Single Versus Dual Platform Analysis for Hematopoietic Stem Cell Enumeration Using ISHAGE Protocol

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Abstract Hematopoietic stem cell transplantation is curative therapy in benign and malignant diseases. Adequate stem cell dose is one of the most important marker of engraftment. Several methods have been developed to enumerate CD34+ cells. We present our data on 147 samples analysis. There was a clear linear correlation between two methods. Both methods were effective. Both single vs dual platform analysis yield similar results. Single platform analysis is easier to perform. In terms of cost reduction dual platform analysis is better.

Keywords Bone marrow transplantation · Hematopoietic stem cells · Flowcytometry · Enumeration · ISHAGE

Introduction

Number of CD34+ pluripotent hematopoietic stem cell relative to weight of the patient, is one of the most important criteria determining success of stem cell transplant [1]. Successful enumeration of CD34 cells require appropriate procedures and protocols to reduce possible errors [2, 3]. In many Laboratories associated with stem cell transplant, CD34+ cell enumeration is done by flow cytometric analysis following the International Society of

Haematotherapy and Graft Engineering (ISHAGE) guidelines [4].

Two common methodologies used to measure CD34+ cells in a given sample are single platform and dual platform methods [5, 6]. In Single platform method, a known number of fluorescent beads are added to the sample and in dual platform method; data is combined from a conventional hematology analyzer and a flow cytometer. In dual platform method, two measurements, preparation of samples can lead to major difference in actual and measured CD34 value. As single platform method uses known number of fluorescent beads, it is much easy to use than routine dual platform methods. Counter checking the single platform assay result with the total leucocyte count obtained from a hematology analyzer can further validate the analysis.

There is lack of clarity if one method is superior to another. Lot of centers around the world use single platform while many others use dual platform analysis. Our work is a comparative study between two methods in mobilized peripheral blood stem cells collected by leukapheresis.

Materials and Methods

Stem cell mobilization was done in patients or healthy donors after 4 days of GCSF or following chemotherapy. Analysis was done on peripheral blood (preharvest) or stem cell bag (post harvest) samples as applicable. All samples were collected in EDTA tubes and kept at room temperature. Cell count of peripheral blood, stem cell samples was done on Beckman Coulter hematology analyzer LH750 in duplication on two equipment. Mean of both the values was

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taken as the actual total cell count (TLC) for the dual platform assay.

Dual Platform Assay

$$\% \text{CD34+ HSC} = \frac{\text{CD34+ HPC Absolute Count (cells/}\mu\text{L) (i.e. region D in Histogram 4, Figure 1)}}{\text{CD45+ Leukocytes Absolute Count (cells/}\mu\text{L) (i.e. Region A in Histogram 1, Figure 1)}} \times 100$$

Reagents

Reagents (Stem-Kit, Beckman Coulter) were used on FC 500 equipment for enumeration of CD34 count on single platform assay and also to calculate the CD34% cells in the samples for dual platform assay. The reagent kit included CD34 PE (Clone 581) and CD45 FITC (clone J33) used for identifying CD34+ hematopoietic stem cells (HSC), along with a 7-Amino-actinomycin D (7-AAD) for cell viability assessment. Lyse solution (10X Ammonium Chloride) was included in the kit to lyse the erythrocytes. Stem cell fluorospheres were also added to the assay for exact enumeration of CD34+ HSC. This assay was run in duplication on the locked protocol based on ISHAGE gating on FC500 and average of two values was taken as final count by single platform [4]. One control tube was also processed to know the validity of the test run.

Sample Preparation

20 μL of CD45 FITC and CD34 PE were added in the two test tubes and 20 μL CD45 FITC/CTRL in one control test tube. 20 μL 7-AAD dye was added in all the three tubes. 100 μL sample was added in all tubes and vortex and incubated for 20 min in dark at room temperature. After incubation 2 ml of freshly prepared 1X NH₄Cl solution was added and quick vortex was done. 100 μL of Stem cell fluorospheres were added to all the tubes and quick vortex done. Samples were analyzed within 1 h of sample preparation.

Gating Strategies and Data Analysis

Triple fluorosphere gating protocol using ISHAGE guidelines was used for calculating the viable CD34+ percentage of the cells and exact count of CD34+ HSC.

Calculations

Single Platform Assay

CD34+ HSC Absolute Count (cells/ μL) = CD34+ HSC Absolute Count (cells/ μL) (i.e. Region D in Histogram 4, Fig. 1).

CD34 Absolute count = % CD34+ HSC $\times$ TLC obtained from Hematology Analyzer (Average of two)

Statistical Analysis

Descriptive statistics were calculated for all variables. Differences in median was used to compare the two groups were assessed using Wilcoxon sign rank when the data was not normal. Relationships between variables were evaluated by Spearman rank correlation. Linear regression analysis was performed to determine factors independently associated with continuous data such as dual platform. For all tests, a 2-sided *P* value of 0.05 or less was considered statistically significant. SPSS 16.0 software was used for the analysis.

Results

Total 147 samples were analysed. Twenty samples were peripheral blood samples which were tested before hematopoietic stem cell harvest. One hundred and twenty seven samples were post harvest samples. Median values of CD34 cell number for single platform method was 498.5 (range: 1–6902) whereas with dual platform method it was 494.5 (range: 0–8796). Analysis of the 146 samples by Wilcoxon sign rank test showed that significant number of values were not similar and had some difference (*P* = 0.031). Linear Regression analysis of two methods gave *R* = 0.967 and *R*² = 0.95 confirming a strong relationship between two methods and little variability. Further analysis between methods demonstrated slope was around 1 (0.923) and though intercept value was 63.3, no significant difference was found (*P* = 0.000) (Fig. 2).

CD34 value with single platform method

$$= 63.3 + 0.923 \times \text{CD34 value by dual platform method}$$

These results show two methods measure same CD34 counts over a wide range of concentration.

Fig. 1 Flow cytometric analysis of PBSC on FC500 with Stem-Kit reagent (by Beckman Coulter) using ISHAGE protocol. Histogram 1, region A includes viable CD45⁺ leukocytes excluding dead 7-AAD cells (region J in histogram 8); appropriate denominator for viable WBC in calculation of the percentage of CD34⁺ cells and region E (lower right) is a lymphocyte specific gate. Histogram 2, plots events from A and J; CD34⁺ cells are gated in gate B. Histogram 3, display events from A, B and J; region C includes CD34⁺ cells with dim to intermediate expression for CD45. Histogram 4, display events from sequential gateings J, A, B, C; Region D identifies a cluster of events meeting all the fluorescence and light scatter criteria of ISHAGE Guidelines for CD34⁺ HPC. Histogram 5, visualize the lower limit of CD45 expression within the CD34⁺ events and Region K (named “listgate -”) to exclude most of the double negative events from the acquisition. Histogram 6, displays events from E and J regions; Verify whether the Forward and Side Scatter gain parameters of the flow cytometer are optimally set for the processed sample. Histogram 7, displays events from region H; Region G encloses the Stem-Count Fluorospheres singlet population. Histogram 8, displays events from region A; Viable events are included within Region J

Fig. 2 Correlation between single vs dual platform methods

Discussion

Hematopoietic stem cell transplant is a curative treatment for many benign and malignant hematological diseases. Adequate CD34⁺ stem cell count remains the most important measurable factor for success of the engraftment success [1].

Over a period of time several attempts are made to simplify the procedure and gating strategies of the CD34⁺ cell enumeration by flow cytometry [7]. The International Society of Hematotherapy and Graft Engineering (ISHAGE) guideline for CD34⁺ cell enumeration is the most

widely used standardized protocol. This protocol is based on ‘lyse no wash’ method. There is no centrifugation step which could result in the selective loss of the cell populations during sample preparations [4]. When developed it was based on dual platform, CD34⁺ cell percentage was obtained from flow cytometric assay and hematology analyzer for total leukocyte count; final count is generated by multiplication of two values. Modifications are made by adding flow count beads in the assay making it a single step assay/single platform assay, in which there is no requirement of hematology analyzer.

Original dual platform assay is criticized for being labor intensive and also for requirement of an additional hematology analyzer in the laboratory, which needs maintenance, calibration and quality control [8]. However, in our study this was not a major hindrance as two hematology analyzers were available in the institute with a work load to justify the regular and stringent QC protocols, calibrations and maintenance [9–11].

Previously published data suggest that a reproducibility of 10% is acceptable for CD34⁺ cell count from the single platform assay, and average of two counts should be used for final reporting. In case, variation in the two values is more than 10%, a repeat test is imperative; increasing the turn-around time to double and also the cost [4, 11]. In our laboratory the reproducibility was better than this level, so we used the single platform assay results as a final count. This variation can be due to variation in CD34⁺ cell percentage or the denominator, i.e. total leukocyte count. Variation in later is more frequent as compared to percentage, which is rare. This variation is largely due to pipetting errors and lack of skilled technical staff (Table 1).

Since platform analysis is costlier. Dual platform analysis is cheaper as there is no need to run controls. There is also resource saving in dual platform analysis. This, however, needs a hematology analyzer which most centers capable of performing bone marrow transplantation are expected to have.

In our study the CD34⁺ count obtained from single platform assay and dual platform assay were comparable. Our study results are similar to previous such studies [2, 5, 6]. The variations in the total leukocyte count from the hematology analyzers were less as compared to flow cytometric assay. We suggest availability of an alternate method to establish denominator in the assay by using hematology analyzer can be useful to prevent the time, resources including the costly reagent of flow cytometry and man-hours [12]. Laboratories having hematology analyzers with well-established QC protocols can add to the quality of CD34⁺ cell enumeration by adding a double

Table 1 Regression analysis between two methods

Method	N	R	R ²	Slope	Intercept	P value
CD34 by single vs dual platform	145	0.967	0.950	0.921	63.3	0.000

check for the total leukocyte count/denominator and reducing the chances of repeat testing, saving time and resources in test failures.

Conclusions

Both single vs dual platform analysis yield similar results. In terms of ease of procedure single platform analysis is better. In terms of cost reduction dual platform analysis is better.

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Compliance with Ethical Standards

Conflict of interest We have no financial relationships to disclose.

Informed Consent Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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