Impaired Colony-Forming Capacity of Circulating Endothelial Progenitor Cells in Patients with Emphysema

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Chronic obstructive pulmonary disease (COPD) is classified into emphysema and chronic bronchitis, which are thought to result from different pathophysiological pathways. Smoking-induced lung parenchymal destruction and inadequate repair are involved in the pathogenesis of emphysema. In addition, decreased expression of vascular endothelial growth factor and increased endothelial cell apoptosis in the lung may participate in emphysema pathogenesis. As stem cells, circulating endothelial progenitor cells (EPCs) may play a key role in the maintenance of vascular integrity by replacing and repairing the damaged endothelial cells in the tissues. To determine whether the lack of appropriate repair by circulating EPCs in cases of smoking-induced endothelial cell injury participates in emphysema pathogenesis, we determined the association between the colony-forming or migratory capacity of circulating EPCs and the presence of emphysema in 51 patients with COPD. The patients were divided into emphysema (n = 23) and non-emphysema groups (n = 28) based on high-resolution computed tomography. Twenty-two smokers with normal lung function and 14 normal non-smokers served as controls. Circulating EPCs isolated from patients with emphysema showed significantly lower colony-forming units (CFUs) than those from patients with non-emphysema group, smokers with normal lung function, and normal non-smokers. EPCs from patients with emphysema showed significantly lower migratory capacity than those from normal non-smoking controls (p < 0.05). On multivariate analysis, the EPC-CFU was independently associated with emphysema (OR 0.944, 95% CI = 0.903-0.987, p = 0.011). Thus, impaired functions of circulating EPCs may contribute to the development of emphysema.

Keywords: chronic obstructive pulmonary disease; colony-forming units; emphysema; endothelial progenitor cell; high-resolution computed tomography


Chronic obstructive pulmonary disease (COPD) is a heterogeneous condition, which is caused by a complicated pathological process; various host susceptibilities probably play important roles in its development (Wedzicha 2000). Originally, COPD was classified into two phenotypes: emphysema (parenchymal destruction) and chronic bronchitis (small airway disease) [Calverley and Bellamy 2000; Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2011]. The chronic airflow limitation that is characteristic of COPD is the consequences of a mixture of these two phenotypes, and their relative contributions towards airflow limitation vary depending on individuals [Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2011]. The two phenotypes are thought to result from differences in sensitivity to smoking, and involve independent pathophysiological pathways (Tatsumi et al. 2004), which interact in a complex manner. However, the reason is unknown why some patients predominantly develop airway obstruction and others predominantly develop parenchymal destruction.

Like many other common chronic diseases, the etiology of COPD is complex and involves multiple genetic and environmental factors. It is now generally accepted that smoking-induced chronic inflammation plays a critical role.
in the development of COPD (Wedzicha et al. 2000); however, there is also a considerable overlap between subjects with COPD and healthy smokers in terms of their inflammatory cell numbers and the concentrations of mediator molecules in biopsy specimens, sputum, and bronchoalveolar lavage fluid (Lacoste et al. 1993; Saetta et al. 1993; Di Stefano et al. 1996; O'Shaughnessy et al. 1997). These observations suggest the involvement of factors other than inflammation in the development of COPD.

Smoking-induced destruction of the lung parenchyma and inadequate repair are considered to be important in the pathogenesis of emphysema. Recent research examined the roles played by vascular endothelial growth factor (VEGF) and endothelial cell apoptosis in the development of emphysema. VEGF is an endothelial survival factor, which is essential for the normal development and maintenance of blood vessels (Wagner 2003). VEGF expression decreases in human lungs with emphysema (Kasahara et al. 2001), and VEGF receptor blockade in an animal model caused airspace enlargement by inducing endothelial and epithelial cell apoptosis (Kasahara et al. 2000). These results suggest that endothelial cell survival plays an important role in the maintenance of lung structure. Several types of aberrant lung tissue repair in the presence of destruction have been suggested as possible pathogenetic mechanisms underlying development of COPD (Bonay et al. 2005).

A number of reports propose that bone marrow-derived stem cells contribute to the repair of injured organs, including the lung (Krause et al. 2001; Theise et al. 2002; Epperly et al. 2003; Ortiz et al. 2003; Abe et al. 2004; Hashimoto et al. 2004; Ishizawa et al. 2004; Yamada et al. 2004; Aliotta et al. 2005). Endothelial progenitor cells (EPCs) that mobilize from the bone marrow are thought to play key roles in the maintenance of vascular integrity, and to act as “repair cells” in response to endothelial injury (Rafii et al. 2000). Cigarette smoking induces physiological and biochemical alterations in endothelial cells, including the impaired endothelium-dependent relaxation of pulmonary arteries (Peinado et al. 1998), cytoskeletal disintegration, and the breakdown of cell-cell junctions and necrosis (Vayssier-Taussat et al. 2001; Tithof et al. 2002). In addition, cigarette smoking is associated with reduced numbers of circulating EPCs, together with impaired EPC differentiation and function (Vasa et al. 2001; Hill et al. 2003). These changes hamper blood vessel healing and growth in smokers.

These observations led us to hypothesize that the lack of appropriate repair of smoking-induced endothelial cell injury by a sufficient number of circulating EPCs may play an important role in the pathogenesis of emphysema. Therefore, the present study aimed to identify possible associations between the colony-forming or migratory capacity of circulating EPCs and emphysema.

**Methods**

**Study Subjects**

Subjects were randomly selected from outpatient clinics in CHA Bundang Medical Center, including patients with COPD and smokers with normal lung function who were followed-up in respiratory clinics. In addition, subjects who visited for routine health check-ups were used as normal non-smoking age-matched controls. COPD was defined according to the GOLD guidelines (forced expiratory volume in 1 second/forced vital capacity; FEV₁/FVC < 70 %) and all patients with COPD had a history of smoking more than 20 pack-years and showed irreversible airflow limitation (reversibility < 12% of the predicted forced expiratory volume in 1 second after 200 µg of inhaled salbutamol).

The patients with COPD were placed into the emphysema or non-emphysema group on the basis of high-resolution computed tomography (HRCT) findings. Emphysema was defined as a lung area greater than 15%, which showed attenuation values lower than -950 Hounsfield Units (HU) at full inspiration (Boschetto et al. 2006). Spirometry (Med Graphics, St. Paul, MN), diffusing capacity, and HRCT were performed in all patients with COPD (emphysema and non-emphysema group) and smokers with normal lung function. For the normal non-smoking control subjects, only spirometry and simple chest X-ray were performed. At the time of their enrolment in the study, the patients with COPD were being treated with inhaled anti-cholinergics, long acting β₂-agonist, and theophylline, and some were receiving inhaled corticosteroids. None were receiving oral corticosteroids and statins. All patients had been clinically stable for at least 6 weeks. Subjects were excluded if they had known or symptomatic cardiovascular disease or had any condition such as cancer or retinopathy, in which neovascularization might be present. Subjects with hypertension and diabetes were included and continued their regular medication. Subjects that showed significant abnormal findings on HRCT or chest X-ray, such as bronchiectasis or sequelae of tuberculosis, were also excluded.

The study was approved by the Institutional Review Board of CHA Bundang Medical Center, and informed consents were obtained from all subjects.

**Measurements**

Spirometry was performed according with the ATS guideline (American Thoracic Society 1995) before and 15 minutes after inhalation of 200 µg of salbutamol via a metered-dose inhaler. The diffusing capacity of the lung for carbon monoxide (DL₅₀) was measured using single-breath carbon monoxide methods at least twice. HRCT scanning was performed using a GE Pro Speed Scanner. One mm slices were taken at 10-mm intervals throughout the thorax while the breath was held at full inspiration with the subjects in the supine position. For each lung at each level, the emphysematous area less than -950 HU and the total area were calculated using the software of the scanner. The overall degree of emphysema for each subject was then expressed as a percentage of the total lung area (Boschetto et al. 2006).

The number of cigarettes smoked daily and the duration of smoking (in years) were recorded, and the cumulative cigarette exposure (in pack-years, cigarettes/day×yrs/20) was calculated. Complete blood count and serum glucose, total cholesterol, low-density lipoprotein-, and high-density lipoprotein-cholesterol and triglyceride levels were also checked.
Isolation of EPCs and colony-forming assays

A 20 ml sample of peripheral venous blood was used to isolate the EPCs. Within 2 hours after collection, the samples were processed using Ficoll-Paque Plus (Amersham, Buckinghamshire, UK) and the peripheral blood mononuclear cells were isolated. The recovered cells were washed twice with phosphate-buffered saline and resuspended in EGM-2 MV Singlequot medium (Cambrex, East Rutherford, NJ), which contains several growth factors, including human VEGF-A, human fibroblast growth factor-2, human endothelial growth factor, insulin-like growth factor-1, and ascorbic acid. The cells were plated on six-well plates (Becton Dickinson, Franklin Lakes, NJ) coated with 2% gelatin (Sigma, St. Louis, MO). The initial seeding density was 4 × 10⁶ cells per well. Non-adherent cells were removed and the growth medium was changed every 3 days. The number of colonies was counted 7 days after plating. An endothelial progenitor cell colony-forming units (EPC-CFUs) consisted of a central cluster of rounded cells from which multiple thin, flat cells radiated. A central cluster alone without associated emerging cells was not counted as a colony. The EPC-CFUs were counted manually in a minimum of four wells by two observers who were unaware of the clinical profiles of the subjects.

EPC characterization

Confirmation of endothelial-cell lineage was performed in samples from five subjects (Vasa et al. 2001; Hill et al. 2003). Briefly, indirect immunostaining was performed by using endothelial-specific markers, including VE-cadherin, platelet endothelial cell adhesion molecule (PECAM), CD34, and a novel hematopoietic stem cell marker, AC133, which is also expressed on subsets of EPCs (Rafii et al. 2000). In addition, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated low-density lipoprotein (LDL) (Molecular probes, Eugene, Ore) at 37°C for 3 hours, and then with fluorescein-isothiocyanate-conjugated Ulex europeaus agglutinin (UEA)-1 lectin (10 µg/mL, Sigma, St. Louis, MO) were used to identify EPCs. The samples were examined with an inverted fluorescent microscope (Zeiss Jena, Germany), and only the cells that exhibited double fluorescence were identified as EPCs (Hill et al. 2003). Fluorescein-isothiocyanate and rhodamine-linked secondary monoclonal antibodies were used. To assess reproducibility, we determined the colony counts twice in two separate blood samples obtained at least one week apart from five subjects.

Migration assay

The migratory function of EPCs, which is essential for angiogenesis (Vasa et al. 2001), was examined by using a modified Boyden chamber technique. A 24-well Transwell apparatus (Coster, Corning, NY) was used. Each well contains a 6.5-mm polycarbonate membrane with 8-µm pores, coated with type I collagen (Sigma, St. Louis, MO). Isolated EPCs were detached by using 1 mmol/L EDTA in PBS (pH 7.4), harvested by centrifugation, resuspended in 500 L EBM, and counted, after which 2 × 10⁵ EPCs were placed on the membrane. The chamber was then immersed in a 24-well plate, which was filled with growth factor-free EBM-2 culture media or EBM-2 with 50 ng/mL of human recombinant VEGFₐβ. After incubation for 24 hours, the membrane was washed with PBS and fixed with 2% paraformaldehyde. For quantification, the cell nuclei were stained with DAPI. The cells that had migrated into the lower chamber were counted manually in four random ×100 microscopic fields (Asahara et al. 1999).

Statistical analysis

All data are presented as means and standard error of mean. Continuous variables were evaluated by nonparametric Mann-Whitney test. Categorical variables were evaluated by Fisher exact test. Univariate correlations were made with Spearman correlation coefficient. Independent predictors of the presence of emphysema in univariate analysis were entered in a multiple logistic regression model to estimate adjusted odds ratios associated with the presence of emphysema. The presence of colinearity was checked by Spearman test. SPSS release 11.0 (SPSS Inc) was used, and differences were considered to be statistically significant at 2-tailed P < 0.05.

Results

Subject Characteristics

The study comprised 51 patients with COPD (emphysema group, n = 23; non-emphysema group, n = 28), 22 smokers with normal lung function, and 14 normal non-smoking controls. The characteristics of the study subjects are summarized in Tables 1 and 2. For statistical analysis, smokers with normal lung function and normal non-smoking controls were only included in the comparison of age and the number of EPC-CFUs.

The two COPD groups were compared with regard to the baseline characteristics. In general, patients with COPD were elderly and had moderate to severe airway obstruction. There was no statistical difference in smoking history. In emphysema group, more patients had stopped smoking than those in non-emphysema group. The emphysema group showed lower obstruction parameters in spirometry, such as FEV₁, FEV₁/FVC and FEF₂₅₋₇₅, than the non-emphysema group. They also showed lower DL₅₀ and DL₄₃ values and a higher degree of emphysema on HRCT than the non-emphysema group.

Number of EPC-CFUs

When cultured on gelatin-coated dishes, peripheral blood mononuclear cells formed distinct colonies comprising a central cluster of round cells and peripheral radiating flat cells. These cells could be shown to uptake acetylated LDL and bind UEA-1 lectin (Fig. 1), and expressed endothelial and hematopoietic stem cell characteristics, including CD34, AC133, PECAM and VE-cadherin (Fig. 2). Patients with emphysema group had significantly fewer EPC-CFUs than patients with non-emphysema group, the smokers with normal lung function, and the normal non-smoking controls (Fig. 3). When all subjects were divided according to smoking status (smokers, n = 73; non-smokers, n = 14), smokers were found to have significantly fewer EPC-CFUs than non-smokers (23.9 ± 2.9 vs 32.2 ± 2.9, P = 0.034, Fig. 4). However, current smokers (n = 32) did not differ significantly in terms of the number of EPC-CFUs compared with ex-smokers (n = 41) (26.9 ± 3.6 vs. 21.5 ± 3.1, P = 0.264).

The relationship between the EPC-CFU number and the degree of emphysema for all patients with COPD and smokers with normal lung function was then assessed. As
shown in Fig. 5, there was a significant inverse correlation between the number of EPC-CFUs and the degree of emphysema on HRCT ($r = -0.351$, $P = 0.004$). In addition, the number of EPC-CFUs did not correlate with age ($r = -0.152$, $P = 0.230$), FEV$_1$, % predicted ($r = 0.079$, $P = 0.534$), or pack-years of smoking history ($r = -0.155$, $P = 0.220$). Finally, multivariate regression analysis revealed that the number of EPC-CFUs, and FEV$_1$ were independently associated with the presence of emphysema (Table 3).

**EPC migration assay**

The migratory function of EPCs in response to VEGF was evaluated in 12 randomly assigned COPD patients (six each from the emphysema and non-emphysema groups), six smokers with normal lung function, and six normal non-smoking controls using a modified Boyden chamber. These subjects were selected randomly on the basis of a random number table. The baseline characteristics of these subgroups were similar to those of the original groups. Patients with emphysema showed a lower baseline EPC migratory function than normal non-smoking controls, whereas patients with non-emphysema group and smokers with normal lung function did not differ significantly from the non-smoking controls. After supplementation with 50 ng/mL of VEGF, all four groups showed significant augmentation of EPC migratory function: for the emphysema group, $13.4 \pm 2.0$ EPCs migrated at baseline per $\times 100$ high-power field compared with $19.7 \pm 2.4$ after the addition of VEGF ($P = 0.016$). For the non-emphysema group, these values were $21.2 \pm 3.0$ and $30.0 \pm 3.6$, respectively ($P = 0.001$), while for the smokers with normal lung function, they were $21.1$
4.2 and 31.6 ± 4.6, respectively ($P = 0.049$). For the normal non-smoking control group, the baseline value was 27.1 ± 4.2 and the value after adding VEGF was 45.7 ± 4.9 ($P = 0.005$). However, compared with normal non-smoking controls, fewer EPCs from patients with emphysema and non-emphysema group migrated after VEGF was added (Fig. 6).

**Discussion**

A major finding of this study was that patients with emphysema had significantly fewer EPC-CFUs than patients with non-emphysema group, smokers with normal lung function, and normal non-smoking controls. Moreover, the EPCs from the patients with emphysema showed impaired baseline migration compared with those from normal non-smoking controls. In addition, analysis of all patients with COPD and smokers with normal lung function revealed that the degree of emphysema on HRCT correlated negatively with the number of EPC-CFUs. These findings suggest that impairment of the colony-forming and migratory capacity of circulating EPCs may contribute to the development of emphysema.

Supporting this are several recent studies suggesting that bone marrow-derived stem cells may play an important role in the regeneration of injured lungs (Krause et al. 2001; Theise et al. 2002; Epperly et al. 2003; Ortiz et al. 2003; Abe et al. 2004; Ishizawa et al. 2004; Aliotta et al. 2005). For example, after whole bone marrow or CD34‘Lin’ cells are transplanted into lethally-irradiated female mice, they differentiate into bronchiolar epithelia and type 2 pneumocytes (Theise et al. 2002). A similar radiation pneumonitis model revealed that, 2 months after bone marrow transplantation, 1-20% of the type 2 pneumocytes were donor-derived cells (Krause et al. 2001).

Ishizawa et al. (2004) reported that in elastase-induced emphysema, bone marrow-derived cells contribute to lung regeneration. These cells were identified at the regenerating alveoli, and expressed epithelial or endothelial markers. Bone marrow-derived circulating EPCs are also regarded as “repair cells”, which arise in response to endothelial injury. Indeed, several studies suggest that EPCs participate in lung repair after pneumonia and acute lung injury (Burnham et al. 2005; Yamada et al. 2005; Kähler et al. 2007; Lam et al. 2008). One of these showed the number of EPCs at the acute phase in patients with pneumonia was significantly higher than those at the recovery phase, and that patients with low EPC counts tended to show persistent fibrotic changes in the lungs after recovery from pneumonia (Yamada et al. 2005). Another study revealed that patients with acute lung injury that showed higher EPC colony counts had a better survival rate. This provided the rationale for a novel therapeutic approach in which EPCs are delivered to the injured lung. Supporting this are recent animal experiments showing that transplanted BM-derived EPCs integrate into injured lung tissue and attenuate lung injury (Kähler et al. 2007; Lam et al. 2008).

Decreases in circulating EPCs contribute to impaired angiogenesis and are ultimately associated with a high risk of cardiovascular disease (Vasa et al. 2001; Hill et al. 2003). Patients with risk factors for ischemic cardiovascular disease have fewer circulating EPCs and impaired migratory capacity, moreover, EPC number and migratory capacity are negatively correlated with Framingham’s cardiovascular risk factor score (Vasa et al. 2001; Hill et al. 2003). Smoking is the major risk factor contributing to decreased numbers of circulating EPCs (Vasa et al. 2001). Given that that cardiovascular disease is the leading cause of hospitalization and mortality among patients with obstructive air-
Fig. 2. Morphology and characterization of EPCs. EPC colonies expressed the endothelial and hematopoietic stem cell markers CD34, AC133, PECAM and VE-cadherin, which are considered to be critical EPC markers.
Fig. 3. The number of EPC-CFUs in the peripheral blood from study subjects. The patients with emphysema (n = 23) had significantly fewer EPC-CFUs after 1 week of culture than patients with non-emphysema group (n = 28), smokers with normal lung function (n = 22), or normal non-smoking controls (n = 14). *P < 0.05.

Fig. 4. The number of EPC-CFUs in the peripheral blood of smokers and non-smokers. Smokers (n = 73) had significantly fewer EPC-CFUs after 1 week of culture than non-smokers (n = 14). *P < 0.05.

Fig. 5. Correlation between the number of EPC-CFUs and the degree of emphysema on HRCT in study subjects. HRCT was performed for all patients with COPD (emphysema group, n = 23 and non-emphysema group, n = 28) and smokers with normal lung function (n = 22). The number of EPC-CFUs correlated inversely with the degree of emphysema on the HRCT scan (r = −0.351, P = 0.004).

Table 3. Multiple logistic regression analysis of factors that correlate with the presence of emphysema in patients with COPD.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Odds ratio</th>
<th>95% CI (upper, lower)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.020</td>
<td>0.934, 1.113</td>
<td>0.662</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>0.944</td>
<td>0.930, 0.989</td>
<td>0.008</td>
</tr>
<tr>
<td>BMI</td>
<td>0.854</td>
<td>0.679, 1.074</td>
<td>0.178</td>
</tr>
<tr>
<td>EPC-CFUs</td>
<td>0.944</td>
<td>0.903, 0.987</td>
<td>0.011</td>
</tr>
</tbody>
</table>

COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 sec; BMI, body mass index; EPC-CFUs, endothelial progenitor cell colony-forming units.
Fig. 6. Migration of EPCs isolated from study subjects. A modified Boyden chamber assay was used with growth factor-free medium (A, C) or VEGF-supplemented medium (B, D). Representative photos show baseline and VEGF-stimulated migration of EPCs from a patient with emphysema (A, B, respectively) and a normal non-smoking control (C, D, respectively). (E) The migration of EPCs before and after VEGF was added was evaluated in 12 randomly assigned COPD patients (six each from the emphysema and non-emphysema groups), six smokers with normal lung function, and six normal non-smoking controls. After VEGF stimulation, the EPCs from all four groups showed significantly augmented migratory function compared with baseline. EPCs from patients with emphysema showed significantly lower levels of baseline migration than those from normal non-smoking controls. EPCs from patients with emphysema and non-emphysema group both showed significantly lower migration after VEGF stimulation than those from normal non-smoking controls. $P < 0.05$; *, baseline vs. VEGF stimulation; †, emphysema group vs. normal non-smoking control group at baseline; ††, normal non-smoking control group vs. the emphysema and non-emphysema groups after VEGF stimulation.
way disease (Anthonisen et al. 2002), and that patients with COPD have a two to three times higher risk of cardiovascular mortality (Camilli et al. 1991; Jousilaiti et al. 1996), low circulating EPC numbers may be another explanation for the apparent link between COPD and cardiovascular events.

Unlike patients with emphysema, patients of non-emphysema group showed similar numbers of EPC-CFUs as normal non-smoking controls. This result is in line with the study by Palange et al., who found that, although patients with COPD generally had fewer circulating CD34+ cells, EPCs, and EPC-CFUs, a subgroup of 22% had normal numbers of circulating progenitor cells (Palange et al. 2006). Palange et al. also reported that these patients had a higher exercise capacity and lower tumor necrosis factor-α levels. However, they did not mention whether these patients were non-emphysema COPD patients, and they did not report the DLco or HRCT findings in these patients.

The innate immune response to the toxic particles and gases in cigarette smoking plays an important role in the pathogenesis of chronic bronchitis (Hogg 2004). Granulocyte monocyte colony-stimulating factor (GM-CSF) is important in such innate immune responses because it induces the production and release of leukocytes. It is also an important degranulation factor that enhances tissue damage induced by granulocytes. In addition, it is a potent bone marrow cell-mobilizing factor (Stanley et al. 1997). Cigarette smoke also directly stimulates granulocyte production in the bone marrow, which may be mediated by GM-CSF released by macrophages (Hogg et al. 1994; Stănescu et al. 1996). Therefore, since chronic bronchitis is associated with strong innate immune responses, one can speculate that patients with chronic bronchitis will have high numbers of circulating EPCs. In this context, it is notable that patients with chronic bronchitis frequently exhibit bacterial colonization, even when their disease is stable; this continues the inflammation, which could be the source of bone marrow stimulation (Sethi et al. 2006).

Peinado et al. (2006) reported that vascular progenitor cells accumulate in injured pulmonary arteries in patients with COPD, and that the number of AC133+ vascular progenitor cells that attach to the endothelium is positively correlated with pulmonary arterial wall thickness. This suggests that circulating EPCs may participate in the development of pulmonary hypertension in patients with COPD. Since patients with non-emphysema COPD had more circulating EPCs than patients with emphysema COPD, this may explain why pulmonary hypertension occurs more frequently and develops earlier in patients with non-emphysema COPD than in patients with emphysema COPD.

This study has some limitations. First, only EPC-CFUs were observed. This is not always representative of what is happening in the bone marrow. Decreased numbers of circulating EPCs could be the result of an imbalance between bone marrow production and tissue utilization, which means that low EPC numbers could be the result of active utilization of EPCs for tissue repair, or the failure of the bone marrow to produce EPCs. Second, only one subpopulation of circulating stem cells was examined. Considering that other stem cells, including hematopoietic stem cells and mesenchymal stem cells, can also participate in lung repair, it is likely that observing other stem cell populations would provide important information. However, stem cells have the ability to cross lineage barriers and adopt the expression profiles and functional phenotypes of cells that are unique to other tissues (Prockop et al. 2003). For example, bone marrow-derived circulating EPCs can acquire the morphology and phenotype of endothelial cells or smooth muscle cells when co-cultured with these cells (Diez et al. 2007). This suggests that circulating EPC numbers may be a good marker of whether there are sufficient numbers of repair cells in the presence of injury. Supporting this is a study by Palange et al. (2006), showing that patients with COPD and low numbers of circulating EPCs also have decreased levels of circulating CD34+ cells, which are the representative hematopoietic stem cells. Third, we measured the number of EPC-CFUs using a colony-forming unit assay, not by FACS analysis, which is more widely used. In most studies, EPCs are identified by flow cytometric identification of cells expressing CD34, CD133, or VEGF receptor 2 (KDR) (Peichev et al. 2000; Reyes et al. 2002). However, because these molecules are also expressed by hematopoietic stem/progenitor populations (Verfaillie 2002; Shizuru et al. 2005; Adams and Scadden 2006; Bryder et al. 2006), it is likely that EPC preparations may be contaminated by hematopoietic cells. By contrast, endothelial colony-forming cells from human peripheral blood, which were harvested by methods similar to our own, displayed robust proliferative potential and formed perfused vessels in vivo (Yoder et al. 2007). This indicates that these cells could be a potentially useful primary therapy for vascular regeneration.

Lastly, while it would have been interesting to measure the serum levels of growth factors or angiogenesis-related markers, these measurements were not made (and now no serum remains). However, it should be noted that there are many discrepancies in the relationship between vascular growth factors and EPC numbers. In patients with rheumatoid arthritis, VEGF and basic fibroblast growth factor levels do not correlate with EPC numbers (Grisar et al. 2005). By contrast, Sala et al. (2010) reported a positive relationship between plasma VEGF levels and the percentage of circulating EPCs ($r = 0.51$, $p = 0.003$) during COPD exacerbations. However, this correlation was not seen in stable COPD patients ($r = 0.20$, $p = 0.22$) (Sala et al. 2010).

The final drawback of the present study was that patients with COPD were classified into emphysema, non-emphysema groups on the basis of HRCT findings and symptoms. This classification is somewhat arbitrary. Other studies that classified the COPD phenotype according to HRCT findings found that the cut-off level of the low atten-
cation area used to separate non-emphysema from emphysema is quite arbitrary, as it ranges from 0% to 25% (Kasahara et al. 2000; Tatsumi et al. 2004). Since emphysema can be identified in smokers without airflow limitation on HRCT (Marsh et al. 2007), the non-emphysema group in the present study included patients with less than 15% of attenuation area lower than -950 HU at full inspiration. In addition, if a patient showed airflow limitation without evidence of emphysema on HRCT, the patient was categorized into the non-emphysema group, regardless of whether symptoms of chronic bronchitis were present. In the present study, three patients without symptoms of chronic bronchitis were included in the non-emphysema group because, by definition, they could not be included in the emphysema group.

In conclusion, patients with emphysema had fewer EPC-CFUs than patients with non-emphysema COPD. In addition, the degree of emphysema on HRCT correlated negatively with the number of EPC-CFUs. These results suggest that emphysema can arise, at least in part, because repair of smoking-induced alveolar injury is impaired due to a lack of circulating ‘repair’ cells. However, it is not clear whether the impaired colony-forming and migratory capacity shown by circulating EPCs is actually the cause of emphysema, or just a by-product of the disease process. Given that this report identified associations, rather than provided solid evidence of, a cause-effect relationship, this conclusion is at best tentative. Further studies will be needed to determine the role of circulating EPCs in the development of emphysema.

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Conflict of Interest
All authors declare no conflict of interest.

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