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The definition of EPCs and other bone marrow cells contributing to neoangiogenesis and tumor growth; Is there common ground for understanding the roles of numerous marrow-derived cells in the neoangiogenic process?

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Summary

Interest in the regulation of blood vessel formation as a mechanism to permit unregulated tumor cell growth was a prescient hypothesis of Dr. Judah Folkman nearly 3 decades ago. Understanding the cellular and molecular mechanisms that effect the recruitment, expansion, and turnover of the tumor microvasculature continues to evolve. While the fundamental paradigms for improving blood flow to growing, injured, diseased, or tumor infiltrated tissues are well known, the potential role of bone marrow derived circulating endothelial progenitor cells (EPCs) to function as postnatal vasculogenic precursors for tumor microvasculature has become a controversial premise. We will briefly review some recently published high profile papers that appear to derive polar interpretations for the role of EPCs in the angiogenic switch and discuss possible reasons for the disparate views in work conducted in both mouse and man.

Keywords

Endothelial progenitor cell; hematopoietic progenitor cell; neoangiogenesis; circulating endothelial cell; endothelial colony forming cell

Introduction

Postnatal vasculogenesis was first proposed in 1997 [1] as a pathway for bone marrow derived cells (BMDCs) to migrate via the circulation to sites for participation in vascular repair and regeneration and has subsequently been reported as a mechanism required for tumor metastasis, neoangiogenesis, and growth promotion.[2–4] This review will first focus on recent published studies conducted in mice, where the results have supported disparate interpretations for the role of circulating bone marrow derived EPCs in tumor neoangiogenesis. We then will review

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recent papers that have attempted to better define human EPCs using conventional flow cytometric approaches and discuss the controversies in quantitating circulating cell populations in human subjects.

Endothelial progenitors support neoangiogenesis and tumor growth in mice

The normal response to meet increased metabolic demands of a tissue or organ is to induce the recruitment of new vessels or enhanced blood flow via existing vessels to the affected site.[5] Vasculogenesis is the formation of blood vessels from angioblast precursors and is the prominent mechanism for establishing the first blood vessels and vascular system during embryogenesis. Angiogenesis is the sprouting of new vessels from the endothelial lining of preexisting vessels. Angiogenesis has long been thought to represent the principle paradigm for neovascularization to maintain homeostasis and for cooptation via the angiogenic switch for tumor growth.[6] Arteriogenesis is the remodeling of arterial vessels to enhance blood flow to the vascular bed downstream. Asahara *et al.* [1] promoted a novel paradigm, referred to as postnatal vasculogenesis, when they reported that progenitor cells for the endothelial lineage could be found in the circulation of human subjects and rodents and the cells displayed the ability to localize to areas of vascular ischemia in vivo. Tissue ischemia or administration of certain cytokines were reported to mobilize the EPCs into the circulation.[7] Subsequent studies identified circulating EPCs as important participants in neoangiogenesis in a variety of cardiovascular injuries or disorders or in tumor angiogenesis in both mouse and man (reviewed in [2–4,8–11]). Several recent studies have evaluated the role of BMDCs in establishing a niche for metastatic tumor cell engraftment and the role of EPCs in progression of small to large tumor masses.[12–14]

To test the question of whether bone marrow (BM) derived EPCs directly contribute to the neoangiogenic progression of small to large tumors, Gao *et al.* [15] obtained Lewis lung carcinoma cells expressing red fluorescent protein and implanted the cells into mice reconstituted with BM cells expressing green fluorescent protein (BM-GFP$^+$). This approach permitted detailed imaging of the growing metastatic cells and the recruited vessels. The authors noted a paucity of vessels in the micrometastatic (< 1mm diameter) foci (day 14), but observed significant vessel infiltration in the macrometastatic (> 1 mm diameter) foci (days 21–28). This change in the extent of vascularization of the tumors suggested a window during which the tumor cells had undergone an angiogenic switch to permit achievement of the large tumor size. While the majority of BMDCs that invaded the tumors belonged to the hematopoietic lineage, some of the BM-GFP$^+$ cells were demonstrated to engraft in the endothelial lining of the tumor vessels, express CD31, and were labeled on the luminal aspect with the systemically perfused lectin *Griffonia simplicifolia* isolectin B4 (GS-IB4) to indicate incorporation into the larger systemic circulation.

Similar results were obtained using another transgenic mouse model (MMTV-*PyMT*) in which polyomavirus middle T antigen expression induces breast cancer with metastasis. To define the role of BMDCs in the growth of the breast cancer metastatic lesions, the MMTV-*PyMT* mice were transplanted with BM-GFP$^+$ cells. As noted with the adoptively transferred lung carcinoma tumors, breast tumors that metastasized to the lungs were initially very small growths and poorly vascularized, but as the metastatic tumors grew over time (16 weeks), vessels became increasingly abundant and up to 12% of the tumor vascular endothelium contained BM-GFP$^+$CD31$^+$ EPCs. Given that putative BM-derived EPCs express vascular endothelial cadherin (VE-cadherin), vascular endothelial growth factor 2 receptor (VEGFR2), Prominin 1 (AC133), and CD31 and lack various hematopoietic markers [14], the authors noted that pulmonary micrometastases contained 5 fold more of the putative EPCs as compared to control mice without micrometastasis. In sum, the authors reported a critical role for the BM-derived EPCs in the angiogenic switch from small to large metastatic tumors.

Endothelial progenitors do not contribute to vessel endothelium or tumor growth in mice

While a role for BM-derived cells in tumor neoangiogenesis appears evident from the above work, other historical data has not supported this hypothesis. De Palma *et al.* [16] found essentially no contribution of transplanted BM-GFP$^+$ cells to the vasculature of implanted tumor grafts in host mice. Gothert *et al.* [17] utilized a transgenic mouse in which an inducible endothelial specific marker system provided evidence that transplanted BM cells do not contribute to tumor vascular endothelium. In this model system, the majority of tumor vessel endothelial cells were derived from vascular endothelium residing in pre-existing tissue vessels. Other work has revealed that murine BM-derived cells contribute to the peri-endothelial macrophage and pericyte populations rather than the endothelium of new vessel.

Purhonen *et al*. [18] recently performed an analysis of the mobilization and differentiation of murine adult BM-derived cells using cell surface markers previously defined on murine EPCs. [7,8] Since tumor cell implantation in C57BL/6 mice has been reported to induce mobilization of BM-derived cells into the circulation and incorporation into tumor vasculature, Purhonen *et al.* [18] implanted B16 melanoma cells into C57BL/6 mice and surveyed affected animals for circulating EPC concentrations. While significant numbers of hematopoietic cells emerged from the marrow and the enhanced peripheral blood white blood cell counts peaked in association with the height of the tumor growth rate, no significant elevation in the circulating BM-derived VEGFR2$^+$ EPC population was observed. Further examination of the tumor vasculature led to the identification of numerous peri-endothelial BM-derived cells but none of the endothelial lining of the tumor vessels contained integrated BM-derived VEGFR2$^+$ cells. Similar results were obtained when B16 melanoma cells were inoculated into transgenic VEGFR2-promoter-lacZ mice, normal mice, or mice engrafted with BM cells from the transgenic VEGFR2-promoter-lacZ mice and the animals were allowed to recover for 7–21 days. While the endothelium of the tumor vasculature in the transgenic VEGFR2-promoter-lacZ mice readily expressed the reporter activity (positive control), none of the vascular endothelium in the tumors implanted in the normal mice (negative control) or mice chimeric for BM from the transgenic VEGFR2-promoter-lacZ mice stained positive for the reporter enzyme (test animals). Thus, data to support the mobilization of BM-derived EPCs into the circulation and incorporation into tumor vascular endothelium was not obtained using multiple approaches in multiple mouse strains. This work did note a robust initial recruitment of BM-derived hematopoietic cells into the nascent tumor microenvironment and incorporation of BM-derived cells into the peri-endothelial space.

Kerbel *et al.* [19] pointed out that some of the differences in the data obtained by Purhonen *et al.* [18] and Gao *et al.* [15] could be related to the type of tumors tested and times at which tumors or implanted matrigel plugs were examined for evidence of BM-derived cell incorporation. Others had noted that bone marrow contributions to tumor vascularion were tumor type and tumor stage dependent.[20] BM-derived cell recruitment into the circulation and incorporation into tumor vessels may also be dependent on treatment of experimental tumor bearing animals with certain chemotherapeutic agents.[21]

Defining EPCs in human subjects

In the human system, like the murine system, no unique identifying marker for an EPC has been reported. Currently EPCs are defined using 3 general approaches. In one approach, peripheral blood mononuclear cells are isolated and plated on fibronectin coated tissue culture plates. After several days, the adherent cells that display the ability to ingest acetylated low density lipoprotein (acLDL) and to bind certain plant lectins are deemed EPC. The putative EPC isolated by this brief adhesion and culture protocol have been studied extensively and an

inverse correlation between the circulating concentration of these cells and an increased risk for developing coronary arterial disease has been reported.[22,23] However, the use of fibronectin coated tissue culture wells has long been realized as method to isolate human blood monocytes for differentiation into macrophages.[24] The fact that human peripheral blood monocyte/macrophages can be cultured in conditions that promote expression of numerous proteins normally expressed by primary endothelial cells, makes determination of EPCs by this method unreliable, as one cannot determine which cell is an EPC and which is a macrophage displaying “endothelial” markers.[25–27]

A second approach utilizes monoclonal antibodies and fluorescence activated cell sorting (FACS) analysis to enumerate putative EPC concentrations in circulating blood or tissues of human subjects. In perhaps the most influential paper in the field, Asahara *et al.* [1] reasoned that some circulating cells may express cell surface markers shared by hematopoietic stem cells (HSC) and progenitors for the endothelial lineage, since endothelial and blood cells share a similar mesodermal origin during embryonic development. Thus, isolation and culture of circulating peripheral blood $CD34^+$ cells (15.7% enriched) on fibronectin coated dishes led to emergence of spindle shaped cells expressing a variety of markers normally displayed by primary endothelial cells. Furthermore, the $CD34^+$ cells or cells expressing VEGFR2 homed to experimentally induced ischemic hindlimb tissues of immunodeficient mice following intravenous infusion. In sum, this paper suggested that $CD34^+$ or $VEGFR2^+$ cells in human adult peripheral blood may function as EPCs in postnatal vascular repair.

Rare endothelial cells circulate in the bloodstream in healthy subjects but increase in number in diseased subjects [28,29] and thus, Peichev *et al*. [30] attempted to devise a protocol to distinguish circulating EPC from circulating mature endothelial cells. In addition to searching for cells expressing CD34 and VEGFR2 (KDR in the human system), these authors also included CD133 as a potential stem cell marker. While CD133 is expressed by progeny from endoderm, ectoderm, and mesoderm and is displayed by cells at all stages of differentiation, the glycosylated form of CD133 forming the AC133 antigen can be found on a variety of stem cell populations including cancer stem cells [31]. Peichev *et al*. [30] reasoned that more primitive EPCs would express CD34, KDR, and CD133 while CD133 and perhaps CD34 expression would be lost as the endothelial cells matured, similar to the modulation in expression of these antigens observed on human HSC as they differentiate into more committed progenitor cells. CD34, KDR, and CD133 were identified in samples obtained from mobilized adult peripheral blood, umbilical cord blood, and human fetal liver tissue. In supportive studies, these antigens were also observed on some cells coating the luminal surface of an implanted left ventricular assist device which had been implanted in human subjects, suggesting that one could use CD34, CD133, and KDR as markers for circulating EPC in human subjects.

CD34, CD133, or KDR or any combination thereof has subsequently been commonly used to enumerate EPCs in human subjects (reviewed in [32]) and circulating EPC concentrations have been correlated with several human disease states.[2,9,11,33] While correlating events on a FACS plot with clinical parameters of a patient’s health may be statistically relevant, few studies have focused on more rigorous determination of which cells are displaying the antigens recorded as the events on the FACS histograms. Indeed, whether examining umbilical cord blood or mobilized adult peripheral blood, cells expressing CD34, CD133, and/or KDR are enriched for hematopoietic colony forming cells and they do not form endothelial cells in vitro or in vivo.[34,35] Thus, CD34, CD133, and KDR expression on circulating human peripheral blood cells fails to uniquely identify a circulating EPC.

Finally, EPCs may also be counted using two different in vitro colony forming cell assays. The colony forming unit-Hill (CFU-Hill) [36] and endothelial colony forming cell (ECFC) assays [37] have recently been directed compared using adult peripheral blood and umbilical cord

blood samples.[38] Mononuclear cells plated in the CFU-Hill assay are recognizable by clusters of round cells overlying adherent spindle shaped cells expressing many proteins similar to primary endothelial cells. However, the adherent spindle shaped cells also express numerous myeloid progenitor cell markers and mature into macrophages that ingest bacteria as readily as control macrophage cells. CFU-Hill cells do not form in vitro capillary-like structures with lumens nor do they proliferate extensively or give rise to secondary colonies in vitro. In contrast, the human peripheral blood and cord blood derived ECFC express cell surface antigens similar to primary endothelium, proliferate at a clonal plating level and replate into secondary and tertiary ECFC, and form capillary-like structures in vitro. The most stringent and remarkable property display by ECFC is the capacity to form human blood vessels in vivo (in immunodeficient mice) and to inosculate with murine vasculature to become part of the murine systemic circulation; a feature not displayed by CFU-Hill or any other putative EPC. Thus, while the CFU-Hill assay identifies hematopoietic cells, only ECFC display all of the properties of an EPC. Since the cord blood ECFC do not express CD45 while the CFU-Hill are $CD45^{+}$, one may use this difference to discriminate these two cell populations. These dramatic differences in putative EPC populations have been confirmed by others.[39–41]

Of all the above methods for EPC identification in human subjects, neither the in vitro adhesion culture nor colony forming cell assay methods are easily applied to clinical research protocols. Thus, numerous FACS protocols for cell identification have been reported as peripheral blood biomarkers for clinical disease (reviewed in [2,32]). In an attempt to develop a robust clinically useful FACS protocol to examine both circulating progenitor cells (CPC) and circulating endothelial cells (CEC), Duda *et al.* [42] reported that CEC can be defined as cells expressing $CD31^{bright}CD45^{-}CD34^{dim}CD133^{-}$ where as CPC are $CD31^{+}CD45^{dim}$ $CD34^{bright}$and $CD133^{+}$. CEC could be identified at a frequency of 0.1–6.0% of peripheral blood mononuclear cells in women with breast cancer and the concentration of CEC was useful in predicting clinical outcome.[43]

Others have questioned the ability of FACS based approaches to enumerate CEC concentrations which may only be accurate to approximately 180 events/mL of peripheral blood whereas immunomagnetic bead separation (IMS) protocols have been useful in identifying 10 or fewer events/mL.[44] The ability to measure minute numbers of CEC is relevant since in normal human subjects, CEC circulate at 0–10 cells/mL.[44] The controversy of whether one must utilize IMS or FACS based protocols for accurate measurement of CEC in human blood and the use of these protocols for assessing tumor progression and clinical outcome remains unresolved. Several recent hybrid protocols that use an IMS pre-enrichment step followed by FACS analysis may be proven applicable but awaits more widespread clinical validation testing. [45,46]

Defining the specific cells that are identified by the CPC markers has also been largely unexplored with various hematopoietic, putative endothelial, cardiac, and even mesenchymal precursors identified by this general term.[47] One wonders whether the specific cells comprising the CPC fraction are indeed consistently present in the various diseases or whether there are a variety of cell types that express the same cell surface antigens (and are recorded as CPC events on the FACS plot) and perform different functions in different diseases.[48] Much more stringent analysis of each putative progenitor type must be completed with functional demonstration of the progenitor properties of the cells as part of the defining of the cell population, before assigning a particular role of the putative progenitor cells in a process of repair or regeneration of a tissue or organ.

Is there common ground for identifying BM-derived cells that regulate tumor angiogenesis in the murine system?

Most of the controversy regarding the role of BM-derived cells in tumor angiogenesis lies in the interpretation of whether or not BM-derived cells integrate into the endothelial layer of the tumor vessels. Prior work by Nolan *et al.* [14] in the murine system, indicates that BM-derived EPCs incorporate for a brief period during the early phases (< 2 weeks) of tumor vascular growth but are scarce after 4 weeks (< 1%). Consistent with the long held views of angiogenesis, longer term tumor vascular endothelium is derived from nearby host vessels. One hypothesis that could serve as a plausible reinterpretation of the disparate results discussed above, is that BM-derived cells are the earliest recruited cells to nascent metastatic foci where they may participate in forming the earliest vasculature, but these BM-derived cells are not specified to maintain a long-term endothelial fate. This could resolve the paucity of BM-derived cells contribution to vessels long term as observed by Nolan *et al*.[14] Only with development of improved cell lineage marking systems will we finally come to a common understanding of which BM-derived cells are contributing to long term engrafted cells in the endothelium of the vasculature. This may help to avoid situations where FACS data suggest that a population of cells is negative for cell surface markers of a particular cell lineage, but the images of the cell morphologies comprising that population indicate otherwise.[14,15]

While the above hypothesis for early and not late involvement of BM-derived cells in neoangiogenesis may resolve some of the issues, it fails to explain the complete lack of contribution of BM-derived cells to vessel enothelial noted by some authors. Ongoing debate as to what constitutes engraftment and function of a BM-derived cell into the endothelial lineage will undoubtedly continue until standardized methodologies are accepted by the field and tools are available for widespread utilization and confirmation by any investigator choosing to do so. This is not a point for disagreement, but one for mutual acceptance similar to standard hematopoietic stem cell (HSC) and progenitor cell definitions which rely on the ability to repopulate all (long term HSC) lineages for more than 16 weeks within a host animal or fail to repopulate long term but give rise to specific in vitro colony forming cells in the presence of specific growth factors (progenitor cells).[49,50] Similar definitions of short or long term engraftment have yet to have been achieved for the endothelial lineage in the murine or human system. For example, one could propose that a donor endothelial cell precursor must engraft, attach to the basement membrane, form tight and adherence junctions with neighboring endothelial cells, display features that are typical of other endothelial cells within that portion of the circulation in that tissue, display some proliferative potential (giving rise to other endothelial cells), and thus become integrated (the number of weeks has yet to be determined for short or long term) with the remaining host endothelial cells at the site of injury.

Is there common ground for better enumeration and identification of human cells that participate in neoangiogenesis?

Most FACS protocols used to identify and enumerate circulating cell subsets involved in neoangiogenesis in human subjects continue to rely on conventional logarithmic scaling of the identified events. Indeed, newer approaches for FACS analysis may need to be employed to more accurately define the cell populations in question as the limitations of logarithmic scaling may be inadequate for the rare putative EPC populations, particularly for those cells with low levels of cell surface protein expression and the number of particular proteins examined simultaneously can compromise accuracy of compensation and gating controls unless using particular approaches.[51] We suggest that future studies should extensively validate each cell population identified or isolated by FACS (using cell morphology, colony assays, or other cell lineage specifics) prior to examining the role that the putative cell population performs in a

particular cell in vitro assay or in vivo process. It would appear that completely relying on a FACS profile to define a cell population is as inadequate and unreliable for the endothelial lineage, as has long been known for defining stem or progenitor cells of the hematopoietic system.[52]

It may also be appropriate to retire the term EPC, since the field has never been able to specify by unique restricted markers what constitutes an EPC. Indeed in the human system, the only cells that display the capacity for postnatal vasculogenesis are the rare circulating blood outgrowth endothelial cells BOEC [53] also called ECFC.[54] ECFC derived from umbilical cord blood or adult peripheral blood display clonal proliferative properties, high telomerase activity, a hierarchical pathway of differentiation, in vitro capillary-like tube formation, and in vivo vessel formation upon implantation in immunodeficient mice. The ultimate origin of the BOEC/ECFC remains undetermined. While Lin *et al*. confirmed that the circulating BOEC with the highest proliferative potential are derived from the bone marrow, this information does not permit determination of the ultimate site of origin within the marrow compartment. It is well known that the bone marrow is a complex conglomeration of blood, mesenchymal, bone, cartilage, fat, and vascular cellular components.[55] In a standard bone marrow transplant setting, many of these cells are transferred in some manner to the host during the 100–200 aspirates collected. Some evidence suggests that ECFC are vessel-derived in both human and rodent systems [56–58] and thus, may be transplantable in the vessel fragments harvested with other marrow constituents. Thus, none of the published studies which examine donor contributions of various cell types to host tissues post-transplant can know with specificity the lineage of origin of the cells coming from the bone marrow.[53] In other words, to say that a cell is BM-derived, fails to specify the origin or stem cell nature of the cell in question; this term simply references a site of derivation, not a lineage or cell state.

Though ECFC may display all the properties of an EPC, there are no reports to identify specific cell surface markers to prospectively define this population. At present, one is able to partially enrich for ECFC activity in cord blood and adult peripheral blood $CD34^{+}CD45^{-}$ cells [34], though no FACS protocol has been reported that can enumerate these cells in normal human subjects or in patients with disease. Future studies will be required to identify specific markers for the ECFC and novel FACS approaches (as noted above) will be required to measure the rare circulating cells with accuracy.

Conclusion

Progress in comparing study results among investigators attempting to define EPCs in the murine system will require improved inducible cell specific marking systems and lineage fate mapping and development of a consensus agreement on the definition of what constitutes endothelial engraftment in vivo. In the human system, advances in defining the role of EPCs in normal subjects or those with disease will require improved FACS protocols that can quantitate rare events, better overall cell validation (not reliance on FACS profile alone to define a cell), and ongoing search for the elusive cell surface markers of the rare circulating endothelial precursors with in vivo vessel forming ability.

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