Chipping away at the surface of the endothelial progenitor cell (EPC) mystery

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Published online: 6 August 2011
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Ever since Asahara et al. published their seminal report describing “*putative progenitor endothelial cells*” in 1997 [1], the idea of circulating cells that can replenish dysfunctional endothelium and promote angiogenesis has captured the imagination of cardiovascular researchers. In the following years, these cells were widely referred to as “*endothelial progenitor cells*” or “*EPCs*,” and the adjective “*putative*” that the authors had astutely used in the initial report was dropped. Numerous *in vitro* and *in vivo* studies were subsequently conducted to elucidate the role of these cells in the pathogenesis of cardiovascular disease and to investigate their therapeutic potential. This resulted in roughly 1,000 publications using the expression “endothelial progenitor cells” within a decade of the initial 1997 publication, and by now, this number has grown to over 2,500. Most studies have found that the number of circulating EPCs is reduced in patients with known cardiovascular risk factors or established cardiovascular disease. This has led to the notion that circulating EPCs may be required to maintain a healthy vasculature.

For certain forms of vascular disease, however, both increased as well as reduced levels of circulating EPCs in patients have been reported when compared to control subjects. In the case of pulmonary artery hypertension (PAH), some studies have shown increased levels of circulating EPCs [2, 3] and pointed out that these increased EPC numbers may reflect an ongoing injury response or increased vascular remodeling that is seen in

PAH. A different study, on the other hand, demonstrated that circulating EPC levels are lower in patients with PAH [4] and may be an indicator of endothelial dysfunction that is observed in PAH. Such discordant findings are most likely due to the fact that the definition of what constitutes “EPCs” varies substantially in the mentioned PAH studies [5].

This problem is not unique to EPC measurements in PAH, because there is little consensus as to the actual definition of EPCs. There are two core approaches as to how EPCs are identified: flow cytometric measurement of circulating cells using predefined surface markers versus enumeration of attached cells in culture dishes after circulating mononuclear cells are plated and cultured in an endothelial medium [6]. To further complicate matters, even the choice of flow cytometry markers and culture conditions often vary between studies. Most studies that use flow cytometry markers to characterize EPCs have employed some combination of hematopoietic stem/progenitor markers such as CD34 or CD133, and co-labeled cells with an endothelial marker such as the VEGF-receptor 2 (VEGFR2 or also known as KDR) or CD31 [6].

Studies that employ cell culture-based approaches to identify EPCs use either short-term culture (frequently 4–7 days) or long-term culture (usually 1–3 weeks) of total mononuclear cells in endothelial culture conditions. As expected, the phenotype of the cells obtained from these distinct conditions varies substantially. Short-term culture tends to yield endothelial-like cells that are minimally proliferative and express myeloid and monocytic surface markers (referred to as “early” EPCs, “early-outgrowth” EPCs, circulating angiogenic cells), which likely promote angiogenesis indirectly by secreting growth factors and not by directly replenishing the endothelium [7]. Long-term culture, on the other hand, can yield highly proliferative

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endothelial colony-forming cells that do not express the myeloid marker CD45 but may replenish the endothelium or form the “building blocks” of angiogenesis. These highly proliferative endothelial cells have been referred to as endothelial colony-forming cells (ECFC), “late” EPCs, or “late outgrowth” endothelial cells [6]. The fact that the expression “EPCs” is used for a plethora of cell types, either defined by flow cytometry of circulating cells or for different types of cultured cells, makes it necessary to replace this generic term “EPC” with more specific descriptions of the cells that are being discussed.

While culture-derived “early” and “late” EPCs have been extensively characterized by flow cytometric surface marker analysis, it is still not clear whether flow cytometry on circulating cells can directly identify the cells that are destined to become culture-derived “early” and “late” EPCs and thus circumvent the need for the cumbersome culturing of cells. To date, one of the best studies that have tried to link surface markers on circulating cells to culture-derived “early” and “late” outgrowth EPCs is the one conducted by Timmermans et al., which demonstrated that the highly proliferative “late” EPCs (or ECFCs) are primarily derived from the CD34+ cell population and proposed to use CD34 and the endothelial marker VEGFR2 as markers of cells that may give rise to “late” EPCs. They also recommended co-staining for the hematopoietic lineage marker CD45, since CD45+ cells primarily give rise to myeloid “early” EPCs that do not form proliferative endothelial colonies [8].

In an article in this issue of the *Journal of Molecular Medicine*, Hansmann et al. use this CD34+VEGFR2+ definition of circulating EPCs and present a novel and convenient way of measuring the number of these cells [9]. They have developed a CD34-antibody-coated microfluidic chip, which “captures” CD34+ cells when EDTA-blood obtained from patients is injected through the device. Following the “capture” of CD34+ cells, subsequent immunofluorescence staining for additional markers, such as KDR (*which is a different nomenclature for VEGFR2*), can be performed to yield CD34+KDR+ cells, and this method also permits co-staining for the myeloid marker CD45 to exclude CD34+KDR+ leukocytes that may have been captured by the chip and do not fit the EPC definition. The authors also demonstrate a remarkable correlation between the number of CD34+KDR+ cells identified by flow cytometry and those enumerated by this new capture chip method, as well as minimal day-to-day variation in the number of circulating CD34+KDR+ cell numbers. From a clinical perspective, the authors are able to confirm the previous report that circulating CD34+KDR+ cells are lower in patients with PAH [4] using the novel capture chip method. Interestingly, this study compares the number of circulating CD34+KDR+ cells with the number obtained by using the even more stringent criteria CD34+/KDR+/

CD31+/CD45−. While these more stringent criteria result in a slight decrease of the number of captured cells, this minimal decrease affects the circulating numbers in both control subjects and PAH patients and therefore does not significantly affect the observed difference between these two groups. Enumeration of circulating CD34+KDR+ cells has also been performed by groups studying patients with atherosclerosis or cardiovascular risk factors; therefore, the utility of this novel capture chip is not limited to PAH patients but may be even more broadly applicable to all patients with cardiovascular disease.

The key advantage of the capture chip is its convenience, since it does not require access to a multi-color flow cytometer. However, it is not clear that this capture chip-based method can replace a functional clonogenic culture assay, which is more cumbersome and time consuming but can help identify highly proliferative colony-forming endothelial cells [6]. The key problem with an assay that is purely based on surface markers such as this novel capture chip assay is that, to date, there is no clear way of distinguishing circulating endothelial progenitor cells that give rise to highly proliferative colonies from mature circulating endothelial cells that may have sloughed off from the vasculature. While Timmermans et al. were able to convincingly show that highly proliferative ECFCs reside within the CD34+ fraction and express KDR, they also pointed out that mature endothelial cells can express both of these surface markers and thus also be found in the CD34+ fraction [6, 8]. Distinguishing progenitors from mature endothelial cells may indeed require clonogenic culture assays or other functional analyses, such as that of cell senescence [10].

As with any interesting paper, the work by Hansmann et al. [9] raises even more questions than it answers. It reminds us how rare circulating CD34+KDR+ cells are: Control subjects had 24–30 cells per 200 μ l of blood, which means that there are only 0.1 to 0.15 cells per microliter of blood. In comparison, the number of circulating monocytes is in the range of 100 to 500 per microliter of blood and thus several orders of magnitude higher than that of circulating CD34+KDR+ cells. Since monocytes can also give rise to endothelial-like cells and contribute to angiogenesis, it is not clear how to assess the relative contribution of the extremely rare CD34+KDR+ cells. The difference in the number of circulating CD34+KDR+ cells between the control subjects and the PAH patients in the present study is also minimal (albeit statistically significant) and may even be reflective of systemic responses to the chronic disease process or the extensive medications in the PAH patients instead of indicating some causal disease mechanism. Further studies are needed to evaluate whether the CD34+KDR+ cell count offers additional prognostic information above and beyond the routine hemodynamic

assessments in PAH. In many ways, this work reminds us of a key problem with EPC research that many of us in the EPC field face and which is best illustrated by the following famous Asian folktale as told by Idries Shah [11]: The Sufi sage Mulla Nasruddin was observed searching for a lost key in the street, and a passerby stopped to help him search for the key. This search was not successful, and after a while, he asked Nasruddin where exactly he had lost the key. Nasruddin responded that he had lost the key in the house, but it was more convenient to search for the key outside of the house because there was more light in the street. Surface marker-based techniques to enumerate circulating EPCs are indeed convenient, and many of us have used such techniques, but such enumerations do not provide the key to understanding what role such circulating cell populations play in the physiological repair of blood vessels and the pathogenesis of vascular disease. Comprehensive mechanistic studies that investigate whether rare circulating EPCs contribute in any significant manner to endothelial repair or disease pathogenesis are still needed. The novel capture chip will not be able to address these core questions, but it will provide a tool to study larger cohorts of patients. This will allow researchers in the field to determine whether measuring the number of circulating cells in patients with vascular disease provides novel prognostic or diagnostic insights.

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