Circulating Endothelial Progenitor Cells in Patients With Eisenmenger Syndrome and Idiopathic Pulmonary Arterial Hypertension

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Background—Impaired endothelial homeostasis underlies the pathophysiology of pulmonary arterial hypertension (PAH). We speculated that PAH patients are deficient in circulating endothelial progenitor cells (EPCs), potentially contributing to endothelial dysfunction and disease progression.

Methods and Results—We recruited 41 patients with Eisenmenger syndrome (13 with Down syndrome), 55 with idiopathic PAH, and 47 healthy control subjects. Flow cytometry and in vitro assays were used to quantify EPCs and to assess cell function. The number of circulating $\text{CD34}^+$, $\text{CD34}^+/\text{AC133}^+$, $\text{CD34}^+/\text{KDR}^+$, and $\text{CD34}^+/\text{AC133}^+/\text{KDR}^+$ progenitor cells was low in Eisenmenger patients compared with healthy control subjects, and those with Down syndrome displayed even fewer EPCs. Reductions in EPC numbers correlated with New York Heart Association functional class, 6-minute walk distance, and plasma brain-type natriuretic peptide levels. The capacity of cultured peripheral blood mononuclear cells to form colonies and incorporate into tube-like structures was impaired in Eisenmenger patients. Idiopathic PAH patients had reduced numbers of EPCs, and the number of circulating EPCs correlated with invasive hemodynamic parameters in this cohort. Levels of immune inflammatory markers, cGMP, stable nitric oxide oxidation products, and asymmetric dimethylarginine were abnormal in patients with PAH and related to numbers of EPCs. Within the idiopathic PAH population, treatment with the phosphodiesterase inhibitor sildenafil was associated with a dose-dependent rise in EPC numbers, resulting in levels consistently above those found with other therapies.

Conclusions—Circulating EPC numbers are reduced in 2 well-characterized forms of PAH, which also exhibit raised levels of inflammatory mediators. Sildenafil treatment may represent a pharmacological means of increasing circulating EPC numbers long-term. (Circulation. 2008;117:3020-3030.)

Key Words: endothelial progenitor cells ■ endothelium ■ heart defects, congenital ■ hypertension, pulmonary ■ pulmonary heart disease

Pulmonary arterial hypertension (PAH) is associated with considerable morbidity and mortality.1,2 Despite differences in the cause and rate of progression, the structural abnormalities found in the pulmonary vasculature of patients with idiopathic PAH (IPAH) and PAH associated with congenital heart disease show remarkable similarities. Endothelial dysfunction also is an integral feature and an early event in the pathogenesis of both conditions.3,4 Restoration of normal endothelial function is the common goal of available treatments.4

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Endothelial progenitor cells (EPCs) are mobilized from the bone marrow and contribute to postnatal vasculogenesis and vascular homeostasis, and the number and in vitro function of circulating EPCs relate to endothelial function.5-7 Recent studies suggest that bone marrow–derived EPCs can incorporate into the pulmonary microvasculature, and augmenting EPC levels by bolus administration may reverse PAH in monocrotaline-treated rats.8 This work is now being trans-
cells coexpressing CD34 and KDR.10,11 We incubated 10⁶ PBMNCs with mouse anti-human KDR (R&D Systems, Abingdon, UK) antibodies for 30 minutes at 4°C according to the manufacturers’ instructions or with appropriate isotype controls. At least 100,000 events were acquired in the lymphomonocytic gate using a FACSCalibur cytometer (Becton Dickinson, Oxford, UK). The number of progenitor cells was expressed as a percentage of all lymphomonocytic cells. EPCs were enumerated using established criteria as CD34+/H11001 and may represent primitive hematopoietic progenitors. In contrast, CD34 cells not expressing the common leukocyte antigen (CD45) were found to yield colony-forming unit endothelial cells in their study. Therefore, we also quantified EPCs as lymphomonocytic cells expressing CD34 with low expression of CD45 (PE-labeled monoclonal mouse anti-human CD45, BD Biosciences) as CD45̄/CD34+ EPCs in accordance with previous studies. To account for possible differing levels of lymphomonocytic cells in the subject groups, EPC levels were corrected for the number of lymphomonocytic cells and compared with the percentages of EPCs in the lymphomonocytic gate.

Colonies were scored as aggregates of three or more cells expressing CD34 with low expression of CD45, and small aggregates were not scored. The value 1.0 was given to the number of colonies scored in each well. In order to determine the number of EPCs, all colonies were counted, and the number that incorporated into tube-like structures was determined as previously described (see the online-only Data Supplement).

Plasma Assays
Plasma levels of chemokine (C-C motif) ligand-2 (CCL)-2 (formerly monocyte chemotactic protein-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), VEGF, cGMP, C-reactive protein (CRP), and ADMA were quantified by use of commercial assays according to the manufacturer’s instructions (see the online-only Data Supplement). The combined concentrations (NOx) of plasma nitrite (NO₂⁻) and nitrate (NO₃⁻), the stable oxidation products of nitric oxide (NO), were determined with an NO chemiluminescence detector (NOA 280, Sievers, Analytix Ltd, Peterlee, UK) as described. Plasma samples were deproteinated by centrifugation in 3-kDa molecular weight cutoff filter units (Ultracel Microcon YM-3, Millipore Ltd, Watford, UK). In addition, a full blood count was made, and brain-type nitriuretic peptide (BNP) levels were measured.

Statistical Analysis
Data are expressed as mean±SD or median and interquartile range (IQR). Statistical analysis was performed with the nonparametric
Subjects
Fifty-five patients with IPAH (44 female; age, 46±14 years), 41 Eisenmenger patients (27 female; age, 37±12 years; 13 with Down syndrome), and 47 healthy control subjects (24 female; age 36±9 years) were recruited. To account for differences in gender distribution between the subject groups, EPC numbers were examined separately in male and female subjects. The Eisenmenger population was younger than the IPAH cohort, and patients exhibited secondary erythrocytosis, with raised hematocrit and hemoglobin levels and cyanosis (Table I of the online-only Data Supplement).

EPC Numbers
Circulating EPC numbers, as defined by the number of double- (CD34+/KDR+) or triple- (CD34+/AC133+/KDR+) labeled cells, were significantly lower in patients with Eisenmenger syndrome or IPAH regardless of gender (Figure 1). Eisenmenger patients with Down syndrome had the lowest number of circulating EPCs. Progenitor cell numbers, defined as single- (CD34+) or double- (CD34+/AC133+) labeled cells, were also lower in the Eisenmenger population compared with control and IPAH subjects (Figure 1). Stratifying subjects by decades of age revealed that differences in EPC numbers between patients and control subjects were maintained throughout all age groups. No significant differences in the lymphomonocyte cell counts were found between the cohorts (Table I). Correcting EPC numbers (expressed as percent of lymphomonocytes) for actual lymphomonocyte cell counts in individual patients did not affect the results of the study, confirming that EPC numbers were significantly lower in Eisenmenger and IPAH subjects compared with control groups (data not shown). In addition, the numbers of CD45low/CD34+ cells were significantly lower in non-Down patients (0.377% versus 1.073%; P=0.003) and Down patients with Eisenmenger syndrome (0.430% versus 1.073%; P=0.02) compared with those of control subjects. Levels of CD45low/CD34+ cells were not significantly different between control and IPAH subjects.

Association Between EPC Numbers and Exercise Tolerance
Within the Eisenmenger population, an inverse relationship was found between circulating EPC numbers and exercise tolerance as judged by New York Heart Association functional class and 6-minute walk distance. EPC numbers were significantly higher in Eisenmenger patients in New York Heart Association functional class II compared with those in class III as determined by the number of CD34+ (median, 0.099 versus 0.062; P=0.03), CD34+/AC133+ (median, 0.032 versus 0.013; P=0.009), CD34+/KDR+ (median, 0.029 versus 0.012; P=0.02), or CD34+/AC133+/VEGFR2+ (median, 0.007 versus 0.002; P=0.008) cells. The number of CD34+/AC133+ cells in Eisenmenger patients also correlated directly with 6-minute walk distance (r=0.64, P=0.008) as shown in the online-only Data Supplement. In contrast, EPC numbers in IPAH patients did not correlate with 6-minute walk distance and did not vary significantly between New York Heart Association classes II, III, and IV (data not shown).

Association Between Treatment for PAH and EPC Numbers
IPAH patients receiving treatment with the phosphodiesterase type 5 (PDE5) inhibitor sildenafil exhibited significantly higher circulating EPC levels than patients not receiving sildenafil (Figure 2A). Furthermore, a dose-dependant rela-
Relationship was found between sildenafil exposure and EPC number, regardless of the surface markers used (Figure 2B and 2C). In contrast, no significant difference in EPC numbers was found in IPAH patients treated with bosentan (125 mg BD) or other targeted therapies compared with those of treatment-naive patients and control subjects (Figure 2A). Similarly, no significant difference was found between the numbers of progenitor cells in Eisenmenger patients treated with bosentan and treatment-naive patients (data not shown). Eleven IPAH patients had also taken an HMG-CoA reductase inhibitor (atorvastatin 20 mg OD, n=1; simvastatin 40 to 80 mg OD, n=10). The circulating EPC numbers in this cohort did not differ significantly from those of control subjects, but they were nonetheless excluded from the analyses of patients receiving sildenafil.

**Colony-Forming Unit Endothelial Cells and Adherent Cells**

Characteristic colony-forming unit endothelial cells were observed after culture of PBMCs in Endocult medium (Figure 3). Consistent with the flow cytometric analysis of circulating progenitors, significantly fewer colony-forming units were obtained from patients with Eisenmenger syndrome compared with control subjects, whereas a trend was found toward more colonies in IPAH patients (Figure 3). Similarly, patients with Eisenmenger syndrome had significantly fewer adherent cells after culture of PBMCs compared with control subjects, whereas IPAH patients had significantly more (Figure 4A).

**Incorporation of Cultured Cells Into Tube-Like Structures**

After 7 days in culture, all adherent cells exhibited Vybrant CFDA SE cell tracer labeling, Dil-Ac–low-density lipoprotein uptake, and immunostaining for von Willebrand factor; a major proportion also displayed UEA-1 binding (Figure 4B). Cells derived from patients with Eisenmenger syndrome had a reduced capacity to incorporate into HUVEC tube-like structures (median, 14.8 cells per field; IQR, 11.5 to 40.7; n=5) compared with cells derived from normal control subjects (51.3 cells per field; IQR, 42.7 to 59.3; n=8; P=0.028) and patients with IPAH (60.3 cells per field; IQR, 44.5 to 71.0; n=8; P=0.019). In contrast, no significant difference was found between control subjects and IPAH patients (Figure 4).

**Circulating Inflammatory Mediators and Indexes of Endothelial Dysfunction**

Plasma levels of inflammatory mediators were significantly higher in Eisenmenger and IPAH patients compared with control subjects, and within the Eisenmenger population, patients with Down syndrome had higher levels of TNF-α, IL-6, CCL-2, and CRP (Figure 5A). Levels of inflammatory mediators correlated with each other and white blood cell count. Furthermore, in the Eisenmenger population, the concentrations of TNF-α and CRP also correlated with combined plasma NOx levels (see the online-only Data Supplement).

Plasma ADMA levels were significantly higher in patients with Eisenmenger syndrome or IPAH compared with healthy control subjects (Figure 5B), and within the Eisenmenger population, those with Down syndrome had significantly higher ADMA levels (median, 1.23 μmol/L; IQR, 0.90 to 1.69) compared with patients without Down syndrome (median, 0.82 μmol/L; IQR, 0.72 to 0.89; P=0.009). Circulating levels of both cGMP and NO metabolites also were significantly elevated in the Eisenmenger and IPAH patients, with markedly higher levels in patients with Down syndrome.
Plasma NOx levels comprised mainly NO\textsubscript{2}/NO\textsubscript{3}, with no differences observed in the levels of NO\textsubscript{2}/NO\textsubscript{3} among the groups (data not shown). Although no direct correlation was found between cGMP and NOx levels, BNP and cGMP levels were positively associated in patients with Eisenmenger syndrome ($r=0.74$, $P=0.0001$), as shown in the online-only Data Supplement. In contrast to the elevated cytokines, plasma VEGF levels were significantly lower in Eisenmenger patients compared with control subjects and IPAH patients (Figure 5B).

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![Figure 4: Number of adherent cells from control, Eisenmenger, and IPAH subjects and number of cells incorporated into HUVEC tube-like structures. B, Adherent cells after 7 days of culture displaying (arrows) Dil-Ac-low-density lipoprotein uptake, FITC-UEA-1 labeling, and Hoechst-counterstained nuclei. C, Incorporation of Vybrant-labeled cells (green fluorescence) in to HUVEC tube-like structures. *$P<0.05$ vs control subjects; †$P<0.05$ vs IPAH patients.](image)

![Figure 5: Plasma levels of TNF-α, IL-6, monocyte chemotactic protein-1 (MCP-1/CCL-2), and CRP. B, Plasma levels of ADMA, cGMP, NOx, and VEGF in control subjects, Eisenmenger (EM) patients with and without Down syndrome, and IPAH subjects. *$P<0.05$ vs control subjects; †$P<0.05$ vs IPAH patients.](image)
Association Between Circulating Cell Numbers and Inflammatory Mediators, ADMA, BNP, and cGMP

Overall, the number of circulating (CD34+/KDR−) EPCs was negatively correlated with levels of TNF-α, IL-6, and CRP, and raised levels of ADMA also were associated with lower EPC numbers (Figure 6). We found a bimodal relationship between cGMP levels and EPC numbers. When subjects were stratified according to diagnosis, a positive association was observed between cGMP levels and circulating EPC numbers in control subjects and, to a lesser degree, in IPAH patients (Figure 7). In contrast, a significant negative association was found in Eisenmenger patients (Figure 7), who had the highest cGMP levels on average (Figure 5). Plasma levels of BNP and circulating EPC numbers also were inversely correlated in the Eisenmenger population, and an inverse correlation was found between BNP and cGMP levels, potentially confounding the relationship between cGMP and EPC numbers in this population (see the online-only Data Supplement).

Association Between Circulating Cell Numbers, cGMP Levels, and Hemodynamic Parameters

Higher circulating EPC numbers and cGMP levels were found to be associated with significantly lower mean pulmonary arterial pressures and higher cardiac index in IPAH patients (Figure 8).

Discussion

The present study investigated the number of circulating EPCs and the function of cultured mononuclear cells in patients with Eisenmenger syndrome and IPAH. Our major findings were that the number of circulating EPCs (CD34+/KDR− or CD34+/AC133+/KDR−) was markedly reduced in patients with Eisenmenger syndrome or IPAH. This reduction was most profound in Eisenmenger patients with Down syndrome. The reduction in circulating EPCs was associated with reduced functional capacity of cultured PBMCs in patients with Eisenmenger syndrome but not in patients with IPAH. Elevated levels of inflammatory mediators, indexes of NO synthesis, and ADMA were found in PAH patients and
correlated directly with EPC numbers. In Eisenmenger patients, a close correlation was found between plasma levels of BNP and cGMP, and BNP related inversely to EPC numbers in this cohort. Notably, EPC numbers also were related to pulmonary hemodynamic parameters in IPAH patients, and long-term sildenafil treatment was associated with dose-dependent elevation of circulating EPC numbers in this cohort.

Both Eisenmenger syndrome and IPAH are associated with pulmonary and systemic vascular endothelial dysfunction.3,4,18,19 In the present study, the reduction in EPC numbers in Eisenmenger patients showed a correlation with disease severity as measured by functional class and 6-minute walk distance, and numbers were lowest in patients with Down syndrome. As a subgroup, Eisenmenger patients with Down syndrome have particularly poor survival prospects20 and show more rapid progression of symptomatic pulmonary vascular disease than other patients with Eisenmenger syndrome.2 These findings are in accordance with observations in various cardiovascular cohorts suggesting that low EPC numbers are associated with a worse prognosis.11,15,16 Patients with IPAH also exhibited significantly fewer circulating EPCs compared with healthy control subjects, and the number of cells correlated directly with cardiac index. In addition, a trend toward lower EPC numbers was found in IPAH patients with higher mean pulmonary arterial pressures, although this association was potentially confounded by the effect of sildenafil treatment. It is possible that mechanistic

Figure 7. Relationship between plasma cGMP levels and circulating CD34+/KDR+ EPCs in control, Eisenmenger, and IPAH subjects. Overall, locally weighted regression analysis indicates a bimodal relationship. Stratification into cohorts reveals a positive association between cGMP levels and EPC numbers in control and IPAH subjects and a negative association in Eisenmenger subjects. Histograms with overlaid density estimates illustrate the higher levels of cGMP in Eisenmenger subjects compared with control and IPAH subjects (see also Figure 5B).
differences (eg, involving chronic cyanosis, erythrocytosis, increased blood viscosity, and associated shear stress) between Eisenmenger and IPAH patients have diverse effects on the number of circulating cells and the function of cultured cells.

The mobilization and function of EPCs are thought to be critically dependent on NO.21 We found that the plasma concentration of ADMA was raised in both Eisenmenger and IPAH patients, which is in keeping with previous studies.22,23 Although increased ADMA levels are associated with reduced NO bioavailability, we observed elevated systemic levels of stable NO oxidation products, which is consistent with earlier reports of raised nitrate levels in adults and children with congenital heart disease.24 A positive correlation was found between cGMP levels and EPC numbers in control subjects and IPAH patients, yet despite high NOx and cGMP plasma concentrations, circulating EPCs not only were deficient in Eisenmenger patients but also exhibited a negative association with plasma cGMP levels. Several potential explanations exist for this phenomenon, but NO and the natriuretic peptides are the major factors stimulating cGMP synthesis in pulmonary vascular tissue.25 In experimental animals, increased pulmonary blood flow and associated pulmonary hypertension are accompanied by raised circulating levels of BNP and cGMP.26 The levels of these 2 factors also were closely correlated in patients with Eisenmenger syndrome, potentially obscuring any direct association between NO and cGMP production. Increased circulating inflammatory mediators also accompanied raised plasma NO levels, and cytokine-inducible NO synthase (iNOS) expression has been described in pulmonary arteries and cardiac tissues of patients with flow-associated pulmonary hypertension and cyanotic congenital heart disease.27,28 This represents a potentially important source of “high-output” NO production that may be associated with oxidative stress and the production of reactive NO species that attenuate the mobilization, function, and survival of EPCs.29,30

Increasing evidence indicates that inflammation has a key role in the pathogenesis of PAH. As in earlier studies,31–33 we found evidence of chronic inflammation with raised plasma levels of the inflammatory mediators TNF-α, IL-6, and CCL-2. Increased pulmonary production of CCL-2 in PAH is postulated to act as a chemoattractant for circulating monocytes,33 whereas both TNF-α and IL-6 have a negative effect on the number and function of EPCs.11,34,35 We also found that plasma CRP was elevated and, together with the inflammatory cytokines, was negatively associated with the number of circulating EPCs. In this regard, CRP has been found to attenuate endothelial NOS (eNOS) expression and the mobilization, differentiation, and survival of EPCs.36,37

An important finding of our study was that IPAH patients treated with the PDE5 inhibitor sildenafil exhibited a selective and dose-dependent increase in circulating EPCs. Studies of erectile dysfunction have indicated that short-term and long-term PDE5 inhibition with vardenafil and tadalafil, respectively, is associated with increased circulating CD34+/KDR+ progenitor cells.38,39 Our study, however, is the first report of the effects of a PDE5 inhibitor on EPC numbers in patients with PAH. Given that eNOS is expressed in cultured EPCs40 and that NO released from organic nitrates can augment circulating EPCs,21,29 it is possible that, unlike other targeted therapies for PAH, sildenafil stimulates an intrinsic NO-cGMP pathway, thereby augmenting EPC numbers. It is unclear whether the effect of sildenafil confers a therapeutic advantage, but active interest exists in using bone marrow–derived cells to treat IPAH.8,41,42 Data obtained from experimental models suggest that these cells may induce regeneration of pulmonary perfusion, leading to improved hemodynamics and survival.8,42 Furthermore, Wang et al9 recently found that the intravenous infusion of cultured autologous cells in IPAH patients was associated with an augmented 6-minute walk test distance and pulmonary hemodynamics 12 weeks later. Nonetheless, a number of unanswered questions about this therapeutic approach remain, and a simpler alternative strategy may be the pharmacological manipulation of EPCs in vivo.

We undertook a careful characterization of circulating progenitor cells using a variety of surface markers.10–14

Figure 8. Correlation between hemodynamic parameters (mean pulmonary arterial pressure [PAP] or cardiac index) and cGMP levels or circulating CD34+/KDR+ cells in patients with IPAH. Parameters were log transformed.
Adherent PBMNCs, after short-term culture, have been widely studied and previously also classified as EPCs, but they are now considered to be mainly of monocyte origin.\textsuperscript{43} Despite their distinct phenotypes,\textsuperscript{12} the colony-forming capacity and in vitro function of cultured PBMNCs have been related to endothelial dysfunction, cardiovascular symptoms, and outcome.\textsuperscript{15,44} In the present study, in vitro analysis of PBMNCs revealed differences between patients similar to those of circulating CD34\textsuperscript{+}/AC133\textsuperscript{+} cells. Thus, although the number and in vitro function of cells from IPAH patients were similar or even greater than in control subjects, both were severely impaired in Eisenmenger patients. It is uncertain whether therapeutic intervention in PAH patients influences cultured and circulating cells, but components of the NO-cGMP pathway are present in PBMNCs\textsuperscript{40,45} and PDE5 inhibition for erectile dysfunction associated with augmented \textsuperscript{46} several cytokines and growth factors are implicated in the mobilization and homing of EPCs. Although levels of inflammatory mediators and ADMA were elevated in both forms of PAH studied, levels of the angiogenic protein VEGF were selectively reduced in Eisenmenger patients. VEGF is a potent stimulus for mobilizing bone marrow–derived EPCs,\textsuperscript{47} and lower levels may be important in this patient group. Indeed, it may be interesting to investigate whether an imbalance exists between other angiogenic (eg, IL-8) and antiangiogenic factors (eg, endostatin) in different forms of PAH.

**Clinical Implications**

Endothelial dysfunction is a hallmark of PAH, and recent evidence suggests that bone marrow–derived cells participate in postnatal blood vessel repair and neovascularization.\textsuperscript{8} The relative deficiency of circulating EPCs in PAH patients may contribute to the pulmonary vascular pathology, whereas chronic pharmacological augmentation with PDE5 inhibitors could offer a novel therapeutic strategy. On the other hand, resistance to apoptosis and proliferation of pulmonary vascular endothelial cells are implicated in the progression of PAH,\textsuperscript{48} and incorporation of circulating progenitors may have adverse long-term consequences. Further studies are needed to understand the therapeutic implications of chronic PDE5 inhibition on circulating EPCs in PAH.

**Study Limitations**

One of the limitations of this study is that it did not investigate the dynamics of circulating EPCs and thus cannot report on whether differences reflect alterations in EPC mobilization, survival, or tissue uptake. Changes in EPCs with time in individual patients would be of interest, particularly when related to clinical status, measures of vascular endothelial function, and the abundance of EPCs in the pulmonary vasculature.

**Conclusions**

Circulating EPCs are reduced in patients with Eisenmenger syndrome or IPAH. Circulating EPC numbers correlated with levels of inflammatory mediators, indexes of NO synthesis, and ADMA production and are related to exercise capacity and central hemodynamic markers. Treatment with sildenafil is associated with a dose-related increase in EPC numbers and may represent a novel pharmacological means of increasing circulating EPCs in PAH patients.

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