Phenotypic Characterization of Endothelial Progenitor Cells from Bone Marrow and Peripheral Blood

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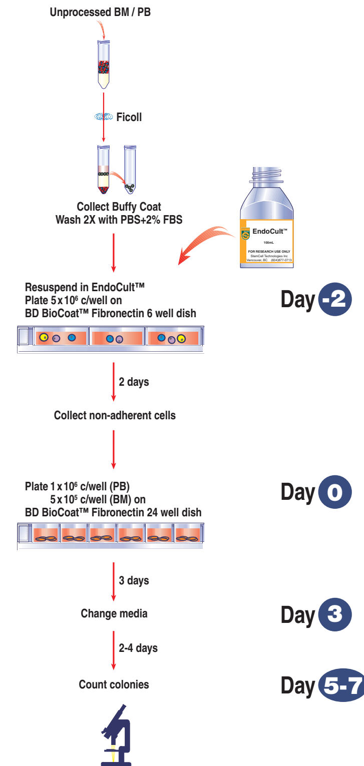
Abstract

Recent evidence of a circulating endothelial precursor cell (EPC) has challenged our understanding of the development of solid tumour vasculature, and introduces new possibilities for anti-angiogenic therapy. Circulating mature endothelial cells (ECs) are elevated in the blood of breast cancer patients (Mancuso *et al*, Blood 97:3658, 2001); however, EPCs have not been evaluated. Hill *et al* (NEJM 348:593, 2003) recently reported an inverse correlation between the number of circulating endothelial colony-forming cells (EPC) and the risk of cardiovascular disease. The phenotype of mature ECs has been defined based on a number of cell surface markers. Less is known about the phenotype of EPCs, although they appear to express some of the same cell surface markers as subsets of hematopoietic cells. We have used the in vitro colony assay described by Hill, in conjunction with flow cytometry, to evaluate the cell surface markers expressed on EPCs. Samples of peripheral blood (PB) or bone marrow (BM) from normal donors were processed by density-gradient centrifugation. The co-expression of CD45 and combinations of CD146, CD144, CD133, CD34, CD105, CD14, and CD3 were analyzed by flow cytometry (100,000 events/tube). Cells were also plated on fibronectin-coated plates in M199 medium containing selected fetal bovine serum (EndoCult™ medium, StemCell Technologies). After 48 hours, the non-adherent cells were re-plated in replicate fibronectin-coated plates, and a sample was also taken for flow cytometric analysis. Colonies were evaluated 5 days later; a colony was defined as a central core of "round" cells with more elongated "sprouting" cells at the periphery (Hill). Immunocytochemical staining for von Willebrand Factor confirmed the endothelial lineage of the cells. The largest population of $CD45^-$ cells in the starting BM was $CD105^+$ ($1.2 \pm 0.6\%$, n=9), possibly reflecting activated mature ECs. Interestingly, these cells did not express the EC markers CD144 (clone F-8) or CD146 (clone P1H12). The percentage of $CD45^-CD105^+$ cells decreased after 48 hours of culture. The percentage of $CD45^+CD14^+$ cells also decreased, reflecting the removal of monocytes. All $CD144^+$ cells were $CD45^+$ $CD105^{+/-}$ and exhibited the scatter characteristics of monocytes. The vast majority of $CD146^+$ cells expressed CD45 and CD3, and may identify subsets of T cells (Pickl *et al*, JI 158: 2107, 1997; Leucocyte Typing VI). CD133 and CD34 were only detected on $CD45^+$ cells (n=3), but all samples contained EPC, as evidenced by growth in the colony assay. $CD146^+$ cells in PB were also $CD45^+CD3^+$, $CD34^-CD105^-$, and all $CD144^+$ cells were $CD45^+$ and had the scatter characteristics of monocytes. PB samples did not contain $CD45^-CD133^+$ cells but did produce colonies in the *in vitro* assay. In one sample, the $CD45^-$ cells did not express any of the other markers evaluated; for another sample the level of expression was extremely low and perhaps insignificant. In summary, in both BM and PB, CD144 was only expressed on $CD45^+$ cells. CD146 was primarily expressed on T cell subsets and not on $CD45^-$ cells. $CD45^-$ cells in PB did not express markers expected to be on EC or possibly on EPC, although the samples did contain EPC. The next step will be to evaluate cells sorted on the basis of their antigenic expression in the colony assay to more clearly define the phenotype of the EPC from BM and PB. Enrichment strategies for EPC will greatly facilitate the characterization of their phenotype.

Methods

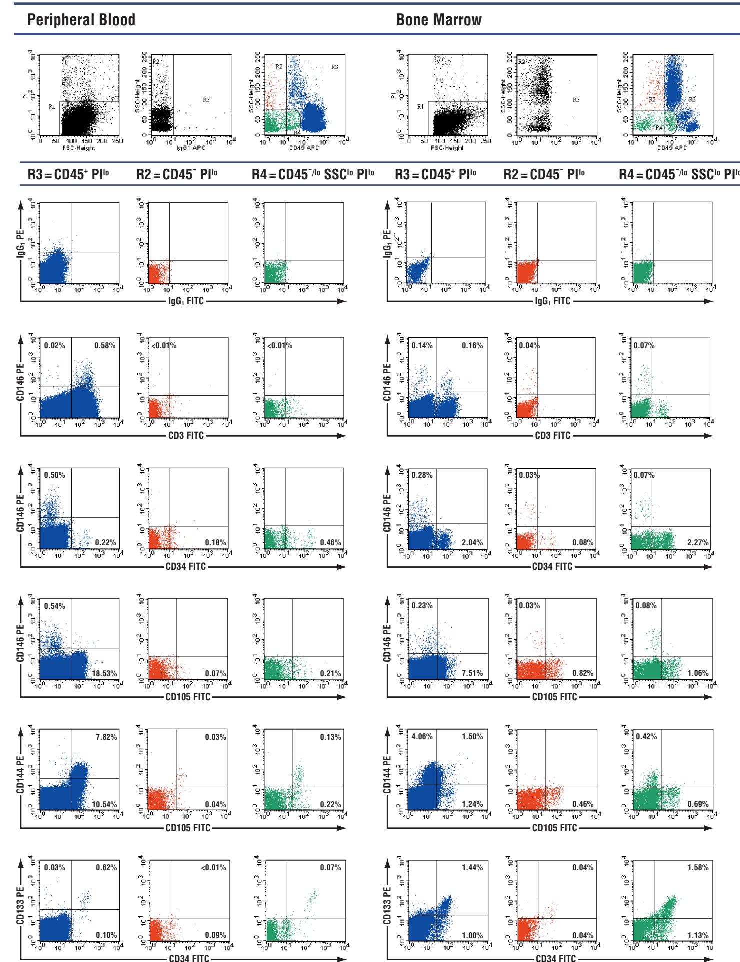
7 Day EPC Colony Assay



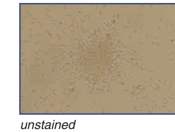
Flow Cytometric Analysis. Peripheral blood or bone marrow samples were lysed with ammonium chloride or ficolled. Cells were stained with fluorescently conjugated antibodies in the dark, and washed with 1 µg/mL propidium iodide (PI). 100,000 events were collected per tube on a FACSCalibur. Isotype controls and gating are as shown. Files were analyzed using CellQuest. Total viable cells were considered to be total PI^{lo} events.

Results

FACS Analysis of Fresh Ficolled Peripheral Blood and Bone Marrow. % indicates % of total viable cells (defined as PI^{lo}) that fall within the given quadrant. PI^{lo} events always exceeded 97% of collected events.



Representative EPC colony after 7 day culture:


unstained

All samples of peripheral blood and bone marrow generated colonies.
Peripheral Blood: ~20 colonies per well (24 well dish)
Bone Marrow: ~40 colonies per well (24 well dish)

Conclusions

- EPC, as defined by a short term colony assay, are present in bone marrow and peripheral blood.
- CD144 / VE-Cadherin, assessed with clone F-8, is only found on $CD45^+$ cells with monocyte scatter characteristics in bone marrow and peripheral blood. Therefore, CD144 may not be a good marker for circulating EC or EPC.
- CD146, assessed with clone P1H12, is expressed primarily on $CD3^+CD45^+$ T cells. Therefore, CD146 is not a good marker for circulating EC or EPC.
- CD133 and CD34 were expressed on $CD45^+$ cells. If these antigens are markers of EPC, as found by others, then EPC are also $CD45^+$, even though mature EC are expected to be $CD45^-$.

References

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