Endothelial progenitor cells in patients with chronic obstructive pulmonary disease

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1British Heart Foundation/University Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom; 2Scottish Centre for Regenerative Medicine, University of Edinburgh, Edinburgh, UK; 3Edinburgh Lung and the Environment Group Initiative/Colt Laboratories, Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom

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Brittan M, Hoogenboom MM, Padfield GJ, Tura O, Fujisawa T, MacLay JD, MacNee W, Mills NL. Endothelial progenitor cells in patients with chronic obstructive pulmonary disease. Am J Physiol Lung Cell Mol Physiol 305: L964–L969, 2013. First published October 18, 2013; doi:10.1152/ajplung.00183.2013.—The pathogenesis of chronic obstructive pulmonary disease is not fully understood. The objective of this study was to compare circulating endothelial progenitor cells in patients with chronic obstructive pulmonary disease to age, sex, and cigarette smoking matched healthy controls. Patients with chronic obstructive pulmonary disease (n = 37) and healthy controls (n = 19) were matched by age, sex, and smoking status. Circulating hematopoietic progenitor cells (CD34+ or CD133+ mononuclear cells) and endothelial progenitor cells (CD34+KDR+ or CD34+CD133+KDR+ mononuclear cells) were quantified by flow cytometry. Endothelial cell-colony forming units from peripheral blood mononuclear cells were quantified in vitro and phenotypic analysis carried out using immunocytochemistry. Patients with chronic obstructive pulmonary disease had more circulating mononuclear cells compared with controls (8.4 ± 0.6 vs. 5.9 ± 0.4 × 10^6 cells/l; P = 0.02). CD34+ hematopoietic progenitor cells were reduced as a proportion of mononuclear cells in patients compared with controls (0.99 ± 0.12 vs. 1.9 ± 0.12%; P = 0.02); however, there were no differences in the absolute number of CD34+, CD34+KDR+, or CD34+CD133+KDR+ cells (P > 0.05 for all). Endothelial cell-colony forming units were increased in patients with chronic obstructive pulmonary disease compared with controls (13.7 ± 5.2 vs. 2.7 ± 0.9 colonies; P = 0.048). In contrast to previous studies, the number of circulating progenitor cells was not reduced in patients with chronic obstructive pulmonary disease compared with carefully matched controls. It seems unlikely that circulating endothelial progenitor cells or failure of angiogenesis plays a central role in the development of emphysema.

circulating endothelial progenitor cells; COPD; endothelial cell-colony forming unit; hematopoietic stem cells

THE PATHOGENESIS OF CHRONIC obstructive pulmonary disease (COPD) is not fully understood. Airway obstruction in COPD results from progressive inflammation and fibrosis of the airways in response to cigarette smoke and air pollution, which in turn leads to loss of elastic recoil mediated by elastolysis, apoptosis, and emphysema. Change in the lung parenchyma (11). Vascular change and angiogenesis is an integral part of the pulmonary inflammatory response (7). Cigarette smoke not only causes inflammation and direct injury to lung tissue, but also inhibits compensatory angiogenesis and thus impairs repair and adaptive mechanisms in a smoker’s lung (16). Furthermore, cigarette smoking is associated with reduced numbers of circulating endothelial progenitor cells (EPC) (6), and EPC are known to increase rapidly and recover their function following smoking cessation (9).

Asahara and colleagues (1) identified a circulating population of EPC with the capacity to form mature endothelial cells and contribute to postnatal angiogenesis, raising the possibility that EPC could be harnessed for vascular regeneration. EPC are commonly identified in circulating peripheral blood using flow cytometry to analyze surface antigen expression. However, a lack of consensus as to which surface markers most accurately identify EPC, and the overlapping expression of markers by EPC and other hematopoietic progenitor cells (HPC), has led to uncertainty regarding the true phenotype of EPC (reviewed in Ref. 15). The endothelial cell-colony forming unit (EC-CFU) assay was once thought to be a measure of EPC function. However, it is now recognized that hematopoietic progenitors, monocytes, and macrophages may contribute to EC-CFU (reviewed in Ref. 18). Although not a measure of EPC, EC-CFU closely correlate with vascular function, are mobilized in response to cardiovascular stress, and are diminished in chronic cardiovascular disease. Although not fully understood, EC-CFU retain utility as a measure of vascular health.

An understanding of the relevance of vascular homeostasis, in particular EPC, to the pathophysiology of COPD is important, given their potential to contribute to endothelial repair and combat vascular remodeling in the emphysematous lung. Emerging evidence from a small number of studies implies that EPC may be reduced in number and in their ability to form outgrowth colonies in COPD (2). However, a failure to match for cigarette smoking in patients with COPD and controls prevents definitive conclusion as to whether COPD per se is associated with a reduction in circulating EPC, or whether these findings are due to the established effects of cigarette smoking. Moreover, the use of CD34 or CD133 expression alone is not sufficient to distinguish EPC from other circulating progenitors (10, 14), and a more comprehensive approach to defining EPC is necessary.

We hypothesized that circulating EPC concentrations are reduced in patients with COPD, independent of their smoking history, and therefore a failure of postnatal angiogenesis contributes to the development of COPD pathophysiology. We compared numbers of both phenotypic and functional subpopu-
lutions of EPC in COPD patients with an age, sex, and cigarette smoking matched healthy control group.

METHODS

Subjects. All studies were performed with local research ethics committee approval (South East Scotland Research Ethics Committee), with written, informed consent of all subjects and in accordance with the Declaration of Helsinki. Patients with COPD (n = 37) were recruited from general practice databases and a specialized outpatient clinic at the Royal Infirmary of Edinburgh, Scotland. COPD was defined using the American Thoracic Society guidelines based upon the combination of typical symptoms and postbronchodilator forced expiratory volume in 1 s (FEV1) <80% predicted, FEV1-to-forced vital capacity ratio <70% and β2-agonist reversibility <15% or 200 mL. Healthy controls (n = 19) were recruited through local medical practice and matched to COPD patients by age, sex, and smoking status. Exclusion criteria were recent exacerbation or hospital admission (within 6 wk), rheumatoid arthritis, inflammatory bowel disease, neoplasia, tuberculosis, or other inflammatory respiratory disorders. Patients with a history of CVD, hypertension, or other chronic respiratory conditions were excluded. COPD patients were matched to COPD patients by age, sex, and smoking status. Exclusion criteria were recent exacerbation or hospital admission (within 6 wk), rheumatoid arthritis, inflammatory bowel disease, neoplasia, tuberculosis, or other inflammatory respiratory disorders. Patients with a history of CVD, hypertension, or other chronic respiratory conditions were excluded.

Blood sampling. Blood samples were drawn from a vein in the anticubital fossa into ethylenediaminetetraacetic acid and processed within 2 h. Differential white blood cell count and platelets were determined using an auto-analyzer in the regional Clinical Haematology Laboratory, Royal Infirmary of Edinburgh.

Flow cytometry. Whole peripheral blood was stained for 30 min at room temperature with FITC-conjugated anti-human CD34 (Becton Dickinson), phycoerythrin-conjugated anti-human CD133 (Miltenyi Biotec), and allophycocyanin-conjugated anti-human kinase insert domain receptor (KDR) (R&D) antibodies. An unstained cell sample, single antibody stains, isotype controls, and fluorescence minus one controls were prepared for each subject. Erythrocytes were lysed (Ur-Lyse, Dako), and samples centrifuged at 1,200 rpm for 5 min at 4°C, washed with phosphate-buffered saline (PBS), and fixed (Cell Fix solution, Becton Dickinson) before analysis. For each sample, 400,000 mononuclear cells (MNC) were collected using a FACS-Calibur analyzer (Becton Dickinson) and data analyses were performed using FlowJo (TreeStar). HPC were defined as CD34+ or CD133+ cells, and EPC as CD34+KDR+ or CD34+CD133+KDR+ cells. Morphological gating was used to positively select MNC and to exclude granulocytes and debris, and antigen expression was assessed to define HPC and EPC populations (Fig. 1).

EC-CFU assay. EC-CFU were quantified as described previously (6). Briefly, peripheral blood MNC (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque, resuspended in EndoCult liquid medium (Stem Cell Technologies, Vancouver, BC, Canada), and seeded onto fibronectin-coated dishes. After 2 days of culture at 37°C, nonadherent cells were collected and replated onto fibronectin-coated 24-well dishes. EC-CFU, defined as a central core of “round” cells with elongated cells at the periphery (6), were counted on day 5 in a minimum of four wells by observers unaware of the subjects’ clinical profiles.

Immunocytochemistry. Immunocytochemical staining of cells within EC-CFU was performed using antibodies to endothelial cell surface antigens CD31, von Willebrand factor, Tie-2, and endothelial nitric oxide synthase, and the pan-leukocyte and hematopoietic cell antigens CD45 and CD68, respectively, as described previously (13). Briefly, cells were fixed with methanol and permeabilized using blocking buffer with added Igepal (Sigma-Aldrich). Colonies were incubated with primary antibodies for 1 h at room temperature and washed with PBS (Sigma-Aldrich). Cells were then incubated with relevant anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 or 647 dyes (Molecular Probes, Invitrogen) for 1 h. Following final washes in PBS and water, nuclei were counterstained with 4′,6-diamidino-2-phenylindole, and colonies were mounted with Permafluor (Thermo Scientific).

Table 1. Characteristics of COPD patients and controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>COPD</th>
<th>Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>37</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>68±7</td>
<td>69±7</td>
<td>0.5</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>19 (73)</td>
<td>10 (63)</td>
<td>0.5</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>4 (15)</td>
<td>2 (13)</td>
<td>0.88</td>
</tr>
<tr>
<td>Ex-smoker, n (%)</td>
<td>22 (85)</td>
<td>10 (63)</td>
<td>0.22</td>
</tr>
<tr>
<td>Nonsmoker, n (%)</td>
<td>0 (0)</td>
<td>4 (25)</td>
<td>0.12</td>
</tr>
<tr>
<td>Cigarettes, pack-yr</td>
<td>48±5</td>
<td>25±6</td>
<td>0.01</td>
</tr>
<tr>
<td>Height, cm</td>
<td>170±1</td>
<td>171±2</td>
<td>0.82</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76±3</td>
<td>77±4</td>
<td>0.85</td>
</tr>
<tr>
<td>BML, kg/m²</td>
<td>26±1</td>
<td>26±1</td>
<td>1.00</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>10 (38)</td>
<td>7 (44)</td>
<td>0.74</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>1 (4)</td>
<td>3 (19)</td>
<td>0.12</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>7 (27)</td>
<td>7 (44)</td>
<td>0.74</td>
</tr>
<tr>
<td>Family history of CVD, n (%)</td>
<td>10 (38)</td>
<td>6 (38)</td>
<td>1.00</td>
</tr>
<tr>
<td>FEV1, liters</td>
<td>2.50±0.13</td>
<td>2.65±0.15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>45±4</td>
<td>98±4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Oxygen saturation, %</td>
<td>95±1</td>
<td>97±2</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Values are number of subjects (percentage) or means±SE. COPD, chronic obstructive pulmonary disease; BML, body mass index; CVD, cardiovascular disease; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity. COPD patients vs. controls were compared using Student’s t-test.
Table 2. Circulating leukocytes in patients with COPD and controls

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin, g/l</td>
<td>141 ± 4</td>
<td>126 ± 4</td>
<td>0.06</td>
</tr>
<tr>
<td>Leukocytes, ×10⁹/l</td>
<td>8.4 ± 0.6</td>
<td>5.9 ± 0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Neutrophils, ×10⁹/l</td>
<td>5.4 ± 0.5</td>
<td>3.6 ± 0.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Lymphocytes, ×10⁹/l</td>
<td>1.8 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>0.60</td>
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<tr>
<td>Monocytes, ×10⁹/l</td>
<td>0.75 ± 0.05</td>
<td>0.51 ± 0.04</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. COPD patients vs. controls were compared using Student’s t-test.

Data analysis and statistics. Continuous variables are reported as means ± SE. Statistical analyses were performed with GraphPad Prism (GraphPad Software) using two-way ANOVA or unpaired t-test. Unpaired t-tests with Welch’s correction for unequal variances were used where data was not normally distributed. The χ² test was used for dichotomous variables. Statistical correlations between clinical data and cell counts were examined by univariate analysis using the linear regression. Statistical significance was assumed if a null hypothesis could be rejected at P ≤ 0.05.

RESULTS

Subjects. Subjects were matched for age, sex, and current smoking history. No significant differences in height, weight, hypertension, diabetes mellitus, hyperlipidemia, or family history of cardiovascular disease were reported (Table 1) (1). While there was no difference in smoking status between patients and controls, COPD patients had a greater number of pack-years. COPD patients had lower FEV₁ and oxygen saturations and increased quantities of CD34⁺ HPC compared with controls (5.2 vs. 2.7 × 10⁹ cells/l; P < 0.05), with increased numbers of peripheral blood monocytes and neutrophils (Table 2).

Circulating HPC and EPC in peripheral blood. Relative quantities of CD34⁺ MNC were reduced in patients with COPD compared with controls (P = 0.02; Table 3); however, absolute concentrations were similar in both patients and controls (P = 0.53). Both relative and absolute concentrations of CD133⁺ HPC, CD34⁺KDR⁺, and CD34⁺CD133⁺KDR⁺ EPC were similar between patients and controls (P > 0.05 for all).

EC-CFU. EC-CFU in patients with COPD (n = 24) were increased compared with controls (n = 16) (3.7 ± 5.2 vs. 2.7 ± 0.9 colonies/100,000 MNC plated; P = 0.048; Fig. 2). A panel of markers was used to investigate the phenotype of cells in EC-CFU using immunocytochemistry (Fig. 3). Staining was similar between patient and control EC-CFU with weakly positive expression of mature endothelial cell markers CD31, von Willebrand factor, Tie-2, and endothelial nitric oxide synthase. The pan-leukocyte marker CD45 and macrophage marker CD68 were strongly expressed in all colonies.

DISCUSSION

EPC are promising biomarkers of vascular health, with therapeutic potential that may be applied in a range of clinical conditions. We hypothesized that failure of postnatal angiogenesis might contribute to the development of emphysema, and that EPC may, therefore, be a future therapeutic target in this condition.

We have quantified multiple circulating progenitor populations in patients with COPD using multiparameter flow cytometric analyses of cell surface antigen phenotype, in addition to quantification of EC-CFU potential, a measure of vascular health. In contrast to previous studies, the number of circulating progenitor cells was similar in patients with COPD compared with carefully matched controls. However, EC-CFU were increased in patients with COPD by fourfold, compared with controls matched for smoking history. Our findings suggest that it is unlikely that circulating EPC defined by CD34⁺KDR⁺ expression play a central role in the development of emphysema. Increased EC-CFU potential in patients with COPD likely reflects increased systemic inflammation.

Consistent with previous studies, we identified an apparent decrease in circulating CD34⁺ cells when expressed as a proportion of all MNC in COPD patients compared with healthy controls (3, 5, 10, 14). However, this disparity is explained by the relative leukocytosis that was present in patients compared with controls. Patients in the present study had total MNC
counts that were approximately twofold higher than in controls. This is consistent with the upregulation of systemic inflammatory pathways known to be important in clinically stable COPD. This observation emphasizes the need to consider both absolute and relative cell counts, particularly in rare event analysis. We found no difference in the absolute number of circulating CD34+ cells between patients and controls. CD34+ and CD133+ cells are HPC, and EPC are more accurately identified by their coexpression of surface antigens common both to stem cells (CD34 and CD133) and vascular cells (KDR/VEGF receptor 2). We did not observe any significant difference in CD133+ HPC or CD34+KDR+ and CD34+CD133+KDR+ EPC in patients with COPD or matched controls, whether these populations were compared using relative or absolute cell counts.

Previous studies have indicated that EPC may be reduced in number and function in COPD. However, it has not always been possible to attribute changes in EPC number to COPD per se, as studies did not reliably match for smoking history, age, and sex, all of which have been shown to significantly influence EPC number and function (9, 12, 17). Unlike previous studies in this field, we have specifically matched groups for age, sex, and smoking history (10, 14). Moreover, our patient sample size is larger than in all previous reports of EPC number in COPD (Table 4). It is likely that the discrepancy between our findings and previous reports is explained by differences in the prevalence of cigarette smoking between patients and controls. Furthermore, we have quantified both relative and absolute numbers of a wide range of hematopoietic and EPC populations and found no difference between patients with COPD and controls. We, therefore, believe the role of circulating EPC in the pathogenesis of COPD has previously been overstated.

In contrast, the number of EC-CFU isolated from PBMC following cell culture was increased in patients with COPD compared with controls. The EC-CFU assay was originally employed to overcome the difficulties associated with accurately quantifying small numbers of circulating cells by flow cytometry. However, while providing a measure of vascular health, it is now known that these colonies do not arise directly from circulating EPC and in fact are the progeny of myeloid cells that indirectly contribute to tissue angiogenesis via paracrine mechanisms (4, 8). EC-CFU are known to express a variety of endothelial characteristics, but are predominantly composed of activated leukocytes of myeloid and lymphocytic lineage. In addition to the marked leukocytosis, peripheral blood monocyte counts were 50% higher in patients with COPD compared with controls. The increased potential to form EC-CFU observed in our patients is probably a result of systemic inflammation and enhanced leukocyte activation in these patients and is consistent with the suggestion that these colonies arise from a subset of angiogenic monocytes (19). As EC-CFU mobilization occurs in response to vascular perturbation, we speculate that the increase in EC-CFU may reflect increased vascular inflammation and contribute to the devel-

Fig. 3. EC-CFU phenotypic characterization by immunocytochemistry. Monoclonal antibodies were used to characterize colony outgrowth from EC-CFU. Secondary antibodies directly conjugated to Alexa Fluor 488 or 647 dyes (green or red, respectively) were used, and DAPI was used as a nuclear dye (blue). Cells showed a positive yet weak expression of endothelial markers CD31 (A), von Willebrand factor (vWF; B), Tie-2 (C), and endothelial nitric oxide synthase (eNOS; D), and more strongly expressed the macrophage marker CD68 (E) and pan-leukocyte marker CD45 (F).
ompement of atherosclerosis in COPD; however, further studies are required to explore this hypothesis.

Differences in study methodology and the criteria used to define circulating EPC may explain discrepancies in findings between our study and previous publications (Table 4). For example, several previous studies have reported reduced levels of circulating EPC in patients with COPD (3, 10, 14). However, these studies refer to numbers of CD34+ or CD133+ cells, which, as discussed, do not specifically identify EPC. In addition, the healthy control subjects included in these studies were not matched for smoking status, which can significantly affect circulating EPC number. Furthermore, each of these studies utilizes a different method of quantifying the cell populations of interest: flow cytometry of whole blood, flow cytometry of PBMC isolated using gradient centrifugation, and magnetic cell sorting of cells in PBMC. It is possible that processing blood using different protocols will affect numbers and/or phenotype of cells, and it is, therefore, difficult to control for these different methodologies. Importantly, our observation that EPC are not reduced in patients with COPD compared with a healthy control population carefully matched for smoking status is actually in agreement with previous findings of Fadini and colleagues (5), who reported no significant difference in CD34+CD133+KDR+ EPC in their patient cohort when smokers were excluded from the analysis.

Our study has a number of limitations that merit discussion. We infer that an impairment of angiogenesis is not central to the pathogenesis of emphysema, as putative CD34+ populations of EPC are not reduced in the circulation of patients with COPD. However, the relative contribution of circulating EPC to angiogenesis in the lung is unknown, and failure of local angiogenic mechanisms in the pulmonary vascular bed may be more important.

**Conclusions.** In contrast to previous studies, we report that the number of circulating progenitor cells is not reduced in patients with COPD compared with controls matched for age, sex, and cigarette smoking status. Instead, we observed that EC-CFU formation is upregulated in COPD, possibly as a consequence of systemic inflammation and hypoxemia. Our observations suggest it is unlikely that circulating EPC or failure of angiogenesis plays a central role in the development of emphysema.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: M.B., G.J.P., T.F., and J.D.M. analyzed data; M.B., M.M.H., O.T., J.D.M., W.M., and N.L.M. interpreted results of experiments; M.B. prepared figures; M.B. drafted manuscript; M.B., M.M.H., G.J.P., O.T., T.F., J.D.M., W.M., and N.L.M. edited and revised manuscript; M.B., G.J.P., J.D.M., W.M., and N.L.M. approved final version of manuscript; M.M.H.,...
G.J.P., O.T., T.F., and J.D.M. performed experiments; G.J.P., W.M., and N.L.M. conception and design of research.

REFERENCES