CORRESPONDENCE

Circulating Endothelial Cell Quantification by Microfluidics Chip in Pulmonary Arterial Hypertension

To the Editor:

Pulmonary arterial hypertension (PAH) is a fatal disease with multiple causes, which is characterized by pulmonary vascular remodeling eventually leading to heart failure (1). Recent studies have unraveled several molecular and cellular pathways that play important roles in PAH and may lead to the development of novel diagnostic and therapeutic approaches. However, the average time between first symptoms and the diagnosis of PAH is >2 years (2, 3). Thus, early diagnosis and adequate risk stratification is pivotal in providing optimal, individualized ("tailored") therapies for PAH. Several molecular and cellular biomarkers have been evaluated in patients with PAH (4). Two cell populations that can be found in peripheral blood and are associated with vascular remodeling have attained particular attention. First, endothelial progenitor cells (EPCs) are bone marrow derived and contribute to vascular repair. We, and others, have shown that EPCs ($CD34^+/KDR^+/CD31^+/CD45^-$) are decreased in PAH (5, 6). Second, circulating endothelial cells (CECs) have been discussed as potential biomarkers in PAH with or without congenital heart disease. CECs originate from the shedding of endothelial cells from damaged vessels and are thought to directly reflect vascular damage. CECs are increased in vascular injury–related disorders such as acute myocardial infarction and unstable angina (7). Data on CEC numbers in PAH are sparse and have been gathered in small patient populations only (8–11).

Current protocols for the isolation and quantification of EPCs and CECs are laborious and time consuming (5, 12). Recently, we developed a disposable microfluidic platform capable of selectively capturing and enumerating EPCs directly from human whole blood (5). Here, we report on the creation of a modified microfluidic chip device for measuring CEC numbers and its application in patients with PAH.

A detailed description of the methods, including the microfluidic chip design (13), can be found in the online supplement.

First, we assessed the reliability of the CEC capture chip by validating the technique against established flow cytometry. We found a strong correlation between the two methods ($r = 0.89$, Figure E1 in the online supplement). To test the clinical applicability of CEC quantification by microfluidic chip, ethylenediaminetetraacetic acid–whole blood was collected from 66 women with PAH (idiopathic PAH, drug-induced PAH, and connective tissue disease-associated PAH) and 6 healthy age- and sex-matched control subjects (Table 1). Individual

patient characteristics are provided in Table E1 in the online supplement.

We found CEC numbers to be increased by three- to fivefold in patients with PAH versus matched control subjects ($P < 0.001$), regardless of the markers used for CEC quantification ($CD146^+$ versus the more stringent $CD146^+/CD31^+$) (Figure 1). Next, we performed a subgroup analysis comparing CEC numbers among different PAH causes (idiopathic, connective tissue disease–associated, drug-induced). Indeed, we found consistent and significant elevation of CECs of similar magnitude in all three PAH subtypes compared with control subjects (Figure 1).

Obesity, insulin resistance, and age are known disease modifiers in PAH (5, 13). Therefore, we tested for correlations between CEC numbers and body mass index and age, but we did not find any according associations (Figure E2).

Cellular biomarkers, such as EPCs and CECs, that reflect vascular repair and remodeling are attractive candidates for monitoring PAH disease severity and progression (4). In contrast to molecular biomarkers, which can be quantified easily by standard assays (e.g., ELISA, polymerase chain reaction) (4), current protocols for the isolation and quantification of EPCs and CECs are complex and time consuming. The standard methods include flow cytometry, magnetic beat-based approaches, and colony-forming assays (5, 12). Here, we developed a microfluidic CEC capture chip and found that the device reliably detected CECs. The CEC capture chip is small ($5 \times 30 \times 0.05$ mm^3), requires only 200–400 µl of whole blood without any preprocessing, and can principally be automated (Figure E3). Therefore, the CEC device represents a potential bedside test for the screening and monitoring of patients with PAH and other diseases related to vascular injury.

Previously, we found lower EPC numbers to be present in patients with PAH compared with control subjects, likely reflecting the reduced vascular regenerative potential in these patients. In addition, we demonstrated that EPCs correlated with body weight and postmenopausal status in patients with PAH. In our current study, we demonstrate a three- to fivefold increase in CEC counts in patients with PAH compared with control subjects, which is in line with previous reports showing increased CEC numbers in adult PAH (8, 10). However, unlike EPCs (5), a correlation between CECs and body weight or age could not be demonstrated. As in our previous EPC-PAH study, we did not observe any differences in CEC numbers between different PAH causes.

Our samples were recruited from the research room of a patient conference, and the vast majority of the patients were in World Health Organization functional classes I and II. Unspecific early vascular injury occurs in PAH and may be associated with increased CECs, regardless of the underlying pathomechanism. Thus, longitudinal data are required to assess whether CECs measured by our device adequately reflect the clinical course in those patients. Regarding this, it is important to note that CECs are increased in pediatric patients with PAH and can be used to discriminate between reversible and irreversible PAH associated with cardiovascular shunts in congenital heart disease (9). Furthermore, the same group showed that CEC numbers in children with PAH are associated with clinical worsening and the treatment efficacy of trepostinil (11).

Table 1. Demographic Characteristics of Control Subjects and Patients with PAH Enrolled

Characteristic	Control Subjects	PAH (total)	IPAH	PAH Associated with CTD	Drug- and Toxin-induced PAH
Number	6	66	42	17	7
Age, yr	46.5	50.4	44.5	52.7	54.1
Height, m	1.68	1.63	1.63	1.62	1.65
Weight, kg	75.6	76.0	69.1	74.2	84.6
BMI, kg/m^2	26.6	28.5	25.9	28.3	31.2
Race/ethnicity					
White	5	54	37	12	5
Black	0	2	0	2	0
Asian	0	1	1	0	0
Hispanic or Latino	0	3	2	0	1
Native of Hawaii/Pacific Islander	0	1	1	0	0
Others	1 (Asian/White)	5	1 (Asian/White)	2 (1 Asian/Pacific Islander plus 1 Asian/Indo-Canadian 1 (no data)	1 (Native American/White)

Definition of abbreviations: BMI, body mass index; CTD, connective tissue disease; IPAH, idiopathic pulmonary arterial hypertension; PAH, pulmonary arterial hypertension.

One limitation of our study is the relatively simple definition of CECs as CD146$^+$ or CD146$^+$/CD31$^+$ cells. Although these markers have been used widely for clinical CEC studies (12), some hematopoietic cells might confound the measurements, and specific cell subpopulations cannot be distinguished by dual labeling only. However, the CEC capture chip allows for additional epitope staining and microscopic evaluation of cell morphology.
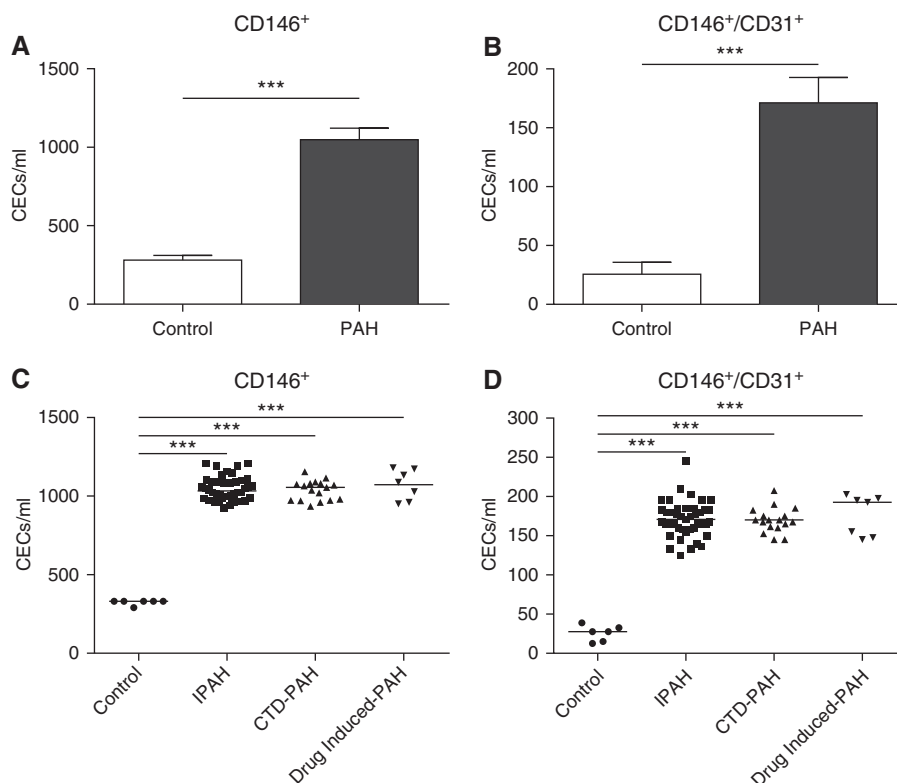

Figure 1. Circulating endothelial cell (CEC) number analysis according to pulmonary arterial hypertension (PAH) subgroup and CEC surface markers. CECs, defined as (*A* and *C*) CD146$^+$, or defined as (*B* and *D*) CD146$^+$/CD31$^+$, were enumerated on the chip. (*A* and *B*) Comparison of patients with PAH with control subjects shows increased overall CEC counts in PAH. (*C* and *D*) Stratification of patients with PAH into subgroups, including IPAH, drug-induced PAH, and CTD-PAH, illustrates that CEC numbers are comparable across PAH subclasses. One-way ANOVA. ***$P < 0.001$. CTD-PAH, pulmonary arterial hypertension associated with connective tissue disease; IPAH, idiopathic pulmonary arterial hypertension.

To date, several studies have identified a variety of markers in PAH, none of which exhibited all the properties of an ideal biomarker (specificity, sensitivity, correlation with disease progression and response to therapy, etc.). Thus, a multiparameter approach is likely needed (4, 14). Further development and validation of simple methodologies, such as the CEC microfluidic capture chip used in our study, are required to allow for a rapid translation of preclinical findings into clinical practice. ■

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