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Original Research Article

CD34+ hematopoietic progenitor cells enumeration and mononuclear cell count: an experience from a tertiary care centre

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ABSTRACT

Background: This study was conducted to assess the CD34+ hematopoietic progenitor cells enumeration by flow cytometry and the utility of performing mononuclear cell count before performing the Stem cell enumeration. Collection was done on two consecutive days with CD34+ hematopoietic progenitor cell enumeration of both the samples. Mononuclear cell counts were done in all the patients. The purpose of the study was to do counts directly from the leukapheresis pack and see the reliability of this practice.
Methods: Samples were collected from the leukapheresis pack and subjected to mononuclear cell count and CD34+ hematopoietic progenitor cells enumeration by flow cytometty before harvesting.
Results: A total of 66 samples from 34 patients were taken up for the study. 76.47% of our cases were that of multiple myeloma and 17.64% of the cases were that of non Hodgkin lymphoma and 2.94% cases each of neuroblastoma and Hodgkin lymphoma. It was noted that the mononuclear cell counts correlated well with the CD34+ HPC in most of the cases with MNC being above 4 x 10^8 per pack per kg body weight in cases where CD34+ HPC counts were more than the desired lower limit of 2 x 10^6 per pack per kg body weight.
Conclusions: It was observed that flow cytometric enumeration of CD34+ hematopoietic progenitor cells directly from the leukapheresis pack gave satisfactory results even without doing peripheral blood CD34+ HPCs enumeration before leukapheresis. Also, in our study we were able to set a limit of mononuclear Cell at 4 x 10^8 per pack/kg BW as counts beyond that always correlated with the more accurate flow cytometric method of CD34+ HPC count of more than 2 x10^6 per pack/kg body weight, therefore acting as a crude method for assessing the mobilization.

Keywords: CD34+, Hematopoietic progenitor cells, Leukapheresis, Mononuclear cells

INTRODUCTION

In Hematopoietic Stem Cell Transplantation (HSCT) the patient is subjected to growth factors followed by myeloabelation and finally hematopoietic stem cells infusion.[1,2] The source for CD34+ Hematopoietic Progenitor Cells (HPCs) can be either peripheral blood, bone marrow or the umbilical cord blood.[1] Peripheral Blood cells after being mobilized from the bone marrow are collected by the process of Leukapheresis using an apheresis machine. This method is used in more than 90% of Autologous Bone Marrow Transplants (BMT).[3] The Peripheral Blood stem cells (PBSCs) are the preferred source for autologous HSCT due to the fact that in comparison to Bone Marrow Stem Cells (BMSCs) it is easier for collection with a faster haematological recovery.[4,5] PBSCs consist of a subpopulation of CD34+ HPCs, which are morphologically difficult to identify.

There are many methods for stem cell quantification after collection but the most common method used today is the flow cytometric evaluation of CD34+ HPC.[6] Other methods such as colony forming units (CFU) of granulocyte-macrophage were also used to estimate stem cell numbers. This method is much less reliable due to the variation in culture techniques, media preparation, and several human factors.[7] Normally, CD34+ HPCs in PB is in the range of 0.01 to 0.05% and in the BM, the concentration is usually less than 1% of the mononuclear cells.[8-11] The exact quantity of Hematopoietic Progenitor Cells required to achieve a desirable engraftment is not clearly defined, but a count of 2 to 5×10^6 CD34+ HPCs/Kg of body weight is considered to be satisfactory to achieve a successful engraftment.[12,13] The protocol that we have been using at our centre is the mobilization of PBSCs using granulocyte colony-stimulating factor (G-CSF) with the use of adjuvants such as Plerixafor in selected cases. After the administration of mobilizing agents the PBSC were collected by leukapheresis.[10] Certain factors have a role in assessing the effective harvesting which include the total leukocyte count, number of lymphocytes and monocytes etc.[14-17] Flow cytometry is the method of choice for monitoring the peripheral blood CD34+ hematopoietic progenitor cell concentration.[13,18] The Mononuclear Cell (MNC) counts were concomitantly done in all the samples received. This is a routine that we employ at our centre and the purpose of doing was to see if any significant correlation existed with the more accurate and reliable CD34+ HPC enumeration by flow cytometry and to see if it was possible to set a minimum MNC count which would correlate with the adequate CD34+HPC cells.

METHODS

This study was conducted in the Department of Hematology along with Department of Oncology at Sher-I-Kashmir Institute of Medical Sciences, a tertiary care centre in Srinagar, Jammu and Kashmir, India. The patients were taken only for autologous bone marrow transplantation with exclusion criteria being an age above 70 years, patients with deranged liver function test, kidney function test, poor cardiac status and patients with a poor lung compliance. CD34+ HPC count was done by Flow Cytometry using the modified International Society of Hemotherapy and Graft Engineering (ISHAGE) protocol.[19] MNC count was done on the leukapheresis sample by doing the Total Leucocyte Count (TLC) on Beckman Coulter LH 750 hematology analyzer and then Differential Leucocyte Count (DLC) was done manually on the peripheral blood film. Leukapheresis was done on 2 consecutive days and then subjected to MNC and CD34+ HPC count immediately. MNC was a practice started at our centre few years back as Flow Cytometry was not available in our city and the samples were outsourced to other places due to which the results would be available only after 2 to 3 days. The idea of doing an MNC was to get a rough idea if the yield would be adequate or not. The purpose of including MNC in our study was to see if it really had a role in knowing the status of collection. Flow cytometric measurement of CD34+ HPCs was done on Beckman Coulter Navios®, a 2 laser and 8 colour instrument by using CD45 (clone-J33) and CD34 (clone 581) monoclonal antibodies (Beckman coulter) conjugated with the fluorochromes Fluorescein Isothiocyanate (FITC) and R Phycoerythrin (PE) respectively along with 7-AAD, a viability dye, stem count fluorospheres and ammonium chloride based lysing solution by using the modified ISHAGE a single platform protocol to identify CD34+ HPCs by flow cytometry. The sample and sheath fluid are taken and the counts adjusted. The dilution factor is to be noted. 20µL of 7-AAD and antibodies are added and mixed well and incubated for 20 minutes. After that lysing solution (prepared fresh by adding 200µL to 1800µL of distilled water) is added and again incubated for 10 minutes. Then stem count fluorospheres are added and mixed well and sample acquired and finally counts done as per modified ISHAGE protocol.

Results were obtained and were analyzed using the Kaluza software (Beckman Coulter, USA). The scatter plots in Figure 1 show quantification of CD34+HPCs. The viability dye 7-AAD was used to separate the non-viable cells which showed positivity for 7-AAD. The region A from Plot A shows the viable events from region K on Plot 6. Plot B shows region B and includes events from region A and K. The region B is adjusted to surround CD34+ HPC. The plot C shows region C and displays events from A and B and K. The region C is adjusted to include cells forming a cluster with characteristic CD34+HPC (cells with low Side Scatter and low to intermediate CD45 staining). The plot D displays events from A and B and C and J. This lymph/blast region (labelled as CD34 Pos on Plot D) identifies a cluster of events meeting all the fluorescence and light scatter criteria of ISHAGE guidelines for CD34+HPC. Once the analysis is complete the absolute count of viable CD34+HPC is established. The plot E displays all events. This plot is useful to visualize the lower limit of CD45 expression within the CD34+ events. Plot F as mentioned earlier is to separate viable cells from non- viable cells. Plot G displays events from region F (see Plot E). The CAL region encloses the stem-count fluorospheres singlet population. This region is labelled as CAL to allow automatic calculation of absolute numbers of CD34+HPC. The correct assayed concentration of the lot of the stem count fluorospheres used is to be entered. In our study the calibration factor of the lot was 964 and 1002. Plot H displayed events from lymph region (see Plot A) and region K. This plot is to see if the forward and side scatter gain parameters of the flow cytometer are optimally set for the processed sample. The CD34+HPCs per pack per kilogram body weight were calculated as follows:

$$\frac{\text{CD34+HPCs/µL x Dilution factor x } 10^{6*} \text{ x Volume of pack (x } 10^{-3}\text{L)}}{\text{Weight of the individual (in Kgs)}}$$

*10^6 in the numerator is for conversion of CD34+HPCs/µL to CD34+HPCs/L.

Mononuclear Cell (MNC) count was done for each leukapheresis pack received. The sample taken from the leukapheresis were collected in EDTA vacutainer and subjected to Total Leucocyte Count (TLC) by hematology analyzer. Then the peripheral blood films were made and subjected to a manual differential count for each sample. The mononuclear cells included lymphocytes, monocytes and blasts. Then the absolute mononuclear count was derived from the TLC. The mononuclear cell count per pack per kg body weight was calculated as follows:

$$\frac{\text{Absolute Mononuclear* Count (x 109/L)xVolume of pack (x } 10^{-3}\text{L)}}{\text{Weight of the individual (in Kgs)}}$$

*10^6 in the numerator is for conversion of CD34+HPCs/µL to CD34+HPCs/L.

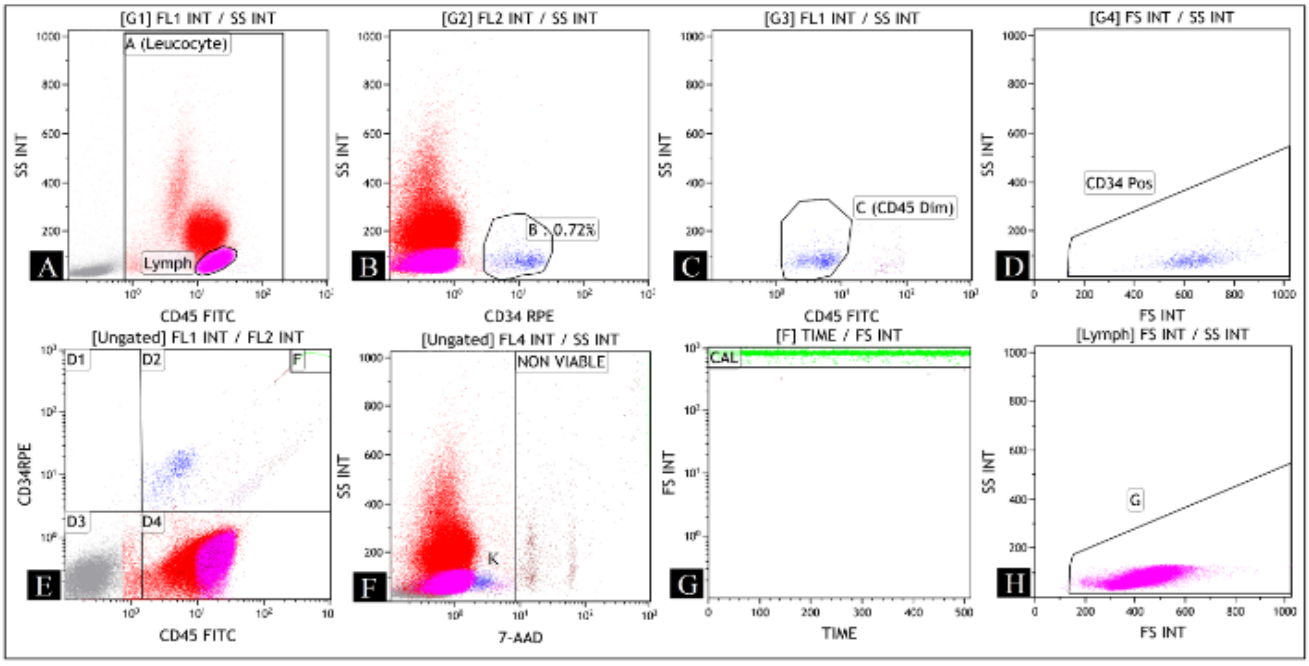

Figure 1: The analysis of apheresis sample stained with stem-kit reagents. A) The region A shows the viable events from region K. B) The plot here shows region B and includes events from region A and K. C) The plot here shows region C and displays events from A and B and K. D) The plot here displays events from A and B and C and J. E) The plot here displays all events. F) This plot displays viable events within the region K. G) This plot encloses the stem-count fluorospheres singlet population. H) This plot displays events from lymph region and region K.

RESULTS

Sixty-six leukapheresis samples from 34 patients were analysed for CD34+ HPC and MNCs. Among the 34 patients taken up for autologous stem cell transplant, 26 patients were that of multiple myeloma with 6 patients of Non-Hodgkin lymphoma and 1 case each of Hodgkin Lymphoma and Neuroblastoma. (Table 1). The patients were started on G-CSF for 4 days with a dose of 10µg per kg body weight. Even on day 5, the day of collection, G-CSF was given. The patient was subjected to leukapheresis on COBE spectra. The sample was sent for MNC and CD34+ HPC enumeration with the details of volume of pack and weight of the individual noted. In an ideal setting a peripheral blood sample is subjected to CD34+HPC enumeration before the leukapheresis to check if mobilization has taken place sufficiently.

Table 1: Diagnosis of the patients subjected to autologous stem cell transplant.

Diagnosis	Number of cases	Percentage of cases (%)
Multiple myeloma	26	76.47
Non Hodgkin lymphoma	6	17.64
Hodgkin lymphoma	1	2.94
Neuroblastoma	1	2.94

In our study we by-passed this step and have employed a direct estimation from the leukapheresis pack. Though we did peripheral blood CD34+HPC enumeration before the leukapheresis in 2 patients of multiple myeloma with counts in one of them being 11.76cells/µL and in the other patient it was 5.37cells/µL and 26.32cell/µL after adding plerixafor. Since we have had a hassle free

operation we continue with this trend of doing CD34+ HPC counts from leukapheresis pack directly with excellent results. G-CSF therapy did not give rise to any serious complication and any side effect we encountered was managed symptomatically. Leukapheresis was done in all patients on two consecutive days irrespective of the CD34+ HPC count on day 1, except in 2 patients of multiple myeloma where in one we had to do it for 3 consecutive days due to insufficient yield; only after adding plerixafor the day 3 sample showed a satisfactory count and in another patient the transplant was started only after one day's collection.

Table 2: Characteristics of the 66 leukapheresis from 34 patients taken up for autologous stem cell transplant.

Gender		Age		TLC (Mean)		MNC (Mean)		CD34+HPC (Mean)	
Male	**Female**	**Median**	**Range**	**Day-1**	**Day-2**	**Day-1**	**Day-2**	**Day-1**	**Day-2**
25 (73.52%)	09 (26.47%)	51	5-70	182.87	170.70	4.59	4.20	4.21	4.03

TLC- Total Leucocyte Count (x 109/L); MNC- Mono Nuclear Cells (x 108 per Pack per Kg BW); CD34+ HPCs (x 106 per Pack per Kg BW). L-litres, Kg-kilograms, HPC-Hematopoietic Progenitor Cells, BW- Body Weight

Table 3: Detailed analysis of the variables used in the study.

Multiple Myeloma								
Weight	**Volume**		**TLC**		**MNCs**		**CD34+ HPCs**	
	Day 1	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**
68	230	230	152.16	123.89	4.61	5.2	2.02	2.26
75	226	225	132.29	73.53	3.94	3.02	3.92	2.5
65	236	230	562.43	178.9	9.56	5.22	8.8	7.1
50	230	230	59.95	165.24	4.62	5.51	6.4	7.3
90	230	230	145.76	267.8	4.81	4.02	3.5	2.2
78	234	242	30.185	78.6	3.23	5.76	2.6	3.6
70	228	236	218.23	320.91	5.18	5.84	5.4	4.6
90	235	235	246.99	240.2	6.4	6.79	5.7	8.2
90	243	242	97.49	142.5	2.9	3.5	0.6	1.56
71	228	230	191.1	142.26	5.09	2.76	9.8	2.0
70	232	232	251.05	256.39	2.83	2.9	1.89	2.03
47	222	222	126.63	190.5	5.1	6.0	3.4	4.2
65	231	230	402.25	303.47	5.43	3.11	8.5	6.8
51	230	230	269.93	150.57	7.42	3.56	9.6	10.7
57	335	330	88.6	123.57	3.49	5.7	11.2	12.55
79	235	235	196.8	205.9	4.04	3.73	2.87	0.23
90	265	275	26.6	150.6	1.86	2.02	0.08	3.4
75	232	232	222	222.7	2.68	2.13	3.2	5.4
74	232	231	113.44	106.6	3.13	2.83	2.53	2.0
58	229	228	60.2	185.4	1.83	4.0	6.06	4.3
64	234	232	174.1	145.12	5.54	3.78	3.7	2.1
69	236	235	116.3	179.6	3.93	5.9	1.43	2.5
67	235	232	263.0	192.5	6.45	3.8	7.5	4.52
55	223	210	150.0	136.5	3.04	2.74	1.45	1.29
57	267	-	149.54	*	5.67	*	2.16	*
62	187	-	310.76	*	3.8	*	1.6	*
Non Hodgkin Lymphoma								
65	226	231	128.44	148.59	3.57	4.2	3.0	3.4
48	224	225	258.44	143.8	6.07	3.6	3.4	1.4
77	230	230	193.4	144.85	3.04	2.5	1.1	2.0
78	235	235	156.0	126.46	3.81	4.1	3.31	4.1
63	235	235	175.4	40.9	6.0	4.89	6.48	5.3
42	224	225	139.5	94.7	2.7	2.94	1.85	2.03
Hodgkin Lymphoma								
81	226	226	233.63	345.31	5.98	5.68	2.3	1.7
Neuroblastoma								
17	205	225	175.0	134.7	8.44	6.81	6.12	5.8

L- litres, Kg-kilograms, TLC- Total Leucocyte Count (x 109/L); MNC- Mono Nuclear Cells (x 108 per Pack per Kg BW); CD34+ HPCs (x 106 per Pack per Kg BW). HPC-Hematopoietic Progenitor Cells, BW- Body Weight.* Only Day 1 sample was collected.# 3 samples were collected.

At the end of day 1 collection G-CSF was given in the evening as well and in the morning of day 2 collection. After 2 consecutive leukapheresis, the patient was subjected to myeloabelation on the same day with melphalan 200mg/m^2 given for multiple myeloma and for lymphomas the BEAM protocol (Carmustine 300mg/m^2 on day 1, etoposide 200mg/m^2/day from day 1 to day 4. Cytarabine 200mg/m^2/day twice daily from day 1 to day 4 and melphalan 140mg/m^2 on day 5) was given. In one pediatric case of neuroblastoma, the myeloabelation was performed by giving busulphan 0.8 to 1mg per kg body weight every 6th hourly for 4 days. Melphalan (140mg/m^2) was given on day 5. Injection lorazepam was given as prophylaxis for convulsions. However, not all patients had a satisfactory CD34+ HPCs after day 1 of leukapheresis. In these patients in addition, an adjuvant was used in form of plerixafor. This was given 12 hours prior to the day 2 leukapheresis and in our study it made a difference in many cases. Finally, after myeloabelation, re-infusion was started after 24 hours. Details of the patients are summarized in Table 2.

Among the 34 cases taken up for the study the proportion of males was 73.52% with a mean age of mean age was 48 years (range: 5-70 years) and the most common indication at our centre for Autologous SCT was Multiple Myeloma (76.47%). The mean TLC was noted to be higher on day 1 sample as compared to day 2 sample and the MNC count was comparable on day 1 and day 2 with a slightly higher MNC on day 1 as compared to day 2. It was also noted that the CD34+HPC count was slightly more on day 1 samples as compared to day 2 sample, though the difference was marginal. In some patients in whom the day 1 CD34+ HPC was low, plerixafor, which augmented the CD34+ HPC mobilization was used before collection on day 2. Plerixafor was not used up front in all cases. It was used in cases where the day 1 collection was sub optimal and also up front in non Hodgkin lymphoma and in patients of multiple myeloma who were heavily treated before transplant. It was noted that in 8 patients on day 1, CD34+ HPC counts were less than 2 x 10^6 per pack per kg body weight with 6 patients of multiple myeloma and 2 patients of NHL. In day 2 samples 5 patients had counts of less than 2 x 10^6 per pack per kg body weight. In 5 of these patients there was an improvement of CD34+HPC counts on day 2 after giving plerixafor.

An interesting finding in our study was that the patients with an MNC counts of above 4x10^8 per pack per kg body weight showed CD34+ HPC counts of above 2 x 10^6 per pack per kg body weight, thus we could establish a cut-off for MNCs as more than 4x10^8 per pack per kg body weight which correlated with a CD34+HPC count of 2x10^6 per pack per kg body weight. Table 3 gives a detailed analysis of the variables taken in the study both on day 1 and day 2 leukapheresis pack which included the weight of the individual, volume of the pack, total leucocyte count, MNC count and CD34+HPCs.

DISCUSSION

The CD34+ HPC counts by flow cytometry is the most important step in stem cell transplant as it helps us to reliably quantify stem cells.[17] The parameter used in our study other than CD34+HPC included MNC counts and TLC. TLC was not considered as a parameter for predicting a satisfactory yield. In many day 2 sample plerixafor was added to augment the mobilization but overall the CD34+ HPCs did not increase on day 2 indicating that the number of CD34+ HPC decrease with time despite the patient on G-CSF. It has been found that the CD34+ HPC count is at peak on day 4 of mobilization.

Thus, the collection on day 1 is expected to have a higher yield of CD34+HPC as compared to the subsequent day. Despite various strategies to produce a satisfactory CD34+HPC collection not all patients achieve a minimum number of CD34+ cells for a successful engraftment which could be due to certain factors as demonstrated by Szmigielska-Kaplon et al. where they explained that due to certain molecular changes there can be a difficulty in stem cell collection depending on the primary disease.[20,21] The G-CSF mobilization regimen used at our centre is widely used but one has to keep in mind the high failure rate associated with it. [22] The failure of stem cell transplant is seen more in patients of non Hodgkin lymphoma (26%) as compared to multiple myeloma (6%).[23,24]

The drug we used in case of poor mobilization was plerixafor which had a synergistic effect when used with G-CSF that can increase the CD34+HPC cells by up to 3.8 times.[22] Plerixafor though not used in all our cases up front, was used in cases of NHL, and heavily treated Multiple Myeloma and cases with poor mobilization on day 1. Plerixafor induces a short lasting rapid mobilization. One major limitation of Plerixafor in our setting was its high cost. A study was conducted by one group with a sample size of 508 patients with 256 multiple myeloma, 270 NHL and 54 Hodgkin lymphoma cases and showed that plerixafor was efficient in CD34+HPC collection (>2×10^6/kg bodyweight) in 81.6% of multiple myeloma patients, 64.8% of NHL patients and 81% of Hodgkin lymphoma patients.[24]

Though our sample size was small but we found in our study that it is a good drug for patients with poor mobilization. The mononuclear cell count done in all the cases in our study. Though a range for CD34+HPC is well defined but no such parameter has been established for the Mononuclear Cell Count (MNC). We in our study wanted to establish a correlation between CD34+HPC count and mononuclear cell counts. Though our sample size was smaller but we were able to establish a rough correlation between the two which needs to be studied extensively with a larger sample size.

CONCLUSION

To conclude, we obviated the need for doing peripheral blood CD34+ HPCs enumeration before leukapheresis, a practice followed by most of the centres and did a direct CD34+HPC estimation from leukapheresis sample which gave satisfactory results. Also, in our study we were able to set a limit of mononuclear cell at $4x10^8$ per pack/kg body weight. This cut-off correlated well with the more accurate flow cytometric method of CD34+ HPC count of more than $2x10^6$ per pack/kg body weight, therefore acting as a crude method for assessing the mobilization. However, the sample size needs to be bigger for a definitive importance of estimation of MNC along with CD34+ HPC cells.

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