Distinct Patterns of Circulating Endothelial Cells in Pulmonary Hypertension

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Abstract

The respective abundance of circulating endothelial cells and endothelial progenitor cells may reflect the balance between vascular injury and repair. As pulmonary arterial hypertension (PAH) and chronic thromboembolic pulmonary hypertension (CTEPH) can share features of pulmonary remodeling, we postulated that the two disorders might be associated with different types of pulmonary endothelial dysfunction.

We studied 25 consecutive patients undergoing cardiac catheterization for suspected pulmonary hypertension. Nine patients had PAH, nine had CTEPH, and seven had normal pulmonary arterial pressure and served as controls. Circulating endothelial cells were isolated with CD146-coated beads. CD34⁺CD133⁺ cells and endothelial progenitor cell numbers were respectively determined by flow cytometry and cell culture, in peripheral vein and pulmonary artery blood. Plasma levels of soluble VEGF, sE-selectin and sVCAM were measured by ELISA.

No difference in progenitor counts or VEGF levels was found across the three groups. Compared to controls, circulating endothelial cell numbers were significantly increased in PAH but not in CTEPH, in keeping with the elevated sE-selectin and sVCAM levels found in PAH alone.

In conclusion, PAH, by contrast to CTEPH, is associated with markers of vascular injury (circulating endothelial cells, sE-selectin and sVCAM) but not with markers of remodeling (endothelial progenitor cells, CD34⁺CD133⁺, VEGF).

Key words: pulmonary hypertension – circulating endothelial cells – circulating progenitor cells - endothelial progenitor cells
INTRODUCTION

Vascular remodeling is observed in several disorders characterized by increased pulmonary vascular resistance and pulmonary hypertension. Recently, the 4TH World Symposium on pulmonary hypertension proposed an updated clinical classification (1), aimed at grouping together different disease manifestations sharing similar pathophysiologic mechanisms, clinical features and therapeutic management. Pulmonary arterial hypertension (PAH, group 1) is a complex and rapidly progressive disorder: without treatment, right heart failure and death ensue within a median of 2.8 years (2). Vessel wall remodeling and cellular hyperproliferation are the hallmarks of severe disease (3, 4). Plexiform lesions in PAH are due to abnormal and disorganized proliferation of monoclonal endothelial cells, that obstruct and eventually occlude the vascular lumen (5-7). Chronic thromboembolic pulmonary hypertension (CTEPH) is characterized by intraluminal thrombus organization, fibrous stenosis, and, in some cases, complete obliteration of pulmonary arteries (1). Pulmonary endarterectomy is the treatment of choice for CTEPH and is the only potentially curative option. However, progressive pulmonary vascular remodeling in the non obstructed vascular bed is thought to be the main pathophysiologic mechanism of CTEPH (8, 9).

Endothelial cell dysfunction is a hallmark both of PAH and of pulmonary hypertension secondary to congenital heart disease (10). Most relevant human data have been obtained with surgical lung biopsy material, through histomorphological studies and generation of vascular structural cell lines. Because lung biopsy is highly invasive, cellular biomarkers of pulmonary vascular remodeling and dysfunction could be very useful for diagnosis and/or prognostication. The ratio between circulating endothelial cells (CEC) and endothelial progenitor cells has raised considerable interest as a marker of the balance between endothelial damage and regeneration, and therefore as a non invasive marker of vascular dysfunction (11).
CEC are non-invasive markers of vascular damage, remodeling and dysfunction (12). CEC are present at very low levels in healthy subjects and have been described as a marker of systemic vascular dysfunction (11). Peripheral CEC counts are elevated in human primary and secondary pulmonary hypertension (13), including pulmonary hypertension secondary to congenital heart disease (14). Several populations of bone marrow-derived cells expressing endothelial markers migrate to sites of neovascularization (15). Hill’s method of counting endothelial progenitor cells is now available in the form of a commercial kit that identifies so-called “CFU-EC”, namely endothelial cells expressing myeloid markers (16). The peripheral blood CFU-EC count is low in patients with cardiovascular risk factors (16). CFU-EC numbers are diminished in chronic obstructive pulmonary disease and correlate negatively with disease severity (17). Angiogenic gene therapy (18) and endothelial progenitor cells transplantation were recently shown to attenuate pulmonary hypertension in a rat model (19, 20), while Wang et al. found that endothelial progenitor cell transplantation improved exercise capacity and pulmonary hemodynamics in patients with idiopathic pulmonary hypertension (21). However, reports on endothelial progenitor cell numbers in pulmonary hypertension are controversial (22-24), but CEC and endothelial progenitor cells numbers have not yet been compared in the same population of adults with pulmonary hypertension.

As the endothelial phenotype is strongly modified in pulmonary hypertension, we postulated that the circulating endothelial compartment, i.e. CEC and CFU-EC, might be differently modulated in PAH and CTEPH. We therefore determined CEC, CD34+CD133+ cells and CFU-EC numbers in both peripheral venous blood and pulmonary arterial blood from subjects with PAH and CTEPH. We then analyzed the results with respect to the clinical and hemodynamic characteristics of the two disorders and to soluble biomarkers of endothelial damage and regeneration.
PATIENTS AND METHODS

Study population

The study was approved by the Necker ethics committee (Comité de protection des personnes Ile de France II), and signed informed consent was obtained from all the patients and controls. We enrolled 25 consecutive patients undergoing right-heart cardiac catheterization at Georges Pompidou European Hospital during routine management of suspected pulmonary hypertension, in keeping with current guidelines. Right atrial pressure, pulmonary artery pressure, pulmonary artery wedge pressure, cardiac output and pulmonary vascular resistance (PVR) were measured in each patient during catheterization. Pulmonary hypertension was defined as a mean pulmonary artery pressure greater than 25 mmHg at rest and a pulmonary artery wedge pressure less than 15 mmHg during cardiac catheterization (25). Pulmonary hypertension was confirmed in 18 patients and ruled out in 7 patients. Following the updated clinical classification of pulmonary hypertension and current guidelines, these 18 patients were classified as having PAH (n = 9; idiopathic: n=3, associated with connective tissue disease (n=3), portal hypertension (n=2) or HIV infection (n=1)) or CTEPH (n = 9). The seven patients who underwent catheterization because they had echocardiographic signs of pulmonary hypertension but whose pulmonary arterial pressure was found to be normal served as controls. All patients had the 6-minute walk test (6WT). All patients with pulmonary hypertension had acute vasodilator testing with inhaled nitric oxide (NO) 10 ppm for 10 minutes, as recommended by the recent European guidelines for the diagnosis and treatment of pulmonary hypertension (26). Only one patient with PAH had an acute response, defined as a reduction in mean PAP ≥ 10 mmHg, reaching an absolute mean PAP of ≤ 40 mmHg with increased or unchanged cardiac output. The BMPR2 gene had been analyzed in
only one patient with idiopathic PAH and was found to be normal. Three patients with PAH were receiving specific drug therapy at the time of right heart catheterization: one patient with idiopathic PAH received a combination of continuous intravenous prostacyclin, sildenafil and bosentan, while the other two patients received bosentan monotherapy. Two patients with inoperable CTEPH were receiving specific drug therapy at the time of right heart catheterization, consisting of a combination of subcutaneous treprostinil, bosentan and sildenafil in one case, and bosentan monotherapy in the other case. Three patients were evaluated twice (at diagnosis and after 3 months of first-line bosentan vasodilator therapy).

**CEC isolation and counting**

Peripheral venous and pulmonary arterial blood samples were collected on EDTA during cardiac catheterization. For CEC quantification and CFU-EC and CD34⁺CD133⁺ counts, the first milliliters of blood was discarded to avoid collecting endothelial cells dislodged by puncture. CEC were counted by an operator who was unaware of the patients’ clinical status. CEC in whole blood were immunocaptured at 4°C with magnetic beads (Dynabeads M-450, Dynal®, Invitrogen, Cergy-pontoise, France) coated with S-Endo 1 (Biocytex®, Marseille, France), a monoclonal antibody against the endothelial antigen CD146. To avoid non specific leukocyte binding to the beads, the cell suspensions were flushed vigorously through the pipette tip during the washing steps and then suspended in acridine orange (3 µg/mL in PBS; Sigma-Aldrich®, Saint-Quentin Fallavier, France) before counting under a fluorescence microscope (λ_exc=490 nm). CEC were identified as >20-µm-diameter cells bearing >10 beads, or as cells with <10 beads but with a well-preserved and recognizable morphology (clear nucleus in a well-defined cytoplasm, and a size compatible with that of endothelial cells). The number of cells in aggregates was determined from the number of nuclei or spherical rosette features. The endothelial nature of the cells thus isolated was confirmed by measuring lectin
Flow cytometric quantification of circulating CD34⁺CD133⁺ progenitor cells in pulmonary hypertension

CD34⁺CD133⁺ cells were quantified in EDTA-anticoagulated peripheral blood and pulmonary arterial blood by means of two-color flow cytometry on a FACScalibur device (Becton-Dickinson, Le Pont de Claix, France). Briefly, 100 μl of blood was incubated with 10 μl of phycoerythrin-cyanin-5 (PC5)-conjugated anti-human CD34 mAb (Beckman-Coulter, Villepinte, France) and 10 μl of phycoerythrin (PE)-conjugated anti-human-CD133 mAb (Miltenyi Biotec, Bergisch-Gladbach, Germany). Non immune mAbs of the same isotype as the immune mAbs and provided by the same manufacturer were used as controls. The number of positive peripheral blood cells was determined by two-dimensional side-scatter fluorescence dot-plot analysis after gating on the lymphocyte population. Each analysis comprised 30 000 events. The number of CD34⁺CD133⁺ cells was normalized and expressed per 1x10⁵ circulating lymphocytes.

CFU-GM quantification

Mononuclear cells (4 × 10⁵ per dish) were seeded in methylcellulose plates (Stem alpha 1F, Stem alpha®, Saint-Genis l’Argentières, France) in medium supplemented with stem cell factor, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, erythropoietin, interleukin 3, and interleukin 6. Granulocyte-macrophage colony-forming units (CFU-GM) were counted after 14 days of culture, by phase-contrast microscopy.

Flow cytometric quantification of CD34⁺CD133⁺KDR⁺ cells
CD34⁺CD133⁺KDR⁺ cells were quantified in EDTA-anticoagulated peripheral blood by flow cytometry on a BD-LSRII® flow cytometer (Becton-Dickinson, Le pont du chaix, France). Briefly, after Ficoll gradient, 100 μl of mononuclear cell suspension was incubated with 10 μl of FITC-conjugated anti-human CD34 mAb (Beckman-Coulter, Villepinte France), 10 μl of phycoerythrin (PE)-conjugated anti-human-CD133 mAb (Miltenyi Biotec, Bergisch-Gladbach, Germany) and 10 μl of APC-conjugated anti-human KDR mAb (Becton Dickinson). Non immune mAbs of the same isotype as the immune mAbs and provided by the same manufacturer were used as controls.

**Sorting of CD34⁺CD133⁺ cells**

CD34⁺CD133⁺ cells were sorted "C:\Program Files\EndNote 9\"with a BD-FACSariaII® flow cytometer (Becton-Dickinson). Briefly, after Ficoll separation, 10 μl of FITC-conjugated anti-human CD34 mAb (Beckman-Coulter) and 10 μl of phycoerythrin (PE)-conjugated anti-human-CD133 mAb (Miltenyi Biotec) were incubated with 2x10⁶ mononuclear cells. After staining, cells were resuspended in PBS-0.5% BSA at 1x10⁶ cells per 100 μL. The suspension was filtered through BD tubes with cell strainer caps (Becton Dickinson) just before cell sorting. CD34+CD133+ cells were collected in tubes coated with BSA.

**Quantification of CFU-EC**

EDTA-anticoagulated blood was diluted 1:1 with PBS, 0.2 M EDTA and overlaid on Histopaque-1077 (Sigma-Aldrich, Saint-Quentin Fallavier, France). Cells were centrifuged at 100 g for 20 minutes. Mononuclear cells (MNC) were collected and washed 3 times in PBS, 0.2 M EDTA. CFU-EC were cultured with the EndoCult® Liquid Medium kit (StemCell Technologies, Vancouver, BC, Canada) as recommended by the manufacturer. Briefly, MNC
were resuspended in complete EndoCult® medium and seeded at 5x10^6 cells/well in fibronection-coated tissue culture plates (BD, Becton-Dickinson Biosciences, Le pont de chaix, France). After 48 h, non adherent cells were collected and plated in Endocult® buffer at 10^6 cells/well in 24-well fibronectin-coated plates, and colonies were counted after another 3 days.

**Measurement of soluble endothelial biomarkers**

Peripheral venous and pulmonary arterial blood was collected during cardiac catheterization in tubes containing 0.105 M sodium citrate (1 vol/9 vol). Plasma was obtained by double centrifugation at 2300 g for 10 minutes and was immediately placed at −80°C until use. Plasma levels of VEGF, sE-selectin and sVCAM were measured with highly sensitive enzyme-linked immunosorbent assays (ELISA) as recommended by the manufacturer (R&D Systems, Minneapolis, MN, USA).

**Statistical analysis**

The non parametric Mann-Whitney \( U \) test was used to identify differences in peripheral markers between the two patient groups and the controls. A paired t test was used to determine the significance of differences between CEC and CD34+CD133+ numbers in the two types of blood sample. Statistical significance was assumed at \( p \leq 0.05 \). All statistical tests were performed with the Stat View software package (SAS, Cary, NC, USA).
RESULTS

Study population

The patients’ and controls’ clinical and hemodynamic characteristics are summarized in table 1. The patients with PAH and CTEPH were comparable in terms of demographic and hemodynamic parameters [mean pulmonary arterial pressure, pulmonary vascular resistance and walking test values: p=0.54, 0.38 and 0.87, respectively].

Circulating endothelial cell (CEC) numbers

Immunomagnetically isolated CEC were observed after staining with the fluorescent probe acridin orange or Ulex europaeus agglutinin I (Figure 1A/B). No significant difference in CEC counts was noted between the two types of blood sample in any of the three groups of subjects (table 2). Peripheral CEC counts were normal (<10 CEC per ml in healthy adults, as defined by the consensus protocol (12)) in both the controls and the patients with CTEPH. In contrast, the mean CEC count was 6-fold higher in PAH patients than in controls (p= 0.01) and 4-fold higher than in CTEPH patients (p= 0.04) (Table 2 and Figure 1B). Moreover, we found no correlation between CEC counts and the NYHA classification or the following hemodynamic data: mean pulmonary pressure [-5.2E-19 (Spearman correlation coefficient p=0.99)], systolic pulmonary pressure [0.139 (p=0.37)], diastolic pulmonary pressure [0.142(p=0.58)], 6-minute walking distance [0.3571 (p= 0.38)], the cardiac index [0.4185 (p= 0.27)] and or pulmonary vascular resistance [-0.2167 (p= 0.58)]. Three PAH patients could be re-evaluated 3 months after diagnosis and initiation of specific treatment with bosentan (Tracleer®), and a marked decrease in CEC counts was observed (Figure 1C).

Circulating CD34⁺CD133⁺ progenitor cell numbers
No significant difference in CD34⁺CD133⁺ counts was found according to the sampling site (Table 2), and the peripheral CD34⁺CD133⁺ counts did not differ significantly across the three groups (controls, PAH and CTEPH) (Figure 2A). CD34⁺CD133⁺ cells were also quantified in two other control groups (48 healthy volunteers and 30 patients without cardiovascular risk factors who were scheduled for valve surgery), and no difference was observed between these groups and the study control group (Figure 2A). CD34⁺CD133⁺ cell counts were not modified by specific therapy in the three treated patients with PAH (data not shown). A subset of CD34⁺CD133⁺ cells (0.4%) expressed the VEGR receptor 2 (KDR), by contrast to the CD34⁺CD133⁻ population, that contained only 0.03% of cells expressing KDR (Figure 2B). CD34⁺CD133⁺-sorted cells were further able to differentiate in CFU-GM in a clonogenic assay, in line with their other features of progenitor cells (Figure 2B). CFU-GM were quantified in each group and, in line with the CD34⁺CD133⁺ cell counts, no difference was observed between the patients and controls (p=0.91 and p=0.66, respectively, for PAH and CTEPH vs controls; and p=0.68 for PAH vs CTEPH) (Figure 2C).

**CFU-EC numbers**

On day 5 of culture, CFU-EC colonies consisted of elongated sprouting cells radiating from a central core of round cells (Figure 3A). These cells expressed von Willebrand factor (Figure 3B), KDR and CD31 (Figure 3C/D) and were able to secrete large amount of the angiogenic growth factors VEGF and SDF-1 (Figure 3D). No difference in CFU-EC numbers was found between the two sampling sites in any of the three groups (Table 2). Peripheral CFU-EC counts were similar in the three groups [median (range): 4 (0-11), 6 (0-37) and 8 (0-23) CFU-EC/10⁶ mononuclear cells in PAH and CTEPH patients and controls, respectively; NS for all comparisons] (Table 2 and Figure 3E). CFU-EC counts were not influenced by specific treatment in the three PAH patients concerned.
**VEGF, sE-selectin and sVCAM plasma levels**

VEGF levels were similar in the three groups [median (range): 20.7 (12.6-115.2), 33.4 (33.3-52.6) and 29.6 (13.9-71.2) pg/ml in PAH, CTEPH and controls, respectively; NS for all comparisons] (Figure 4A). By contrast, sE-selectin levels were significantly higher in PAH patients than in CTEPH patients (p = 0.04) and in the controls (p = 0.03) [median (range): 45 (32-57), 33 (19-53) and 29 (14-71) ng/ml, in PAH and CTEPH patients and controls, respectively] (Figure 4B). In line with the results for sE-selectin, levels of sVCAM, another biomarker of endothelial lesions and/or activation, were significantly higher in PAH patients than in CTEPH patients (p = 0.02) and in the controls (p = 0.006) [median (range): 1167 (455-3454), 544 (402-1347) and 486.5 (279-1084) ng/ml, in PAH and CTEPH patients and controls, respectively] (Figure 4C).
DISCUSSION

This is the first study to quantify the different components of the circulating endothelial compartment (CEC, CFU-EC, CD34⁺CD133⁺) and to compare their abundance in two different forms of pulmonary hypertension. In PAH, contrary to CTEPH, we found elevated CEC numbers and high levels of sE-selectin and sVCAM, both of which are recognized markers of vascular damage or activation, whereas markers of endothelial remodeling (CFU-EC, CD34⁺CD133⁺ and VEGF) were normal in both settings.

One important result of this study is the similar numbers of CEC, CD34⁺CD133⁺ and CFU-EC in peripheral and pulmonary blood. When we started this study, we had no knowledge of CEC clearance from peripheral blood, and therefore studied local release of CEC in blood sampled from the pulmonary artery. We were surprised to find that peripheral blood gave identical results. However, in a previous study of children with pulmonary hypertension secondary to congenital heart disease, we also found similar CEC numbers in peripheral blood and pulmonary artery blood (14). This suggests that peripheral blood is a reliable proxy for the central compartment, despite conjecture that the latter would be more appropriate for detecting markers of endothelial dysfunction in pulmonary hypertension. If this is confirmed, further studies of endothelial status in pulmonary hypertension would require only minimally invasive sampling procedures.

CEC are a novel marker of endothelial damage, and their numbers correlate with other markers of endothelial function such as flow-mediated dilation and von Willebrand factor and tissue plasminogen activator levels (27). CEC counts are increased in coronary heart disease, renal vascular disease (28) and transplantation (29) and are a marker of progression and survival in acute coronary disease (30). Very few data are available on CEC counts in pulmonary hypertension. Bull et al. found that CEC numbers were increased in adults with
various types of pulmonary hypertension (13). We recently showed that preoperative CEC numbers distinguished two groups of children with PAH secondary to congenital heart disease, namely those with markedly increased CEC numbers at baseline, in whom PAH became irreversible after surgery, and those with low CEC counts and reversible PAH (14). The latter study established CEC as a biomarker with strong prognostic value in this setting. For CEC quantification in the present study, it should be noted that we discarded the first milliliters of blood in order to avoid collecting endothelial cells dislodged by puncture.

CEC counts were increased in subjects with PAH and normal in patients with CTEPH. In addition, we found no correlation between the CEC count and hemodynamic parameters (mean pulmonary pressure, PVR, cardiac index) in either patient group. It is also important to note that the two groups were comparable in terms of hemodynamic and clinical parameters (Table 1).

Taken together, these data suggest that high pulmonary pressures are not the main trigger of endothelial cell detachment from vessel walls and their subsequent circulation. Rather, the supernumerary CEC observed in PAH might result from increased vascular activation and/or remodeling. From a histopathological standpoint however, the both disorders are associated with distal microvascular remodeling, which includes smooth muscle cell proliferation and arteriolar muscularization, fibrosis, endothelial cell proliferation, intimal thickening and **in situ** thrombosis. Plexiform lesions have a similar histologic aspect in idiopathic and secondary PAH. Also, actively proliferating endothelial cells are found in both forms of the disease, with no evidence of apoptosis. One particular characteristic of idiopathic PAH might however be critical, namely the nature of the endothelial cell expansion observed in plexiform lesions: it is monoclonal in idiopathic PAH (6, 7), whereas monoclonality has never been reported in other forms of PAH. Whether or not this reflects a particular capacity of endothelial cells to be shed from vessels in PAH remains to be investigated. Moreover, Moser and Bloor (9) conducted a
comprehensive and systematic histopathologic analysis of small pulmonary arteries in patients with CTEPH and concluded that these patients displayed a full range of distal histopathology changes indicating advanced vessel remodelling, including plexiform lesions, smooth muscle cells hypertrophy, and intimal proliferation/fibrosis. These lesions are also found in pulmonary arterial hypertension, without obstructive vessels specifically due to pulmonary embolism. We found that bosentan, an endothelin receptor antagonist, was associated with a decrease in CEC numbers after 3 months of treatment in all three patients we were able to study. It has been postulated that CEC are released upon uncontrolled endothelial cell proliferation or activation in pulmonary hypertension. By regulating vascular resistance, bosentan might reduce endothelial activation and, thus, CEC release.

Regarding the distinction between CEC and CD34⁺CD133⁺, the method we used to quantify CEC, based on their size and the number of coated beads, allowed us to distinguish CEC from CD146-expressing CD133⁺ cells. Delorme et al. found that progenitor cells coexpressing CD146 and CD133, an antigen associated with immaturity, looked significantly different from mature CEC, which had morphologic features of differentiated cells and were more than 20 μm in diameter (31). It is therefore unlikely that the CEC we counted using the IMS method included progenitor cells.

As endothelial progenitor cells are involved in healing processes (15), and as neoangiogenesis is consistently observed in PAH, we suspected that endothelial progenitor cells numbers might be elevated in this setting. Because of the observed EC monoclonality, and as endothelial progenitor cells exhibit clonogenic properties (32, 33), we postulated that they might be partly responsible for the endothelial proliferation observed in PAH. As there is no consensus method for accurately quantifying endothelial progenitors in small samples, we chose to quantify circulating CD34⁺CD133⁺ progenitor cells, that comprise a subpopulation of endothelial progenitors and have been described as the source of endothelial progenitor cells.
in culture (34-36). In experimental models of hypoxia-induced PAH, endothelial progenitors appear to be involved in pulmonary vascular remodeling (37). However, human studies have variously shown increased CD34⁺CD133⁺ numbers in patients with PAH (22) or no correlation between PAH and CD34⁺CD133⁺ (24). In another study, numbers of endothelial progenitors isolated by cell culture were elevated in PAH (23). In the present study, CD34⁺CD133⁺ and CFU-EC counts did not differ between the pulmonary hypertension and control groups (Figures 2 and 3). Further studies are needed to confirm the observed lack of EC progenitor mobilization in pulmonary hypertension, based on the assessment of different types of endothelial progenitor (CD34⁺CD133⁺ cells expressing KDR and/or CD146) and late-outgrowth endothelial progenitor cells obtained in culture. However, it is noteworthy that, although angiogenic and inflammatory marker levels have been found to correlate with the abundance of CD34⁺CD133⁺ and endothelial progenitors (38, 39), VEGF levels were similar in our patient groups and controls, further supporting the lack of circulating EC progenitor mobilization. The VEGF levels found here are similar to those found in healthy male and female volunteers (40). In the present study, VEGF was quantified in plasma and not in serum, in order to rule out any potential influence of platelet VEGF granule content release. Indeed, it has been reported that VEGF of platelet origin can interfere with the pathophysiology of pulmonary hypertension (41) and with VEGF quantification in serum (42).

The source of the EC involved in the vascular remodeling associated with pulmonary hypertension is unclear. One possibility is that vascular progenitors may be recruited from a stem cell niche within the lung, as opposed to resulting from systemic progenitor mobilization. Finally, sE-selectin and sVCAM, two adhesion molecule inducible on endothelial cells, have also been linked to endothelial damage and/or activation. Recently, E-selectin was described as a pivotal molecule for endothelial progenitor cell homing (43), whereas sVCAM plasma
levels in pulmonary hypertension have been shown to correlate with endothelial activation reflected by endothelial microparticle detection (44). The elevated plasma levels of sE-selectin and sVCAM that we observed in PAH could, along with the excess of CEC, reflect intense endothelial activation, which might participate in local pulmonary recruitment of progenitor cells, although this needs to be demonstrated in larger studies.

In conclusion, this study shows that levels of circulating biomarkers of endothelial activation differ markedly between patients with pulmonary arterial hypertension and patients with chronic thromboembolic pulmonary hypertension of comparable severity. The value of CEC as a biomarker of clinical outcome and/or the treatment response in PAH remains to be determined.
Acknowledgments:

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Conflicts of interest: none
Table 1. Characteristics of the patients

Data are presented as medians and [25th – 75th percentiles]

PAH: pulmonary arterial hypertension; CTEPH: chronic thromboembolic pulmonary hypertension; NYHA: New York Heart Association functional class; 6’WT: 6-minute walk test, RAP: right atrial pressure; mPAP: mean pulmonary artery pressure; sPAP: systolic pulmonary artery pressure; dPAP: diastolic pulmonary artery pressure; CI: cardiac index; PVRi: indexed pulmonary vascular resistance; acute responder: acute response to inhaled NO testing; RHC: right heart catheterization

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<th>Controls (n=7)</th>
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<th>CTEPH (n=9)</th>
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Table 2. Comparative CEC, CD34+CD133+ and CFU-EC counts in pulmonary artery and peripheral vein blood samples obtained during catheterization

Data are geometric means and confidence intervals (95%CI). As the sampling site and interaction effects were not statistically significant, the groups were further compared independently of the sampling site.

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<th>CTEPH (n=9)</th>
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<td>Versus PAH (PV)</td>
<td></td>
<td></td>
<td>*p=0.04</td>
</tr>
<tr>
<td><strong>CD34+CD133+ counts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(/10^5 lymphocytes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral vein (PV)</td>
<td>53.3 (23;79)</td>
<td>43.3 (3;86)</td>
<td>20 (16;89)</td>
</tr>
<tr>
<td>Pulmonary artery (PA)</td>
<td>50 (20;99)</td>
<td>31.6 (0;79)</td>
<td>36.6 (16;86)</td>
</tr>
<tr>
<td>PV versus PA</td>
<td>p=0.84</td>
<td>p=0.81</td>
<td>p=0.19</td>
</tr>
<tr>
<td>Versus controls (PV)</td>
<td>p=0.85</td>
<td></td>
<td>p=0.17</td>
</tr>
<tr>
<td>Versus PAH (PV)</td>
<td></td>
<td></td>
<td>p=0.13</td>
</tr>
<tr>
<td><strong>CFU-EC counts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cells/10^6 mononuclear cells)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral vein (PV)</td>
<td>8.4 (0;23)</td>
<td>4 (0;11)</td>
<td>6.3 (0;3)</td>
</tr>
<tr>
<td>Pulmonary artery (PA)</td>
<td>8 (1;28)</td>
<td>3.7 (0;10.2)</td>
<td>4 (0;19.1)</td>
</tr>
<tr>
<td>PV versus PA</td>
<td>p=0.43</td>
<td>p=0.25</td>
<td>p=0.31</td>
</tr>
<tr>
<td>Versus controls (PV)</td>
<td>p=0.19</td>
<td></td>
<td>p=0.48</td>
</tr>
<tr>
<td>Versus PAH (PV)</td>
<td></td>
<td></td>
<td>p=0.36</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. CEC morphology and counts in peripheral venous blood of patients with pulmonary hypertension.

A/B/C: Identification of CEC by fluorescent microscopy after immunomagnetic separation and staining with *Ulex europaeus* agglutinin I (UEA I) or acridine. The arrows indicate A- a CEC after staining with UEA I, B- a CEC after staining with acridine, C- a lymphocyte after staining with acridine.

D: CEC counts in peripheral venous blood of PAH and CTEPH patients. CEC counts were significantly increased in PAH (*p=0.01 and *p=0.04, respectively, versus controls and patients with CTEPH).
E: CEC quantification in 3 PAH patients before and after a 3-month course of first-line bosentan therapy.

Figure 2. CD34+CD133+ counts in peripheral venous blood in PAH and CTEPH patients.

A. CD34+CD133+ were quantified by flow cytometry. The patients did not differ significantly from the controls. (p=0.85 and p=0.17, respectively, for PAH and CTEPH vs controls and p=0.13 for PAH vs CTEPH).
B. 0.4% of CD34⁺CD133⁺ were positive for KDR. CD34⁺CD133⁺ cells sorted with a BD-FACSariaII® flow cytometer (Becton-Dickinson) were able to differentiate into CFU-GM in a clonogenic system (methyl cellulose).

C. CFU-GM were quantified with a clonogenic assay (methyl cellulose). The patients did not differ significantly from the controls (p=0.91 and p=0.66, respectively, for PAH and CTEPH vs controls and p=0.68 for PAH vs CTEPH).
Figure 3. CFU-EC quantification in PAH and CTEPH

A. Representative CFU-EC. Note the central core of rounded cells with spindle-shaped cells sprouting through the periphery.

B/C/D: CFU-EC immunohistological characterization (von Willebrand factor, inset: isotypic control), cytometric analysis (KDR) and real-time quantitative PCR (CD31, VEGF, SDF-1 and CXCR-4)

E. Numbers of CFU-EC colonies in PAH, counted with Endocult®. Results are the number of adherent cell clusters per $10^6$ mononuclear cells. Results are means ± SEM (p=0.19 and p=0.48, respectively, for PAH vs controls and p=0.36 for PAH vs CTEPH).
Figure 4. Plasma levels of VEGF, sE-selectin and sVCAM in PAH and CTEPH.

Plasma levels of VEGF (pg/ml, A), sE-selectin (ng/ml, B) and sVCAM (ng/ml, C) were measured with ELISA. sE-selectin and sVCAM were increased in PAH (sE-selectin: *p=0.04 and *p=0.03, respectively, for PAH vs CTEPH and controls; sVCAM: *p=0.02 and *p=0.006, respectively, for PAH vs CTEPH and controls).
REFERENCES


