Circulating Endothelial Cells
A New Candidate Biomarker of Irreversible Pulmonary Hypertension Secondary to Congenital Heart Disease

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Background—Congenital heart disease can be complicated by pulmonary arterial hypertension (PAH), the reversibility of which is often difficult to predict. We recently reported a lung biopsy study showing impaired apoptotic regulation of endothelial cells in irreversible PAH. The objective of the present study was to identify noninvasive biomarkers of endothelial turnover that could be used to identify congenital heart disease patients at risk of irreversible PAH.

Methods and Results—Circulating endothelial cells (CECs) isolated with CD146-coated beads and circulating CD34+/CD133+/H11001 progenitor cells (CPCs) were quantified in peripheral vein, pulmonary artery, and pulmonary vein blood samples from 26 patients with congenital heart disease (16 with reversible PAH [median age 2 years] and 10 with irreversible PAH [median age 9 years]) and 5 control patients. Surgical lung biopsy was performed in 19 cases. As expected, endothelial remodeling was observed in irreversible PAH but not in reversible PAH. CEC numbers were each similar in the 3 types of blood samples. CEC numbers were significantly higher in patients with irreversible PAH (median 57 CEC/mL) than in patients with reversible PAH and control subjects (median 3 CEC/mL in the 2 groups). In contrast, CPC numbers did not differ among patients with irreversible or reversible PAH and control subjects (median 84, 64, and 44 CPC/10^5 lymphocytes, respectively, in the 3 groups).

Conclusions—Irreversible PAH in congenital heart disease is associated with endothelial damage and with increased circulating endothelial cell counts. The present study suggests that CECs could be a valuable tool to define therapeutic strategies in congenital heart disease patients with PAH.

Key Words: hypertension, pulmonary ▪ pediatrics ▪ endothelium ▪ endothelial cells ▪ progenitor cells

Pulmonary arterial hypertension (PAH) is a common complication of left-to-right shunts. According to the type of defect, the reversibility of PAH after surgery is often difficult to predict. Why PAH should be reversible in some patients with congenital heart disease (CHD) and not in others is poorly understood. Furthermore, CHD patients with PAH form a heterogeneous group that is inadequately defined in the Venice classification of pulmonary hypertension. Indeed, in some cases, pulmonary pathophysiology and natural history of left-to-right shunt cannot identify patients whose PAH will be irreversible after surgical repair of the underlying cardiac defect. We recently observed neoangiogenesis in surgical lung biopsy samples from patients with reversible PAH due to CHD, together with a proliferative apoptosis-resistant endothelial phenotype. Because lung biopsy is a very invasive tool, we sought to determine whether less invasive biomarkers could help to distinguish reversible from irreversible PAH in CHD.

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Circulating endothelial cells (CECs) are a noninvasive marker of vascular damage, remodeling, and dysfunction. Thanks to a consensus definition of CEC and a standardized protocol for identifying these cells, good agreement now exists among laboratories with regard to normal CEC counts. CECs are present at very low levels in healthy subjects, whereas elevated levels have been reported in various pathologic situations, including coronary heart disease, infectious diseases, immunologic disorders, and cancer (for review, see Blann et al). CECs have both diagnostic and prognostic significance.
Endothelial dysfunction is strongly involved in the pathophysiology of PAH, but only 1 study has shown an increase in CEC numbers in idiopathic PAH.14 No data are available on CEC levels in PAH secondary to CHD. Because the endothelial phenotype is strongly modified in this setting,5 we postulated that CEC numbers might be increased in a subgroup of CHD patients at risk of irreversible PAH. Here, we quantified CECs in blood sampled from the pulmonary artery, pulmonary vein, and peripheral veins during catheterization of subjects with CHD and irreversible or reversible PAH. We also quantified the CD34+ CD133+ population of circulating progenitor cells (CPCs) that originate from bone marrow, as an index of hematopoietic and endothelial mobilization capacity. CEC and CPC counts, as indicators of endothelial lesions, repair, or both, could provide an estimate of individual vascular competence in irreversible PAH complicating CHD.

We postulated that elevated CEC counts may identify a subgroup of CHD patients with PAH. The aim of the present study was to determine CEC and CPC counts in CHD patients with reversible and irreversible PAH with identifiable vascular remodeling in surgical lung biopsy and to evaluate their possible contribution to current diagnostic strategies.

Table 1. Patient Characteristics and Follow-Up

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age, y*</th>
<th>Diagnosis</th>
<th>PAP (S/D/M), mm Hg</th>
<th>QP/QS</th>
<th>Decision</th>
<th>PAP at 6 mo (S/D), mm Hg</th>
<th>Follow-Up, mo</th>
<th>QP/QS After Surgery</th>
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</thead>
<tbody>
<tr>
<td>Reversible PAH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>4</td>
<td>VSD</td>
<td>90/48/63</td>
<td>1</td>
<td>1.6</td>
<td>Banding</td>
<td>30/15</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>2</td>
<td>VSD</td>
<td>85/35/45</td>
<td>1.3</td>
<td>1.5</td>
<td>Banding</td>
<td>28/15</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>2</td>
<td>VSD</td>
<td>95/55/66</td>
<td>1.3</td>
<td>1.7</td>
<td>VSD closure</td>
<td>30/12</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>13</td>
<td>VSD</td>
<td>75/40/53</td>
<td>1.5</td>
<td>1.5</td>
<td>Banding</td>
<td>30/15</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>0.5</td>
<td>VSD</td>
<td>65/40/54</td>
<td>1.2</td>
<td>1.7</td>
<td>VSD closure</td>
<td>25/15</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>3</td>
<td>VSD</td>
<td>55/35/43</td>
<td>1.8</td>
<td>2.7</td>
<td>VSD closure</td>
<td>30/15</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>0.4</td>
<td>ASD + VSD</td>
<td>63/33/45</td>
<td>1.7</td>
<td>1.9</td>
<td>Banding</td>
<td>397/43</td>
<td>6</td>
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<tr>
<td>8</td>
<td>F</td>
<td>1</td>
<td>Truncus</td>
<td>60/40/50</td>
<td>1.8</td>
<td>2.3</td>
<td>Repair</td>
<td>39/12</td>
<td>10</td>
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<tr>
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<td>VSD</td>
<td>80/15/35</td>
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<td>2</td>
<td>Banding</td>
<td>25/15</td>
<td>8</td>
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<tr>
<td>10</td>
<td>F</td>
<td>6</td>
<td>VSD</td>
<td>90/40/65</td>
<td>1.1</td>
<td>1.4</td>
<td>VSD closure</td>
<td>30/15</td>
<td>6</td>
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<tr>
<td>11</td>
<td>F</td>
<td>1</td>
<td>ASD + VSD</td>
<td>116/20/58</td>
<td>1</td>
<td>1.1</td>
<td>VSD closure</td>
<td>32/15</td>
<td>8</td>
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<tr>
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<td>F</td>
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<td>VSD</td>
<td>100/53/71</td>
<td>1.7</td>
<td>2.3</td>
<td>VSD closure</td>
<td>25/15</td>
<td>6</td>
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<tr>
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<td>M</td>
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<td>VSD</td>
<td>70/40/53</td>
<td>2</td>
<td>2</td>
<td>Banding</td>
<td>35/20</td>
<td>6</td>
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<tr>
<td>14</td>
<td>M</td>
<td>4</td>
<td>VSD</td>
<td>95/45/61</td>
<td>1.2</td>
<td>1.6</td>
<td>VSD closure</td>
<td>48/20</td>
<td>6</td>
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<tr>
<td>15</td>
<td>M</td>
<td>0.3</td>
<td>VSD</td>
<td>90/37/65</td>
<td>1.3</td>
<td>1.8</td>
<td>VSD closure</td>
<td>28/16</td>
<td>6</td>
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<tr>
<td>16</td>
<td>F</td>
<td>0.5</td>
<td>VSD</td>
<td>110/45/65</td>
<td>1.1</td>
<td>1.8</td>
<td>VSD closure</td>
<td>35/20</td>
<td>6</td>
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<tr>
<td>Irreversible PAH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>17</td>
<td>F</td>
<td>8</td>
<td>VSD</td>
<td>95/50/60</td>
<td>1</td>
<td>1.2</td>
<td>Banding</td>
<td>90/40</td>
<td>12 (Alive)</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>32</td>
<td>Single ventricle</td>
<td>100/60/70</td>
<td>0.8</td>
<td>1.1</td>
<td>Medical treatment</td>
<td>90/38</td>
<td>12 (Alive)</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>0.8</td>
<td>VSD</td>
<td>78/16/45</td>
<td>1.6</td>
<td>2</td>
<td>Banding</td>
<td>50/30</td>
<td>12 (Alive)</td>
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<tr>
<td>20</td>
<td>M</td>
<td>15</td>
<td>AVSD</td>
<td>80/38/60</td>
<td>1.3</td>
<td>1.8</td>
<td>Medical treatment</td>
<td>95/50</td>
<td>10 (Alive)</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>4</td>
<td>VSD</td>
<td>114/78/58</td>
<td>0.8</td>
<td>1</td>
<td>Medical treatment</td>
<td>90/40</td>
<td>12 (Alive)</td>
</tr>
<tr>
<td>22</td>
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<td>10</td>
<td>Single ventricle</td>
<td>88/40/55</td>
<td>1</td>
<td>1</td>
<td>Banding</td>
<td>110/55</td>
<td>10 (Alive)</td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>14</td>
<td>Single ventricle</td>
<td>71/44/55</td>
<td>2.5</td>
<td>2.5</td>
<td>Banding</td>
<td>85/40</td>
<td>0 Died after surgery</td>
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<tr>
<td>24</td>
<td>M</td>
<td>0.75</td>
<td>VSD</td>
<td>120/46/60</td>
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<td>2</td>
<td>VSD closure</td>
<td>110/50</td>
<td>10 (Alive)</td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>14</td>
<td>VSD</td>
<td>107/63/78</td>
<td>1.3</td>
<td>1.6</td>
<td>Medical treatment</td>
<td>95/40</td>
<td>6 (Alive)</td>
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<tr>
<td>26</td>
<td>F</td>
<td>4</td>
<td>Patent ductus arteriosus</td>
<td>87/64/77</td>
<td>1.2</td>
<td>1.9</td>
<td>Ductus closure</td>
<td>95/45</td>
<td>12 (Alive)</td>
</tr>
</tbody>
</table>

ASD indicates atrial septal defect; PAP (S/D/M), pulmonary arterial pressure (systolic/diastolic/mean); QP, pulmonary blood flow; QS, systemic blood flow; F, female; M, male; VSD, ventricular septal defect; and NA, not available.

*Age at catheterization.
Table 2. Characteristics of the Patients

<table>
<thead>
<tr>
<th></th>
<th>Reversible PAH</th>
<th>Irreversible PAH</th>
<th>p</th>
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<tr>
<td>Age, y</td>
<td>2.0 (0.3–13.0)</td>
<td>9.0 (0.75–32.0)</td>
<td>0.0322*</td>
</tr>
<tr>
<td>Saturation, %</td>
<td>98.0 (91.0–100.0)</td>
<td>100.0 (91.0–100.0)</td>
<td>0.6725</td>
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<tr>
<td>Mean PAP</td>
<td>53.5 (35.0–71.0)</td>
<td>60.0 (45.0–78.0)</td>
<td>0.0741</td>
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<td>QP/QS</td>
<td>1.0 (1.0–2.0)</td>
<td>1.0 (0.8–2.5)</td>
<td>0.3127</td>
</tr>
<tr>
<td>QP/QS after iNO</td>
<td>2.0 (1.1–2.7)</td>
<td>1.5 (1.0–2.5)</td>
<td>0.3838</td>
</tr>
</tbody>
</table>

PAP indicates pulmonary artery pressure; QP/QS, pulmonary to systemic blood flow ratio; and iNO, inhaled nitric oxide.

Data are expressed as median (95% CI). Baseline characteristics were compared between groups with Wilcoxon rank sum test for nonnormally distributed variables (age) and Student unpaired test otherwise.

*P<.05, reversible vs irreversible PAH.

Methods

Study Population

The present study was approved by the Necker ethics committee, and signed informed consent was obtained from all patients and control subjects. Twenty-six consecutive CHD patients with PAH who underwent cardiac catheterization at Necker-Enfants Malades Hospital were enrolled with their parents’ consent between July 2007 and January 2008. Five patients catheterized for coronarography after the arterial switch operation for transposition of the great arteries served as control subjects. None of these patients had PAH.

Table 1 shows the patients’ clinical and hemodynamic characteristics. All patients had measurements of pulmonary artery pressure and shunt determination by oximetry before and after nitric oxide inhalation. Preoperative hemodynamic investigations failed to establish whether PAH would be reversible after pulmonary artery banding or cardiac defect repair (Table 2); therefore, the patients underwent either complete repair or palliative surgery based on our usual clinical and hemodynamic criteria. Four patients with irreversible PAH were not submitted to surgery and received medical treatment. Lung biopsy was performed in 19 patients for histomorphometric and immunohistochemical analysis of pulmonary arteries. Patients were retrospectively separated into 2 groups based on pulmonary artery pressure 6 months after surgery. Sixteen patients had normal pulmonary artery pressure (reversible PAH), and 10 still had elevated pulmonary artery pressure with pulmonary vascular resistance (irreversible PAH). Pulmonary artery pressure was similar in the 2 groups, but the patients with irreversible PAH were older (Table 2). Patients with symmetric irreversible PAH received sildenafil and/or bosentan. Patients with trisomy 21 (Down syndrome) were not included because of a possible overlap between the CPC count and abnormal bone marrow hematopoietic cells.15 Furthermore, patients with Eisenmenger physiology and Down syndrome have particularly poor survival prospects16 and show more rapid progression of symptomatic pulmonary vascular disease than other Eisenmenger patients.17

Vascular Remodeling

Lung biopsy was performed during cardiac surgery in 19 cases. Paraffin-embedded tissues were cut into 4-μm slices and subjected to immunohistochemical studies and histomorphometric analysis of the pulmonary vascular bed. Apoptosis of vascular cells and inflammatory cells was assessed by means of immunohistochemistry with anti-Bcl-2 as the primary antibody (monoclonal anti-human BCL2 oncoprotein, Dako Corp, Glostrup, Denmark). Vascular expression of the angiogenic vascular endothelial growth factor was assessed by endothelial cells in irreversible PAH (F). Late EPCs isolated from cord blood were used as a control for bcl-2 (G) and VEGF (H) staining. Inset: detailed view of a negative control.

Figure 1. Lung biopsy sections showing histological changes in intra-acinar pulmonary arteries of patients with reversible and irreversible PAH. Increased wall thickness is associated with intimal thickening in irreversible PAH. The antiapoptotic protein bcl-2 is not expressed in reversible PAH (C), but it is expressed by endothelial cells of severely damaged arteries in irreversible PAH (D). VEGF staining is weak in reversible PAH (E) and strong in endothelial cells in irreversible PAH (F). Late EPCs isolated from cord blood were used as a control for bcl-2 (G) and VEGF (H) staining. Inset: detailed view of a negative control.

(negative control). The slides were then incubated for 15 minutes with a biotinylated secondary antibody and stained with peroxidase-labeled streptavidin as recommended by the manufacturer (Dako Corp). The slides were then counterstained with Harris hematoxylin.

Late endothelial progenitor cells (EPCs) derived from cord blood were used as a positive control (Figure 1G and 1H). Late-EPC culture and characterization have been described in detail elsewhere.18–21 For these experiments, EPCs were fixed in formalin, included in paraffin blocks, and treated with the same protocol as lung biopsy samples in paraffin. A negative control with a nonimmune monoclonal antibody of the same isotype is shown in the inset of Figure 1H.

CEC Counting After Immunomagnetic Separation

Peripheral venous and pulmonary arterial and venous blood samples were collected on EDTA during the first cardiac catheterization. CECs were counted by an operator unaware of the patients’ clinical features. Immunocapture of CECs from whole blood was performed at 4°C with magnetic beads (Dynabeads M-450, Dynal, Invitrogen, Carlsbad, Calif) coated with S-Endo 1 (Biocytex, Marseille, France), a monoclonal antibody raised against the endothelial antigen CD146. To avoid nonspecific binding of leukocytes to CD146-coated beads, the cell suspensions were flushed vigorously through the pipette tip during the washing steps and then suspended in acridine orange (3

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μg/mL in PBS; Sigma-Aldrich, Saint-Quentin Fallavier, France) before being counted under a fluorescence microscope (λ<sub>em</sub>=490 nm). CECs were identified according to their morphological criteria, ie, >10 beads bound to >20-μm cells or cells with <10 beads but with a well-preserved and recognizable morphology (clear nucleus in a well-delineated cytoplasm and a size concordant with endothelial cells). The number of cells in aggregates was determined from the number of cells with spherical rosette features. The endothelial nature of the isolated cells was confirmed by measuring lectin Ulex europaeus agglutinin-1 (UEA-1) expression. The number of CECs was expressed as cells per milliliter of blood.

Flow Cytometric Quantification of Circulating CPCs
CPCs were quantified in peripheral blood and in pulmonary arterial and venous blood obtained during the first cardiac catheterization by means of 2-color flow cytometry on a FACScalibur device (BD, Franklin Lakes, NJ). Briefly, 100 μL of blood was incubated with 10 μL of phycoerythrin-cyanin-5–conjugated anti-human CD34 monoclonal antibody (Beckman-Coulter, Villepinte, France) and 10 μL of phycoerythrin-conjugated anti-human-CD133 monoclonal antibody (Miltenyi Biotec, Bergisch-Gladbach, Germany). Nonimmune monoclonal antibodies of the same isotype and the same fluorochrome as the immune monoclonal antibodies and provided by the same manufacturer were processed as negative controls to set appropriate regions. Before analysis, the flow cytometer was thoroughly cleaned to remove residual particles. The number of positive peripheral blood cells was determined by 2D side-scatter fluorescence dot-plot analysis of the sample after gating on the lymphocyte population. Each analysis included 30,000 events. Double-positive events scored with the isotype control were subtracted from CD34<sup>+</sup>CD133<sup>+</sup> events. The percentage of CD34<sup>+</sup>CD133<sup>+</sup> cells counted within the lymphocyte gate was converted into the absolute number of cells per 10<sup>5</sup> lymphocytes using the white blood cell count obtained with an automated cell counter (LH750, Beckman-Coulter). This normalized expression allowed us to avoid the bias due to the elevated lymphocyte count observed during childhood, which begins to normalize after 4 years of age.

Statistical Analysis
A calculated sample size of 5 subjects per group was needed to detect a 10-fold increase in CEC numbers between normal and pathological subjects, with expected mean CEC numbers of 0.15 and 2.45, respectively, on a log scale (geometric means of 1.16 and 11.6 CECs, a 10-fold increase in CEC numbers between normal and pathological subjects). The statistical power was calculated with the power of 80% (nQuery Advisor software, Statistical Solutions, Inc, Boston, Mass).

Baseline characteristics of the subjects were compared between the groups with Wilcoxon rank sum test on nonnormally distributed variables and Student unpaired test otherwise. The effect of the sampling site, the group, and their interaction on CEC and CPC variability was tested by 2-way repeated-measure ANOVA on log-transformed CEC and CPC numbers. Statistical results are expressed as the ratio of geometric means between groups, with the associated 95% confidence intervals (CIs) and P values adjusted with the Bonferroni method. Correlations were detected with the Spearman correlation coefficient. Sensitivities and specificities are presented with their exact binomial 95% CIs. All statistical analyses were performed with StatView or SAS statistical software (Cary, NC). P values <0.05 were considered to denote significant differences.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Histomorphometric Features and Markers of Apoptosis and Neoangiogenesis
Among the 26 consecutive CHD patients with PAH, 19 underwent a lung biopsy (6 patients with irreversible PAH and 13 with reversible PAH). In the 13 patients with reversible PAH, histomorphometric examination of pulmonary vessels showed medial hypertrophy without intimal damage. All pulmonary vessels were negative for the antiapoptotic marker Bcl2. Lung biopsy samples obtained during pulmonary banding or shunt closure in 6 patients with irreversible PAH showed intimal damage with Bcl2 immunostaining of endothelial cells, which indicated that endothelial cells had an apoptosis-resistant phenotype. In addition, vascular endothelial growth factor was strongly expressed by endothelial cells in arteries of all sizes in biopsy specimens from patients with irreversible PAH (Figure 1). This was consistent with our previous description of active endothelial remodeling in irreversible PAH.<sup>5</sup> Cells used as positive controls for vascular endothelial growth factor and Bcl2 expression<sup>18</sup> were EPCs derived from cord blood, which have been shown to form neovessels in preclinical models and for which the phenotype is the same as that of newly formed vessels after injection of a cell therapy product in human adults.<sup>23–24</sup>

Circulating Endothelial Cells
After immunomagnetic separation, CECs were observed after staining with the fluorescent probe acridine orange or UEA-1 (Figure 2). No significant difference in CEC counts was noted among the different types of blood samples in any of the 3 patient groups, as tested by ANOVA on log-transformed CEC numbers (Table 3). CEC counts in peripheral blood were similar in control subjects and patients with reversible PAH and were consistent with normal values defined by consensus (<10 CEC/mL, similar to the number found in normal subjects, as defined with the consensus protocol<sup>19</sup>). In contrast, the CEC count was 10-fold higher in irreversible PAH.
P/H110050.0005 and P/H110210.0001, respectively, versus control sub-
jects and patients with reversible PAH; Table 3; Figure 3).

CEC counts ranged from 12 to 166 per milliliter. The patient
with 12 CEC/mL was a 4-year-old twin boy with bronchopul-
monary dysplasia born prematurely at 30 weeks and referred
to our institution for a ventricular wall defect. Thus, with the
limits due to the small cohort size, values higher than the
consensus cutoff of 10 CEC/mL\(^1\) had a sensitivity of 100%
(95% CI 69.2% to 100%), a specificity of 87.5% (95% CI 61.7%
to 98.4%), a negative predictive value of 100% (95% CI 76.8%
to 100%), and a positive predictive value of 83.3% (95% CI 51.6%
to 97.9%). We found no correlation between CEC numbers and age,
regardless of the territory explored (Spearman correlation coeffi-
cient: peripheral vein 0.30 [\(P=0.15\]), pulmonary artery 0.16
[\(P=0.53\]), and pulmonary vein 0.14 [\(P=0.57\)].

CPC Numbers
As observed with CECs, no significant difference in CPC
counts was found according to sampling site in any of the 3
groups (Table 3). Peripheral CPC values in the patients with
reversible and irreversible PAH were not different from the
control value (\(P=1.0000\) for reversible and irreversible PAH
versus control subjects; Table 3; Figure 4). Moreover, we
found no correlation between CPC and CEC numbers, re-
gardless of the territory explored (Spearman correlation
coefficient: peripheral vein 0.22 [\(P=0.24\]), pulmonary artery
0.27 [\(P=0.24\]), and pulmonary vein 0.1 [\(P=0.72\)]. This
absence of correlation confirms that no CPC increase oc-
curred from bone marrow to repair the potential endothelial
damage observed with an elevated CEC count.

Discussion
Mechanisms that determine the reversible or irreversible
nature of PAH that commonly complicates left-to-right

![Figure 3](http://circ.ahajournals.org/)

Figure 3. CEC counts in peripheral venous blood of CHD
patients with pulmonary hypertension. CEC counts were signifi-
cantly increased in irreversible PAH. Effects of the sampling
site, the group, and their interaction on CEC variability were tested
with ANOVA (*\(P=0.0005\) and *\(P<0.0001\), respectively, versus control sub-
jects and patients with reversible PAH; Table 3; Figure 3).

![Figure 4](http://circ.ahajournals.org/)

Figure 4. CPC counts in peripheral venous blood of CHD
patients with pulmonary hypertension. Number of CPCs deter-
ned by FACS analysis. Neither group of patients differed sig-
nificantly from the control subjects. Effects of the sampling site,
the group, and their interaction on CPC variability were tested
with ANOVA (*\(P=1.0000\) for both irreversible and reversible PAH).

### Table 3. CEC and CPC Counts in Blood Samples Obtained by Catheterization of the Pulmonary Artery,
Pulmonary Vein, and Peripheral Vein in Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (n=5)</th>
<th>Reversible PAH (n=16)</th>
<th>Irreversible PAH (n=10)</th>
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<td>CEC counts in blood samples, cells/mL</td>
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<td></td>
</tr>
<tr>
<td>Peripheral vein</td>
<td>3.0 (1.3–7.1)</td>
<td>3.0 (1.9–4.9)</td>
<td>49.3 (26.7–90.9)</td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>4.8 (1.8–12.8)</td>
<td>4.3 (2.5–7.5)</td>
<td>59.1 (27.9–125.1)</td>
</tr>
<tr>
<td>Pulmonary vein</td>
<td>3.3 (0.6–19.8)</td>
<td>6.2 (2.4–15.8)</td>
<td>62.7 (18.6–211.6)</td>
</tr>
<tr>
<td>All sampling site</td>
<td>3.6 (1.3–10.4)</td>
<td>4.3 (2.4–7.7)*</td>
<td>56.7 (26.9–119.8)*†</td>
</tr>
<tr>
<td>Difference vs controls</td>
<td>1.2 (0.3–5.3); (P=1.0000)</td>
<td></td>
<td>15.6 (3.1–77.8); (P=0.0005)</td>
</tr>
<tr>
<td>Difference vs reversible PAH</td>
<td></td>
<td></td>
<td>13.2 (4.1–42.8); (P&lt;0.0001)</td>
</tr>
<tr>
<td>CPC counts, cells/(10^5) lymphocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral vein</td>
<td>51.2 (13.9–189.1)</td>
<td>37.6 (17.7–79.9)</td>
<td>50.2 (19.9–126.4)</td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>54.5 (14.0–212.6)</td>
<td>41.3 (18.8–90.5)</td>
<td>67.7 (25.2–181.9)</td>
</tr>
<tr>
<td>Pulmonary vein</td>
<td>51.2 (12.2–214.0)</td>
<td>39.1 (17.8–85.8)</td>
<td>54.6 (20.3–146.8)</td>
</tr>
<tr>
<td>All sampling site</td>
<td>52.3 (14.7–185.5)</td>
<td>39.3 (19.0–81.2)</td>
<td>57.0 (23.2–140.1)</td>
</tr>
<tr>
<td>Difference vs controls</td>
<td>0.8 (0.1–4.6); (P=1.0000)</td>
<td></td>
<td>1.1 (0.2–7.5); (P=1.0000)</td>
</tr>
<tr>
<td>Difference vs reversible PAH</td>
<td></td>
<td></td>
<td>1.5 (0.3–6.1); (P=1.0000)</td>
</tr>
</tbody>
</table>
Irreversible PAH is difficult to predict on the basis of preoperative testing and has adverse long-term consequences. We recently reported a hyperproliferative endothelial phenotype and active neoangiogenesis in lung biopsy samples from patients with irreversible PAH due to CHD. The present study was designed to confirm our histological findings and to identify a noninvasive marker of vascular remodeling and regeneration, focusing on CECs and CPCs.

CECs are a novel marker of endothelial damage, and their number correlates with other markers of endothelial function, such as flow-mediated dilation, von Willebrand factor, and tissue plasminogen activator levels. CEC counts are increased in coronary heart disease, renal vascular disease, and transplantation and are a marker of progression and survival in acute coronary disease. Few data are available on CEC counts in pulmonary hypertension except the work of Bull et al., who showed that the CEC count correlates with pulmonary artery pressure in adult patients with different causes of pulmonary hypertension. In the present study population, pulmonary pressure was similar in patients with reversible and irreversible PAH. None of the clinical and hemodynamic criteria could clearly identify patients with reversible or irreversible PAH in the present study. According to our previous findings showing endothelial damage in irreversible PAH, we sought to determine whether CEC counts in patients with PAH due to CHD could provide information about the reversibility of PAH in this category of patients.

CECs were easily quantified in this pediatric population, and counts in the 5 pediatric control subjects were close to those described in adults by the consensus network. The only adult patient included in the present study (patient 18, 32 years old) was referred to our institution for PAH-associated CHD, present since infancy. The CEC count was high in this patient (66 per mL) and did not differ from that of younger patients with PAH (mean 61.7 and 61.1 CEC/mL, calculated with and without patient 18, respectively).

The endothelium is a highly dynamic tissue in equilibrium with a circulating compartment, offering new opportunities for noninvasive exploration. CEC and CPC counts may provide an estimate of individual vascular competence that results from the equilibrium between injury and repair and may yield prognostic clues in vascular diseases. However, we do not know whether a gradual alteration of the equilibrium between injury and regeneration of the vascular endothelium contributes to endothelial dysfunction along with age. The first information arising from the present work is that CEC numbers in peripheral blood were similar to those in central blood (pulmonary artery or pulmonary vein). Second, CEC numbers appeared to reflect pulmonary endothelial remodeling, because a high CEC increase was found in children with irreversible PAH secondary to CHD, which was associated with major endothelial damage, compared with control subjects and patients with reversible PAH. However, the source of the supernumerary CECs associated with irreversible PAH, described for the first time in the present work, remains unclear; it might result from endothelial lesions or newly formed vessels, as in cancer. One limitation of the present study is the absence of normative pediatric data across a range of ages.

Irreversible PAH is associated with neoangiogenesis (Figure 1). Because EPCs are involved in healing processes, we hypothesized that EPCs could be associated with the neoangiogenesis process observed in irreversible PAH. Because no consensus method exists for accurately quantifying EPCs in small samples, we chose to quantify circulating CPCs, which constitute a subpopulation of EPCs and have been described as the source of EPCs in culture. In experimental models of hypoxia-induced PAH, EPCs appear to be involved in pulmonary vascular remodeling. In a recent study, CPC numbers were found to be higher in patients with idiopathic PAH than in control subjects; however, another recent study showed no correlation between PAH and CPC numbers, whereas a correlation was found between idiopathic PAH and EPC numbers isolated by cell culture. In the present study, CPC counts were similar in the peripheral vein and in pulmonary artery and vein blood in patients with PAH secondary to CHD (Table 3). In addition, we found no significant difference between control subjects and PAH patients. Nevertheless, further studies are needed to explore the involvement of the different types of EPCs (CD34+/CD133+ expressing KDR [kinase insert domain protein receptor] and EPCs obtained in culture) in irreversible pulmonary hypertension secondary to CHD. Although angiogenic and inflammatory markers have been found to be correlated with CPCs or EPCs, we did not find any differences between the 3 groups in terms of vascular endothelial growth factor, placental growth factor, or interleukin-6 levels (data not shown). This result is in line with the absence of difference in CPC levels between patients. Finally, this observation strongly suggests that the modification of pulmonary vasculature that occurs during PAH is not the consequence of a systemic mobilization of progenitor cells.

In the method we used to quantify CECs, the size and number of particles around the cells allowed us to differentiate CECs from CPCs expressing CD146. Furthermore, Delorme et al. found that progenitor cells coexpressing CD146 and CD133, an immature antigen, looked significantly different from mature CECs, the latter of which presented with morphological features of differentiated cells and a size larger than 20 μm. We can thus speculate that the CECs we counted using the immunomagnetic separation method did not include progenitor cells. Moreover, the similar CPC counts found in the control subjects and the patients with reversible and irreversible PAH suggest that CPCs were not involved in the observed differences in CEC counts.

In conclusion, the present study suggests that CEC count in peripheral blood could help to predict the reversibility of PAH associated with CHD. Whether CEC count could replace lung biopsy to predict long-term outcome in CHD patients with PAH must be confirmed by a longer follow-up and a larger number of patients.

Acknowledgments

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Disclosures

None.

References


Pulmonary arterial hypertension (PAH) can complicate several types of congenital heart disease, and its reversibility is often difficult to predict. We postulated that the numbers of circulating endothelial cells (CECs) and circulating progenitor cells (CPCs) might serve as noninvasive biomarkers of endothelial turnover and thus help to identify patients with congenital heart disease who are at risk of irreversible PAH, thereby avoiding the need for lung biopsy. Indeed, we recently observed impaired apoptotic regulation of endothelial cells in lung biopsy samples from patients with irreversible PAH. CEC and CPC numbers were each similar in peripheral vein, pulmonary artery, and pulmonary vein blood samples, whether from patients with reversible PAH (n=16), patients with irreversible PAH (n=10), or control subjects (n=5). CEC numbers were significantly higher in patients with irreversible PAH than in patients with reversible PAH and control subjects, whereas CPC numbers did not differ among the 3 subgroups. The CEC count did not correlate with age or the CPC count. CEC counts could thus be used for individual evaluation of vascular competence and serve as noninvasive biomarkers of endothelial lesions and as treatment decision aids in congenital heart disease patients with irreversible PAH.
Circulating Endothelial Cells: A New Candidate Biomarker of Irreversible Pulmonary Hypertension Secondary to Congenital Heart Disease

David M. Smadja, Pascale Gaussem, Laetitia Mauge, Dominique Israël-Biet, Françoise Dignat-George, Séverine Peyrard, Gabriella Agnoletti, Pascal R. Vouhé, Damien Bonnet and Marilyne Lévy

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