15 age-matched smokers (10 light smokers [<20 cigarettes per day] and 5 heavy smokers [≥20 cigarettes per day]). All subjects were apparently healthy men. Nonsmokers were recruited from healthy volunteers and smokers were recruited from among a group of people who wanted to quit smoking. Subjects were not using any medications such as statin, antidiabetic drugs, or antihypertensive drugs. Smokers quit smoking on their own with (n=8) or without (n=7) nicotine patches (Nicotinell TTS; Novartis Pharma K.K.). Unexpectedly, all smokers were only able to quit smoking for 1 month, after which they resumed smoking habit. The study protocol is shown in Figure 1. Baseline characteristics of the subjects are shown in Table 1. This protocol was approved by the Institutional Review Board at Nagoya University Graduate School of Medicine, and informed consent was obtained from all subjects.

Quantification of EPCs

We first examined circulating EPCs in a cell culture assay as reported previously. In brief, peripheral blood (PB) (20 mL) was obtained and mononuclear cells (MNCs) were isolated by a density-gradient centrifuge method. MNCs were cultured on gelatin-coated 6-well plates in EBM medium supplemented with EGM SingleQuots (Clonetics) and 20% fetal calf serum. At day 7, EPCs were characterized by dual staining for DiI-acetylated low-density lipoprotein incorporation (2.4 μg/mL; Molecular Probes) and fluorescein isothiocyanate (FITC)-labeled Ulex europaeus afflutinin I (lectin, 10 μg/mL; Sigma). Attaching double-stained cells were counted manually in 10 random microscopic fields. We further examined circulating PCs and EPCs using cell surface antigen. In previous reports, circulating mononuclear cells with CD34−CD133+/CD34−CD133+/VEGFR2− were quantified as tentative PCs/EPCs.

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<th>TABLE 1. Baseline Clinical Characteristics of Nonsmokers and Smokers</th>
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<td>Nonsmokers (N=9) Mean (SE) Smokers (N=15) Mean (SE) P Value</td>
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<tr>
<td>Age (y) 43.9 (2.0) 38.9 (2.0) NS</td>
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<tr>
<td>Systolic blood pressure (mm Hg) 127.9 (4.1) 117.6 (4.1) NS</td>
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<td>Body mass index (kg/m²) 23.7 (0.6) 22.2 (0.6) NS</td>
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<td>White blood cells (×10⁹/L) 5477 (484) 7193 (544) P=0.013</td>
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<td>Fasting blood sugar (mmol/L) 4.52 (0.26) 5.12 (0.13) NS</td>
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<tr>
<td>Total cholesterol (mmol/L) 5.56 (0.17) 5.01 (0.17) NS</td>
</tr>
<tr>
<td>Triglyceride (mmol/L) 1.85 (0.31) 1.55 (0.31) NS</td>
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<tr>
<td>Nicotine (ng/mL) Not detected 24.9 (2.5) —</td>
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<td>Cotinine (ng/mL) Not detected 246.0 (17.6) —</td>
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Unpaired t test or Mann–Whitney U test.
Therefore, 4 mL of PB was drawn and white blood cells were stained with allophycocyanin (activated protein C)-conjugated anti-CD45 monocolonal antibody (mAb) (Caltag Laboratories), PC5-conjugated anti-CD34 mAb (Beckman Coulter, Inc), phycoerythrin (PE)-conjugated anti-CD133 mAb (Miltenyi Biotec GmbH), and anti-VEGFR2 (VEGFR2) mAb (Sigma), whereas FITC-conjugated anti-CD133 mAb (Miltenyi Biotec GmbH), and anti-CD45 low CD34 cells were quantified and expressed as number of cells per 10^6 total events or number of cells per milliliter of blood. The numbers shown in Table 1. There were no differences in age, blood pressure, body mass index, or biochemical measurements. Only white blood cell count was significantly higher in smokers than in nonsmokers. We cultured attaching EPCs from peripheral blood from nonsmokers, light smokers, and heavy smokers and compared the EPC count among these 3 groups. The number of attaching EPCs was greater in nonsmokers than in light smokers (Figure 2). We could not culture EPCs at all from heavy smokers in repeated series of cell culture experiments (Figure 2). The peripheral MNCs from heavy smokers had become swollen and perished in the same culture conditions as the EPC culture from nonsmokers and light smokers. We therefore adopted another method for the measurement of EPCs in peripheral blood. As previously reported, EPCs are mainly derived from CD45^−CD34^−CD133^− progenitor cells.20,21 Thus, we decided to use the circulating CD45^−CD34^−CD133^−/CD45^−CD34^−CD133^−/CD45^−CD34^−/CD133^−/VEGFR2^− cells to evaluate PC/EPC levels in peripheral blood.

Biochemical Measurements

PB (7 mL) was collected from nonsmokers and smokers. PB was also collected from smokers at 0, 2, and 4 weeks after cessation and at 4 weeks after they resumed smoking. Complete blood counts, cholesterol, fasting glucose levels, and other biochemical markers were examined as routine tests.

Statistics

Data are expressed as means±SE. The means for nonsmokers were compared with those for smokers with the use of an unpaired t test or the Mann–Whitney U test. The PC and EPC levels were compared among nonsmokers, light smokers, and heavy smokers using the Mann–Whitney U test analysis. The change in PC and EPC level concomitant with smoking cessation was compared with the Friedman test. The change in PC and EPC levels after subjects resumed smoking was tested with the Wilcoxon signed rank test. The difference in the change in PC levels between light smokers and heavy smokers after smoking was tested with the Mann–Whitney U test. All statistical analyses were performed with Statview 5.0 (SAS Institute Inc). Differences of P<0.05 were considered significant.

Results

Clinical characteristics for nonsmokers and smokers are shown in Table 1. There were no differences in age, blood	

![Figure 2. Characterization of EPCs. a, Overlay images of Dil-AcLDL uptake and lectin binding of isolated EPCs from nonsmokers, light smokers, and heavy smokers. Double-positive cells appear yellow. Representative images are shown from at least 3 experiments. b, Dil AcLDL/FITC-lectin double-positive attaching cells in 10 fields were counted. Data are mean±SE.](http://atvb.ahajournals.org/issue/1444/Arterioscler-Thromb-Vasc-Biol/August-2004/11006)
CD45lowCD34+/H11001CD133+ cells. In fact, CD45lowCD34+/H11001CD133+ cells gave rise to EPCs in culture conditions, as demonstrated by DiI-acetylated LDL uptake and lectin staining (Figure 3d). However, CD45lowCD34+/H11001CD133+VEGFR2+ cells did not proliferate but survived without any morphological change for 4 weeks (data not shown).

PC levels in light smokers and heavy smokers significantly decreased compared with nonsmokers (P<0.0001, P<0.0001, respectively) (Figure 3b). EPC levels were also reduced in light smokers (P=0.037, P=0.038, respectively) (Figure 3c). Duration of smoking period and/or the sum of cigarette pack-years were not correlated with the number of circulating EPCs (data not shown).

Effects of Smoking Cessation on Inflammatory Markers and Lipid Parameters
We examined the change of inflammatory markers and lipid parameters during the period of smoking cessation. There were no differences in these parameters (Table 2).

Effects of Smoking Cessation on Circulating EPCs
We next examined the change in circulating PCs/EPCs during the period of smoking cessation. The levels of PCs (CD45lowCD34+/H11001CD133+ cells) increased rapidly 2 weeks after smoking cessation in chronic smokers (Figure 4a). EPCs (CD45lowCD34+/H11001CD133+VEGFR2+ cells) showed a similar change as PCs (Figure 4b). CD45lowCD34+/H11001CD133+vegfr2+ cells. In fact, CD45lowCD34+/H11001CD133+ cells gave rise to EPCs in culture conditions, as demonstrated by DiI-acetylated LDL uptake and lectin staining (Figure 3d). However, CD45lowCD34+/H11001CD133+VEGFR2+ cells did not proliferate but survived without any morphological change for 4 weeks (data not shown). PC levels in light smokers and heavy smokers significantly decreased compared with nonsmokers (P<0.0001, P<0.0001, respectively) (Figure 3b). EPC levels were also reduced in light smokers (P=0.037, P=0.038, respectively) (Figure 3c). Duration of smoking period and/or the sum of cigarette pack-years were not correlated with the number of circulating EPCs (data not shown).

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also showed similar patterns of change (data not shown). The increase of PCs or EPCs continued for 4 weeks after cessation, reaching a level equivalent to that of nonsmokers. Interestingly, the levels of PCs and EPCs again rapidly decreased after smoking was resumed (Figure 4a, 4b).

**Effects of Nicotine Patch on Smoking Cessation-Induced Increase in EPCs**

We compared the magnitude of increase in EPC levels after smoking cessation between the subjects who used nicotine patches to quit smoking and those who did not. The magnitude of increase in EPC counts after smoking cessation was slightly greater in the nicotine patch users than in the nonusers, but this difference was not statistically significant (Figure 4c).

**Smoking Cessation-Induced Increase in EPCs Between Light and Heavy Smokers**

We finally compared the magnitude of increase in EPC counts between light and heavy smokers. Percent increase of the number of EPCs was much greater in light smokers (<20 cigarettes per day) than in heavy smokers (≥20 cigarettes per day) (Figure 4d).

**Discussion**

The major findings of the present study are that the number of circulating PCs/EPCs was significantly lowered as the number of cigarettes consumed increased; in the smokers group, PCs/EPCs increased rapidly after smoking cessation and, then after smoking was resumed, again decreased to the level similar to that before smoking cessation; use of nicotine patches slightly increased the magnitude of the increase in PCs after smoking cessation; and, finally, the magnitude of the increase in PCs after smoking cessation was greater in light smokers than in heavy smokers.

In the present study, chronic smokers had fewer circulating PCs/EPCs than did nonsmokers at the beginning of the study, consistent with the findings of Vasa et al. However, attaching EPCs from heavy smokers died during early phase of culture. This phenomenon was not presented in Vasa’s study, and the reason for this discrepancy is unknown. In contrast, the measurement of PCs/EPCs by cell surface antigen is not affected by culture conditions. Furthermore, identification of PCs/EPCs using CD45<sup>+</sup>CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup> allowed us to assess the direct effect of smoking on circulating PC/EPCs. We identified PCs/EPCs after gating CD45<sup>+</sup>CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup> because our preliminary studies revealed that CD45<sup>+</sup> cells contained CD34<sup>+</sup>CD133<sup>+</sup> cells, and this gating could minimize intra-experiment variation in counting CD45<sup>+</sup>CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup> cells. Using CD45<sup>+</sup>CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup> markers, our present study further revealed that smoking cessation rapidly increased the number of circulating PCs/EPCs. This quick recovery of PCs/EPCs after quitting smoking was one of the most surprising findings in the present study.

The possible mechanisms for these findings are as follows. First, smoking may affect bone marrow environment, and PC/EPC mobilizations from the bone marrow could be decreased by smoking. In fact, smoking is a well-known factor inhibiting the release of physiological amounts of nitric oxide produced by endothelial nitric oxide synthase, and a recent study has demonstrated that endothelial nitric oxide synthase is important for EPC mobilization from bone marrow. Second, change in PC/EPC levels associated with smoking status is possibly related to the fact that chronic smokers have endothelial dysfunction. Because smoking cessation rapidly improves endothelial function, the changes in EPC levels and endothelial function would be parallel. Recent studies also showed that EPCs accelerated re-endothelialization of injured endothelium. Taken together, one possible explanation is that injured vessels in smokers may use EPCs to maintain endothelial function, and that the increase in circulating EPCs after smoking cessation may be the result of a decreased number of injured vessels after cessation. These 2 hypotheses could account for the phenomenon that resuming smoking substantially reduced PCs/EPC levels in peripheral blood in the present study.

There are 2 additional findings in the present study. First, the magnitude of the increase in EPCs after smoking cessation was slightly higher among nicotine patch users than nonsmokers. However, this difference was not statistically significant. Recently, Heeschen et al demonstrated that nicotine stimulates angiogenesis at sites of ischemia. Therefore, the effect of nicotine may account for the small increase in EPC levels in the nicotine patch users. However, studies by Heeschen et al were performed in a nonsmoking setting and the effect of nicotine on EPCs in chronic smokers remains unknown. Second, the magnitude of the increase in EPCs after smoking cessation was smaller in heavy smokers than in light smokers. The latter finding suggests that light smokers may have a better chance of recovery in terms of circulating EPCs after smoking cessation. This fact also indicates that bone marrow or endothelium in heavy smokers is irreversibly damaged, at least in part.

There are several limitations in the present study. Although most EPCs are differentiated from CD45<sup>+</sup>CD34<sup>+</sup>CD133<sup>+</sup> cells, some EPCs may differentiate from other fractions of mononuclear cells, such as CD14<sup>+</sup>-mononuclear cells. Therefore, we could not apply this study’s conclusions to the EPCs derived from other than CD45<sup>+</sup>CD34<sup>+</sup>CD133<sup>+</sup> cell fractions. Smokers generally tend to cheat in this kind of study, and we might have underestimated the change in EPC levels. However, this possibility is less likely because we strictly monitored serum nicotine levels.

In summary, “on-and-off” cigarette smoking markedly influenced the number of circulating PCs/EPCs in apparently healthy chronic smokers. Because the decreased number of EPCs in peripheral circulation is a strong predictor for cardiovascular risk, our new findings provide further evidence for the consensus that smoking cessation is to be highly recommended for the prevention of cardiovascular diseases, and the measurement of PCs/EPCs in peripheral blood is a new tool to monitor smoking-related cardiovascular burden in chronic smokers.

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References

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