Variation of CD34+ Cell Enumeration of Cord Blood with Additional CD14 Gating by Flow Cytometry

Hsin-Tsung Ho, MD, PhD, FCAP, Chun-Chun Lin, MT(ASCP), Johnson Lin, MD

1Department of Laboratory Medicine and the 2Department of Hematology and Oncology, Mackay Memorial Hospital, Taipei, Taiwan

DOI: 10.1309/4G0WKRCLX1RFFGGL

The enumeration of CD34+ cells is crucial for evaluation of apheresis products and its optimal timing of harvest. However, a consensus for CD34+ cell enumeration in peripheral blood stem cell (PBSC) transplants has not emerged.

Using 3-color flow cytometry and monoclonal antibodies against CD45, CD34, and CD14, we evaluated the variation of CD34+ cell enumeration in 36 cord blood samples.

With additional CD14 gating, the enumeration of CD34+ cells following the ISHAGE guidelines was significantly decreased, and the decrease correlated to the amount of monocytes expressing CD34.

The enumeration of CD34+ cells plays an important role in the analysis of PBSC content to optimize timing of apheresis and “online” quantitation of the PBSC yield during apheresis. In 1995, the research group at Toronto Hospital was asked by the International Society of Hematology and Graft Engineering (ISHAGE) to develop clinical guidelines for the enumeration of CD34+ cell in peripheral blood. However, a consensus for performing the “rare event” analysis of CD34+ cells in the PBSC transplants settings has not emerged. Comparing the results from multicenter studies, the choice of different denominators, use of different gating strategies, and methods of calculating absolute CD34+ cell numbers have generated divergent data. Sample variability remains a significant problem with respect to numbers of red cells, platelets, platelet aggregates, non-specifically stained adherent cells, and cellular debris, all of which may be faithfully recorded by flow cytometers. Many of these events can not be excluded merely by optimizing the forward angle light scatter threshold. Since it is crucial to obtain an accurate CD34+ cell count that clinicians rely upon in assessing the engraftment potential of a particular sample, we would like to understand the correlation and degree of impact of these factors affecting the enumeration of CD34+ cells which may explain the inter-laboratory variation. To our knowledge, there has not been any sort of study addressing the problem of sample variability caused by nonspecific adhesion of CD34 antibodies with monocytes, platelets, or non-viable cells. In this study, we were particularly interested in the variation resulted from additional gating of monocytes.

Materials and Methods

In our hospital, cord blood samples were routinely obtained from newborns for the serologic test of syphilis. A small portion of cord blood collected in sodium heparin tubes (Becton Dickinson M, Franklin Lakes, NJ) from 36 randomly chosen newborns of full term (37-41 weeks) were used in this study. The blood was stored at 4°C and processed within 8 hours postpartum. Duplicate blood specimens of 100 microliters were labeled with 2 sets of monoclonal antibodies against CD45 (leukocytes), CD34 (stem cells), and CD14 (monocytes). The monoclonal antibodies were selected with 3 fluorescences at the following combinations: the experimental set of CD45-FITC/CD34-PC5/CD14-PE and the control set of CD45-FITC/Isotype-PC5/Isotype-PE. The blood and antibodies were incubated at room temperature for 30 minutes, followed by the lysis of red cells. The remaining cells were then washed twice with PBS and resuspended in 0.5 mL 1% paraformaldehyde. List mode data were collected on a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter M, Fullerton, CA) and analyzed with Coulter SYSTEM II software. The color display of graphic dots was designated as follows. The green, red, and blue colors represent the events of CD45-FITC, CD34-PC5, and CD14-PE, respectively.

The enumeration of CD34+ cells was accomplished according to the 4-parameter flow methodology adopted by ISHAGE in which the strategy did not include CD14 gating [F1 and F2]. The CD34+ cells were also quantified with the same method plus additional CD14 gating [F3 and F4]. The leukocyte count of each blood specimen in duplicate was performed by the hematology cell counter (Coulter Gen S). Following the basic ISHAGE protocol calculation, absolute number of CD34+ cells = (average CD45+ cell count – isotype control) / average CD45+ events counted x leukocyte count. Student t test and linear regression were applied in the statistical analysis.

Results

In order to understand the effect of CD14 gating upon CD34+ cell enumeration, both ISHAGE strategy and the same
method plus additional CD14 gating were performed for each specimen.

First, the quantitation of CD34+ cells was accomplished by ISHAGE method. Here we demonstrated 1 of the 36 newborn cord blood specimens with its flow cytometric graphs and statistics as an example [F1 and F2]. In this case, there were 241 CD34+ cells as well as 6 isotypic control cells among 50,000 leukocytes. The corresponding leukocyte count was 10.37 x 10^6/mL. Following the basic ISHAGE protocol, the percentage of CD34+ cells per leukocytes was calculated as (average CD34+ cell count – isotype control) / average CD45+ events counted = (241 – 6)/50,000 = 0.47%. The absolute number of CD34+ cells was equal to 10.37 x 10^6 x 0.47% = 48,739. By analyzing all 36 specimens, the average number of CD34+ cells per mL cord blood was 40,411.

Second, the specimen of the same case was quantitated with ISHAGE method plus additional CD14 gating [F3 and F4]. There remained 143 CD34+ cells together with 5 isotypic control cells among 50,000 leukocytes. The percentage of CD34+ cells per leukocytes was equal to (143 – 5)/50,000 = 0.276%. The absolute number of CD34+ cells became 10.37 x 10^6 x 0.276% = 28,414. Meanwhile, the number of monocytes expressing CD34 per 50,000 leukocytes was estimated as the CD34+CD14+ cell count minus its isotype control = 116 – 9 = 107. Thus, with additional CD14 gating we found that the average number of CD34+ cells per mL cord blood reduced to 34,524.

Apparantly, the average CD34+ cell count was lower with additional CD14 gating. The percentages of decreases ranged from 0.9 to 41.7% with a mean 15.1%. The decrease was significant, analyzed by 1-tail paired Student t test (P=0.015). The corresponding numbers of CD34+ monocytes per 50,000 leukocytes were also quantitated with a mean of 33 and a range of 5 to 116. The relationship between the decreased percentages of CD34+ cell counts (mean 15.1%, range 0.9%-41.7%) and the corresponding numbers of CD34+ monocytes (mean 33, range 5-116) per 50,000 leukocytes was further studied by linear regression. The adjusted R was 0.808.

Discussion

The flow cytometry-based CD34+ cell analysis, since it can be performed in less than an hour, would be suitable for the determination of optimal timing for apheresis collections and the “online” evaluation of the apheresis products. So far the technique of flow cytometry has been widely used in 2 different potential sources of stem cells other than bone marrow, namely PBSC and cord blood. The lack of standardized methodology makes the analysis difficult to determine the quantity of stem cells and compare the results of clinical trials between institutions. In multicenter studies of PBSC, a wide range of results was found although the same antibodies and lysis techniques were used. The major source of variation was the gating strategy used for cytometric data acquisition and analysis. In this study, we looked into the degree of impact resulted from the additional CD14 gating, which had not been evaluated before.

Compared to the traditional settings the ISHAGE protocol, this method has the following two advantages. First, by including only CD45+ events in the analy-
sis, red blood cells, their nucleated precursors, platelets and cellular debris, which are variably present in peripheral blood and apheresis preparation and which are recorded by flow cytometers, are excluded from subsequent analysis. Second, the blast gating technique (CD45 staining versus side scatter) provides a means of delineating specifically stained CD34+ stem cells (dim CD45) from nonspecifically stained debris (CD45 negative) and normal lymphocytes (bright CD45). As shown in F3, monocytes are identified as a part of CD45+ events, and their distribution overlaps the blast gating area. Monocytes within the blast gating area were recorded by the ISHAGE protocol. Our results show that the coexistence of these monocytes expressing CD34 is significantly related to the variation of CD34+ cell enumeration.

CD34+ cells can be found in the peripheral blood of normal individuals but are extremely rare (consensus range is 0.01%-0.1%). CD14+ cells collected from peripheral blood of healthy donors usually represent mature monocytes. Ferrero and colleagues stated that a small proportion of these cells, ranging from 5% to 10%, coexpressed the CD34 progenitor marker and could migrate across the endothelial cell monolayer. Since CD34 is usually lost during leukocyte maturation, the CD14+CD34+ cell population

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Number</th>
<th>%Total</th>
<th>%Gated</th>
</tr>
</thead>
<tbody>
<tr>
<td>UnGated→CD45+</td>
<td>50000</td>
<td>75.81</td>
<td>75.81</td>
</tr>
<tr>
<td>UnGated→Low CD45</td>
<td>1281</td>
<td>1.94</td>
<td>1.94</td>
</tr>
<tr>
<td>CD45+→CD34+</td>
<td>18</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>CD34+→CD45+</td>
<td>18</td>
<td>0.03</td>
<td>100.0</td>
</tr>
<tr>
<td>CD34+→Low CD45</td>
<td>7</td>
<td>0.01</td>
<td>38.9</td>
</tr>
<tr>
<td>Low CD45→Low SSC</td>
<td>6</td>
<td>0.01</td>
<td>85.7</td>
</tr>
</tbody>
</table>

Percentage of CD34+ cells per leukocytes: \(\frac{(241 - 6)}{50000} = 0.47\%\)

Number of CD34+ cells per ml cord blood: \(10.37 \times 10^6 \times 0.47\% = 48739\)
might represent an intermediate stage of differentiation toward mature monocytes. In our cord blood specimens, about 0.2% to 2.3% of monocytes are CD34 positive and account for the 0.9% to 41.7% decrease of CD34+ cell counts with additional CD14 gating. The CD14+CD34+ events may represent the monocytes in the intermediate stage of differentiation, or the nonspecific binding effect between monocytes and anti-CD34 antibodies, or both.13 From the point of quality assurance, establishment of laboratory proficiency testing program or automated gating software may be helpful to minimize this major variation.10,14

**Conclusion**

Our study showed that the quantitation of cord blood CD34+ cells following the ISHAGE guidelines was significantly decreased with additional CD14 gating. The decreased percentage of CD34+ cells correlated to the amount of monocytes expressing CD34. Thus, the nonspecific binding effect of monocytes should be considered in CD34+ cell enumeration. This finding may partially explain the interlaboratory variation of CD34+ cell enumeration by different gating strategy.