

Standardization of a Colony Assay to Further Characterize Endothelial Precursor Cells in Blood and Bone Marrow

SM Faulkes¹, C Pereira¹, CE Peters¹, TE Thomas¹, AC Eaves² and E Clarke¹

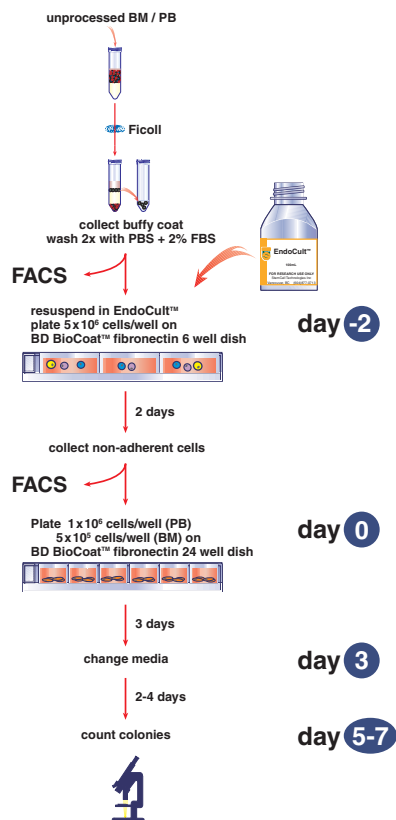
¹StemCell Technologies Inc, Vancouver, Canada, and ²Terry Fox Laboratory, BC Cancer Agency, Vancouver, Canada

Background

Blood vessel development is a regulated process involving the proliferation, migration, and remodelling of endothelial cells from adjacent pre-existing blood vessels (angiogenesis) or the differentiation of endothelial progenitor cells (EPCs) from mesodermal precursors (vasculogenesis). EPCs or angioblasts were originally thought to be present only during embryonic development, however accumulating evidence in the past several years suggests that they can persist in adult life. This has generated interest in the use of EPCs for neovascularization of ischemic or injured tissue and for the clinical assessment of risk factors for various diseases. There is currently no standardized procedure for the isolation and *in vitro* culture of EPCs. The most commonly used isolation method is culturing mononuclear cells on a variety of substrates. There has been no systematic study regarding the physiological variations in the number of EPCs in healthy individuals. Recently, Hill *et al.* (NEJM 348:593, 2003) described an *in vitro* colony assay that showed an inverse correlation between the number of circulating endothelial colony forming cells (CFU-EC) and the risk of cardiovascular disease. We have used this assay in conjunction with flow cytometry to characterize the CFU-EC in normal peripheral blood and bone marrow and evaluated the cell populations removed following adherence on fibronectin coated plates. Although there is a general consensus of the phenotype of the mature endothelial cell, less is known about the phenotype of the circulating CFU-EC. We further attempted to evaluate the cell surface markers expressed on the circulating CFU-EC using a panel of antibodies.

Methods

Figure 1. 7 day EPC colony assay



Flow cytometric analysis (FACS) was performed on ficolled bone marrow (BM) and peripheral blood (PB) samples prior to culture and following 48 hours of adherence on fibronectin coated plates.

Results

Table 1. Flow cytometric analysis on bone marrow samples

cell phenotype	Ficoll'd BM prior to culture (% mean ± SD)	Ficoll'd BM following adherence (% mean ± SD)
CD14 ⁺	7.8 ± 1.6	0.97 ± 0.50
CD105 ⁺ /CD45 ⁻	2.0 ± 0.49	0.28 ± 0.14
CD146 ⁺ /CD45 ⁺ /CD3 ⁺	0.54 ± 0.29	0.42 ± 0.18

Incubation for 48 hours on fibronectin coated plates removed 33.1 ± 5.3 % of mononuclear cells including monocytes (CD14⁺) and mature endothelial cells (CD105⁺CD45⁻).

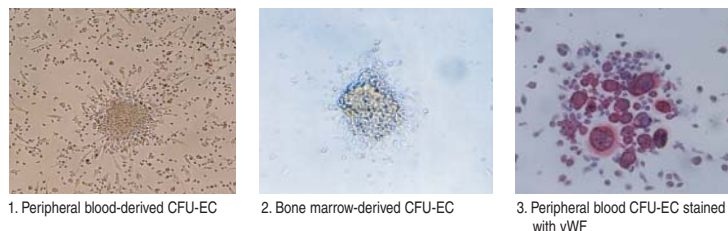
Table 2. Frequency of endothelial progenitors in bone marrow and peripheral blood

sample	number	frequency of CFU-EC
bone marrow	5	1:10,000 ± 2500
peripheral blood	16	1:120,000 ± 170,000

Bone marrow samples were purchased from Cambrex, MD. All samples were obtained from young volunteer donors and cultured as described in Figure 1. Peripheral blood samples were obtained with consent from male donors (age range 20-40 years) and cultured as described in Figure 1. The data in Table 2 suggests bone marrow samples have a higher frequency of CFU-EC, although the relative low sample number and limited age range may explain the low variability in this data. Peripheral blood results suggest a significant lower frequency of CFU-EC. The large variability in the data maybe due to the broader age range and unknown clinical situation.

Figure 2. Representative colonies after 7 days of culture

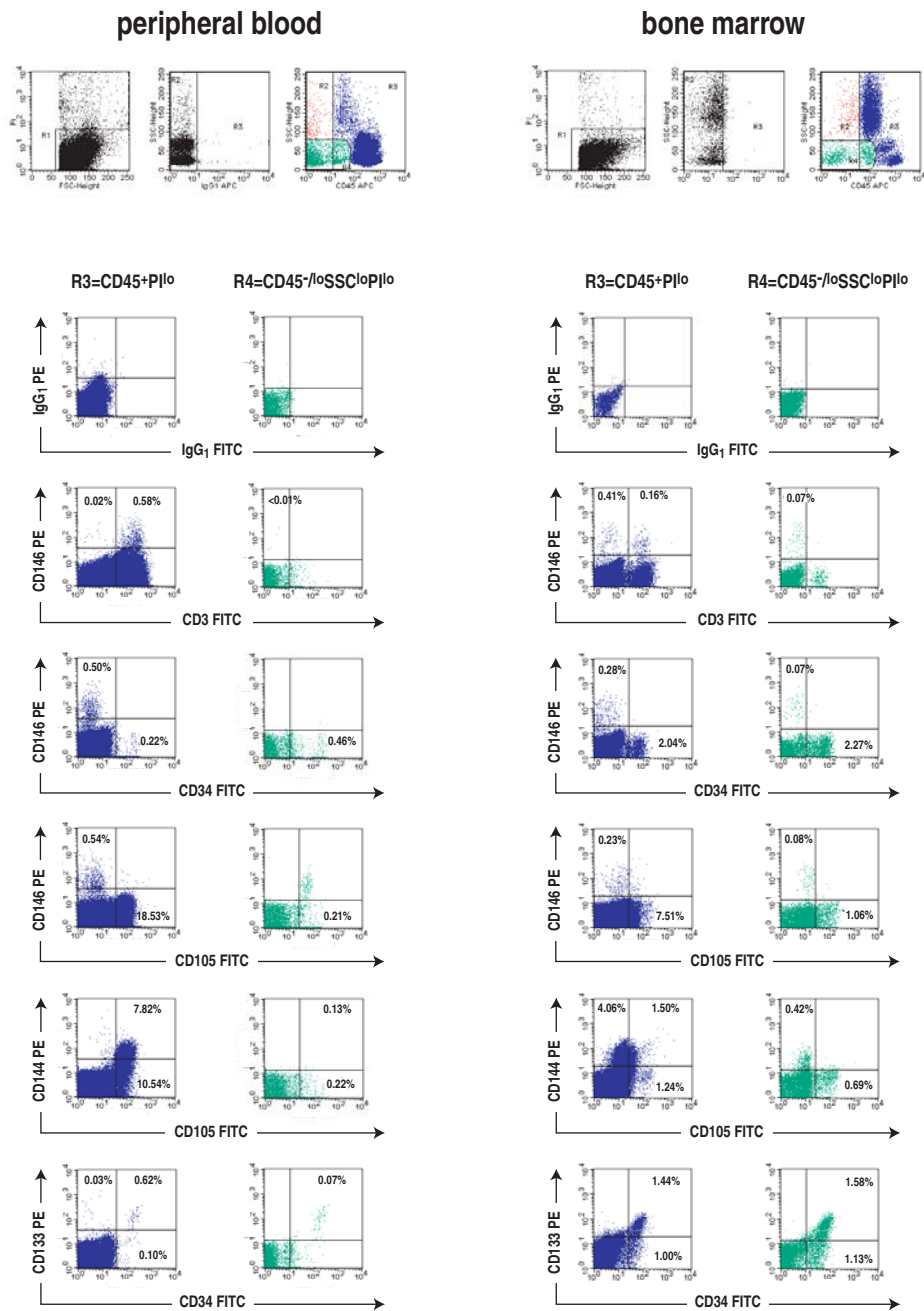
All photographs were taken at 125X magnification on day 7. Photograph 3 shows von Willebrand Factor (vWF) staining using the APAAP procedure in which colonies were fixed with 4% paraformaldehyde and stained at 1:50 dilution with vWF (clone 2F2-A9 from BD Pharmingen).



Which cell is the circulating CFU-EC?

Although there is a general consensus on the phenotype of the mature endothelial cell, the phenotype of the circulating progenitor remains incompletely defined and may depend on the assay used. We attempted to evaluate using a panel of antibodies, specific cell populations in ficolled peripheral blood or bone marrow that may contain candidate endothelial cell precursors. Since the frequency of CFU-EC in bone marrow is approximately 10 times higher than that of peripheral blood, we specifically looked for a population of cells from both sources showing similar proportions (Figure 3).

Figure 3. FACS analysis of fresh ficolled peripheral blood and bone marrow



Cells were stained with antibodies in the dark, and washed with 1 µg/mL propidium iodide (PI). 100,000 events were collected per tube on a FACSCalibur and files analyzed using CellQuest. % indicates % of total viable cells (defined as PI^o) that fall within the given quadrant. Antibodies used: CD146 clone P1H12 (BD Pharmingen), CD105 clone SH2 (StemCell Technologies Inc), CD144 clone F8 (Santa Cruz Biotechnology), AC133 (Miltenyi).

Conclusions

- CFU-EC frequency is higher in bone marrow than peripheral blood
- Pre-plating on fibronectin coated dishes results in a decrease in CD14⁺ and CD105⁺CD45⁻ populations
- Although CFU-EC morphology was variable, all colonies expressed von Willebrand Factor
- Phenotypic characterization remains difficult since antibodies associated with endothelial cells may be co-expressed on other cell populations

