

Critical Role of Flow Cytometry in Evaluating Peripheral Blood Hematopoietic Stem Cell Grafts

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In the late 1950s, Thomas et al. (1) successfully transplanted bone marrow cells into supralethally irradiated recipients, and until the 1990s, the majority of autologous and allogeneic hematopoietic stem/progenitor cell transplants were performed utilizing bone marrow as a source of stem cells. In the mid-1980s, reports demonstrated the feasibility of obtaining clinically useful numbers of peripheral blood stem/progenitor cells (HPSC) from cancer patients recovering from chemotherapy (2). By the early 1990s, the availability of a number of hematopoietic cytokines used either singly or in combination and/or with chemotherapy facilitated the harvesting of peripheral blood stem cells (PBSC) (3). This development, coupled with clear data that time to hematopoietic reconstitution is significantly shorter with PBSC compared to bone marrow, has led to the widespread use of PBSC for autologous and, increasingly, allogeneic transplantation (reviewed in 4). More recently, cord blood has provided a source of HPSC, with an estimate of over 2,500 transplants performed worldwide since the first one in 1988 (5). Traditionally, the absolute mononuclear count in relation to patient body weight was used to predict the engraftment potential of bone marrow and, more recently, cord blood. However, due to the variable HPSC content of peripheral blood, this number is unreliable. Initially, colony-forming cell (CFC) assays were used as a surrogate for PBSC, but this test has the limitation of requiring 10–14 days to perform, making it unsuitable for planning apheresis schedules. In addition, CFC assays are subjective and lack standardization in both methodology and reagents.

FLOW CYTOMETRIC ENUMERATION OF CD34⁺ CELLS USING MULTICOLOR BOOLEAN GATING

By the late 1980s, it was established that virtually all of the CFC activity and engraftment potential of marrow or peripheral blood samples was contained in the small population of cells bearing the CD34 antigen (reviewed in 6), and a large number of clinical studies have established that CD34⁺ cell transplants are safe, durable, and potentially therapeutically effective (7,8).

Siena et al. (9) were the first to describe a flow cytometric method to measure the percent of CD34⁺ cells in peripheral blood samples. Several groups sub-

sequently proposed variations of this method (10,11). While various approaches had been applied to the enumeration of CD34⁺ cells, it was apparent by the poor performance seen in multi-institutional quality control (QC) programs (12) that a consensus method based on the science of the CD34 molecule and the cells that express it was required. Sutherland et al. (13) had developed a sensitive and accurate multiparameter flow methodology that utilized four parameters; forward and side light scatter and the intensity of CD34 and CD45 staining. Unique to this method was the use of Boolean gating strategy, coupled with cluster analysis that was amenable to use on a variety of sources of hematopoietic stem cells (13). This method formed the basis of a clinical guideline for CD34⁺ cell enumeration, now known as the International Society of Hematotherapy and Graft Engineering (ISHAGE) protocol, now the International Society for Cellular Therapy (ISCT) protocol (14). In independent multicenter studies of the effects of different gating strategies, Chang and Ma (15) demonstrated that of those tested, only the ISHAGE protocol gave reproducible results from all centers of within $\pm 10\%$ of the median CD34⁺ cell value on both PB and PBSC collections. By incorporating a known number of fluorescent counting beads in the flow cytometric analysis, and assessing the ratio between the number beads and CD34⁺ cells counted, an absolute CD34⁺ cell count can be generated using a single instrument platform. This eliminates the need for a nucleated cell count as performed by a hematology analyzer. Two commercial kits, Becton Dickinson Biosciences (BDB) Pro-COUNT™, and the Beckman Coulter Stem-Kit™ that is based on the single platform ISHAGE method developed by Keeney et al. (16) are available commercially to enumerate CD34⁺ cells. Recent interinstitutional studies have shown improved precision and reproducibility of this key measure of graft adequacy when the stan-

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standardized single platform ISHAGE protocol (17) is correctly deployed (18).

Baseline peripheral blood CD34⁺ cell counts (prior to mobilization) are generally <5/ μ l or <0.1%. Following mobilization, CD34⁺ peripheral cell counts are a reliable predictor for harvesting adequate numbers of HPSC for transplantation and may increase to several hundred per microliter. Studies have shown that the rate of platelet engraftment is generally rapid in patients given as few as 2×10^6 CD34⁺ cells/kg body weight (19). As flow cytometry can produce results in under 1 h, serial assessment of peripheral blood CD34 content can be used to determine optimal timing of apheresis, and the required number of apheresis procedures (20).

The Need to Evaluate Viable CD34⁺ Stem Cells

There is increasing acceptance of the need to include viability dyes in the enumeration of CD34 cells, particularly when cord blood, bone marrow, or manipulated (i.e., held overnight, postselected, or postthawed) samples are assessed. Inclusion of the viability dye 7-AAD in the single platform ISHAGE method allowed necrotic cells to be excluded from analysis (16,17). Recent studies by Allan et al. (21) correlated the number of non-necrotic (7-AAD negative) cells infused postcryopreservation with time-to-engraftment. In this study of patients with a variety of hematological diseases, those receiving less than 2×10^6 CD34⁺ cells/kg body weight had significantly longer median time to platelet engraftment (17 days) than those receiving from $2\text{--}5 \times 10^6$ (12 days) or greater than 5×10^6 (10 days). This method has also been shown to provide accurate CD34⁺ counts in postcryopreserved cord blood samples (22).

By adding Syto-16 (distinguishes viable cells [which stain Syto-16 bright] from apoptotic cells [which stain Syto-16 dull]) to the single platform ISHAGE method, De Boer et al. (23) have shown that in postthawed samples, a variable number of 7-AAD-/Syto-16 dull apoptotic cells can be detected. In this study, the non-necrotic (7-AAD-) nonapoptotic (Syto-16 bright) fraction contained all the viable cells as measured by CFU and transwell migration assays (23). Additionally, Syto-16 staining was superior to annexin V for excluding apoptotic cells. Even more sensitive detection of early apoptosis on precryopreserved samples can be performed by detecting caspase activation using carboxyfluorescein valyl-alanyl-aspartyl fluoromethyl ketone (z-VAD) (Greco, personal communication).

Current recommendations from the College of American Pathologists (CAP) require that apheresis samples over 4 h old be tested for CD34⁺ cell viability. This recommendation was adopted to exclude dead cells in precryopreservation products. Loss of viability can occur due to delays in processing and/or poor quality control during processing. Although clearly possible in a research setting, the routine deployment in clinical labs of methods that simultaneously detect early apoptotic cells in addition to necrotic cells in thawed

products might be considered premature at this time. Such methodologies also run the theoretical risk of increasing the numbers of dead/apoptotic cells detected via "laboratory-induced apoptosis." This is particularly pertinent to busy clinical labs where samples may have to wait a considerable time between preparation and analysis. Nevertheless, it is clear that when appropriately performed, enumeration of non-necrotic, nonapoptotic CD34⁺ cells infused into a patient may provide a better prediction of engraftment potential in apheresis products with marginal CD34⁺ content and in postcryopreserved cord blood samples.

The enzyme aldehyde dehydrogenase (ALDH) has previously been shown to have high levels in hematopoietic progenitor cells (24). Recently there has been renewed interest in ALDH due to improvements in the assay system to detect the enzyme (25). In apheresis samples, the ALDH⁺ population has been shown to contain CFC and long term culture (LTC) initiating cells, with expansion in both primary and secondary LTC. These cells are also capable of multilineage differentiation in vitro and the number of ALDH⁺ cells infused to transplant recipients has been shown to correlate to time-to-engraftment of platelets and neutrophils (24). Technically, the staining of ALDH may be reduced in the presence of significant numbers of red blood cells (cord blood, bone marrow, or peripheral blood). The utility of measuring ALDH⁺ cells in these sources of stem cells remains to be proven.

Immunological Characterization of CD34⁺ Stem Cells

A variety of sophisticated in vitro and in vivo models of hematopoiesis have shown that the cells responsible for sustained multilineage engraftment are contained in the most primitive subsets of CD34⁺ cells (and their precursors). Such subsets of CD34⁺ cells in bone marrow express very low levels of antigens associated with lineage commitment (lin⁻) and are found in the CD34^{hi}, Thy-1 (CD90)⁺, CD133⁺, CD38/CD71/HLA-DR^{dull/negative} fraction (26,27). Following on this work, Negrin et al. (28) demonstrated that as few as 8×10^5 highly purified lin⁻/CD34⁺/Thy-1⁺ PBSC-derived cells per kilogram body weight effected timely engraftment in metastatic breast cancer patients [28]. While this work showed that highly purified subsets of CD34⁺ cells can be effective in certain circumstances, selection and high speed sorting of CD34⁺ subsets is expensive and currently technically demanding for routine deployment in the clinical setting. Since virtually all patients receiving the minimum dose of 2×10^6 CD34⁺ cells/kg body weight engraft in a timely manner, it is unlikely that monitoring subsets in patients receiving the target dose (or greater) will provide any additional clinical information. While the main indications for subtyping of CD34 cells remains in the research setting, it may be informative to analyze the immunological profile of CD34⁺ subsets in patients with an inadequate dose of CD34⁺ cells collected and/or in poor mobilizers who

receive salvage marrow collections. Several studies of the latter have shown that patients engraft only slowly, if at all (29). Our own preliminary data suggest that this slow engraftment is due to the lack of primitive subsets of CD34⁺ cells in such transplants. Other potential clinical applications for immunological characterization of CD34⁺ cells include measuring qualitative differences in response to different cytokine regimens and quality assuring selected stem cell products during cell processing.

CONCLUSION: LOOKING FORWARD

The basics of CD34 cell enumeration techniques have generally reached consensus; that is, the use of CD34 and CD45, single platform methodology, and a viability dye when appropriate. To this point, many quality assurance programs have used stabilized whole blood samples for logistical reasons. A joint approach by the Diagnostic Immunology Resource and Blood Transfusion Committees of the CAP will provide viable apheresis samples beginning in the Spring of 2004, which will allow laboratories to evaluate samples similar to those seen in clinical practice. Over the last few years, the number of clinical applications for stem cell transplantation has expanded considerably. Stem cells are obtained from increasingly diverse sources, including marrow, peripheral blood, and cord blood, for both autologous and allogeneic transplantation. Ex vivo manipulations have been developed to "engineer" the graft to suit specific clinical requirements, including "positive" selection techniques to purify CD34⁺ cells, "negative" purging techniques to remove residual tumor cells in the autologous setting, or T lymphocytes in the allogeneic setting. There is considerable interest in ex vivo expansion methodologies and gene therapy protocols. Flow cytometry provides an excellent technology to accurately monitor the qualitative and quantitative consequences of such procedures on stem cell products. Currently, while evidence of trilineage engraftment is used as the primary endpoint of transplant, it is clear that posttransplantation immune reconstitution is an important predictor of disease-free progression and overall survival (30). In summary, flow cytometric enumeration of CD34⁺ cells plays a critical role in identifying the optimum product for transplantation, and this technology will continue to have a major role in defining and evaluating the most suitable product in an increasingly diverse set of transplantation therapies.

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