CHARACTERIZATION OF CD34+ HEMATOPOIETIC STEM CELLS FROM HUMAN PERIPHERAL BLOOD BY MAGNETIC CELL SORTING

Irfan Ahmed Siddiqui[1]and Dr. K. Babu Rao[2]

[1]Research Scholar, CMJ University, Shillong, Meghalaya

Principal Donbasco College Pharmacy, Guntur District, AP

Abstract

*CD34+ cells, present at a frequency of 0.18 ± 0.052% among leukocytes from peripheral blood (PB), can be rapidly and efficiently en- riched to a frequency of 38.6-87.1% (&4*4 by high-gradient magnetic cell separation (MACS) for Immunopheno- typing, characterization in colony-forming cell assays, and further purification to homogeneity (>98%) by multiparameter fluorescence-activated cell sorting (FACS). Enriching PB-CD34+ cells for immunophenotyping allows the detection of small subpopulations, ex- pressing the B-cell antigens CD10, CD19, and CD20, the T-cell antigens CD45RA and CD7, and a small subpopulation expressing high levels of CD34 which mostly coexpress CD19 CD20 ; and CD38*

. All PB-CD34 $^{+}$ cells express elevated levels of CD71 (transfer-rin receptor), with a subpopulation of high expressing cells, and CD38. Some cells express CD33. MACS-enriched PB-CD34^{+} cells show "normal" hematopoie-tic colony formation in vitro. The ease and efficiency of purification of large numbers of CD34^{+} cells from PB by MACS is not only relevant for the characterization of migrating stem cells but also opens new possibilities for stem cell transplantation & genetic manipulation of the hematopoietic system.

***Key terms**: Migrating stem cells, flow cytometry, cell sorting, fluorescence-activated cell sorting, immunophenotype, colony-forming assay*

INTRODUCTION

Stem cells capable of restoring Haematopoietic are known to circulate in peripheral blood (PB) (1,6,23,24). Haematopoietic stem cells obtained from PB (PB-stem cells) by leukapheresis have been used for transplantation as an alternative to bone marrow-derived stem cells (BM-stem cells), the haemopoietic "potential" of PB-stem cells being almost equivalent to that of BM-stem cells (3,7,10,12,13,16,24,26). However, little is known about the phenotype and biology of PB-stem cells, their origin, and the reason for their migration (10). For clinical application in peripheral blood stem cell transplantation (PBSCT), one would also like to know the frequency of malignant cells in PB and in purified PB-stem cells, the frequency of granulocyte/ macrophage colonies (CFU-GM), the timing and technique of PB-stem cell collection, and the potential for long-term reconstitution (10,24,32). The investigation of cells from blood, capable of hematopoietic reconstitution, has been hampered by their low frequency.

Powerful cell sorting methods are required to purify hematopoietic progenitor cells for cellular and molecular analysis, for autologous transplantation, and for genetic manipulation of the hematopoietic system. The isolation of rare cells, like PB-stem cells, is a challenge for any cell sorting technology. Positive selection, i.e., isolation of the rare cells according to the marker molecules they express, is superior to negative selection, i.e., depletion of all other cells according to the markers they express, since usually discriminative markers are not available for all other cells, especially

In case of malignant cells. Fluorescence-activated cell sorting (FACS), the most powerful technology for positive selection of cells, according to multiple optical and immunological markers, for isolation of high numbers of rare cells has the drawback of long processing times, because the cells are analyzed and sorted serially. To overcome this limitation, we have recently developed a fast, parallel, and efficient magnetic cell sorter (MACS) (17), which ideally complements flow cytometry and FACS by effective enrichment of rare cells according to one immunological parameter, for analysis of pheno-type and biological potential. Here we describe the purification of rare $CD34^+$ cells from PB by MACS for immunophenotyping by flow cytometry, analysis of the hematopoietic potential, and purification by FACS.

Review of literature

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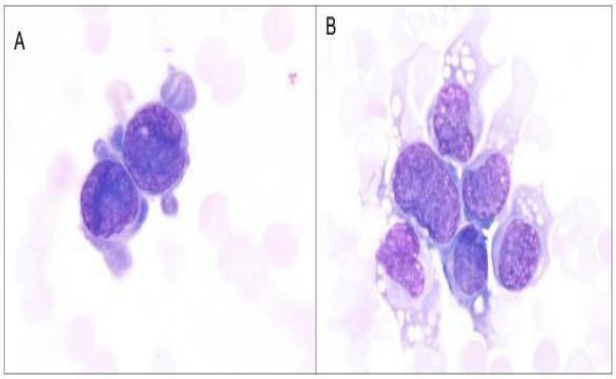
1.20 ± 0.21% ofWl $CD34^+$ cells. Of the CD34 highly positive cells, the majority were positive for CD19 and CD38 and some were positive for CD20. In the sample shown in Figure 2, 2.15% of the CD34 ' population stain brightly for CD34. Within the CD34 intermediately positive cells, 1.6% were CD20'", whereas among CD34 highly positive cells, 64.7% were $CD20^+$ (Fig. 2, Table 1). CD34 highly positive cells contained 91.9 ± 9.05 (n - 3)% of CD19 + cells, 64.8 ± 14.4% of CD20~ cells, and 84.5 ± 10.3% of $CD38^+$ cells, respectively.

The light scatter of CD34 highly positive cells, with a mean relative intensity (MC) of 502 ± 12.1 (channels) and a coefficient of variation {CV) of 13.5 ± 1.10%, was lower than that of CD34 intermediately positive cells (MC: 584 ± 11.9, CV: 12.4 ± 1.09%). The mean relative intensity of side scatter also was lower for CD34 highly positive cells (MC: 101 ± 2.10, CV: 33.8 ± 2.69%) than for CD34 intermediately positive cells (MC: 142 ± 5.23, CV: 22.8 ± 1.83%). Thus, by means of scatter, CD34 highly positive cells were smaller and contained less fractile cytoplasm than CD34 intermediately stained cells. To verify the phenotype by microscopy, we isolated CD34 highly positive cells by FACS and stained them with May-Griinwald-Giemsa. The sorted cells were viable and had small lymphoid and lymphoblas-toid morphology.

Materials and Methods

Collection and Preparation of Human PB

Human PB was obtained from the blood bank PB (500 ml) from healthy adults was drawn with 500 U heparin, and mononuclear cells were obtained by centrifugation over Ficoll-Paque (19). PB mononuclear cells (PBMC) were incubated in RPMI 1640 containing 10 mM leucin-methyl-ester (Sigma Chemical Co., St. Louis, MO) for 45 min at room emperature to deplete monocytes and NK cells (29). After washing in phospate buffered saline (PBS), 4-6 x 10^8 cells were left. Live and dead cells, stained with 0.5% Trypan Blue in saline, were counted in a Neubauer chamber.



Mononuclear cells

STAINING PROCEDURES AND MAGNETIC CELL SORTING WITH MACS

PBMC. suspended in PBS containing 1% bovine se-nim albumin (BSAj and 0.01% sodium-azide, were incubated for 10 min at 4°C with 1 mg/ml IgG3, K antibody 'HPCG, F. Kearny, Birmingham, AL), as an inhibitor of unspecific staining, and subsequently for 10 min at 4"C with biotinylated anti-CD34 antibody IOM34, and was biotinylated and titrated for flow cytometry as previously reported (17). The cells were washed once in PBS and labeled with superpara-magnetic strepta vidin-microparticles diluted 1:10, for 5 min at room before adding 3 fxg/ml phycoerythrin streptavidin mJabeted cells were separated in a high etic field, generated in a steelwool ma-nto the field *of* a permanent magnet as detail <17i. The positive ceils the column outside of the magnet. All *SK* performed in a laminar flow hood the efficiency of separa-fl#w cytMbetrv. astag a FAC- can and add-ing 10 |o.g/ml propidium iodide to the cell suspension for exclusion of dead cells

Incubator

FLOW CYTOMETRY AND FACS

Cell populations enriched for CD34^ cells by MACS were further characterized by dual-color imrnunofluo-rescence using the following fluorescein isothiocyanate (FITC)-conjugated human monoclonal antibodies: My9 (CD33) (CD14) (CD38), (CD7), and (CDt3)(CD45RO) anti- Leu- 5b (CD2), anti-Leu-4 (CDS), anti-Leu-1 (CDS), anti-CALLA (CDIO), anti-Leu-12 (CD19), anti- Leu-16 (CD20), anti-Leu-Ml (CD15), anti-Leu-HA (CD16), anti-Leu-18 (CD45RA), and anti-transfer in receptor (CD71) A FACStar+ or FACScan with 488 nm argon laser excitation was used with collection of forward and orthogonal scatter (linear amplification) and fluorescence of FITC, PE, and propidium iodide (logarithmic amplification)Data were recorded and analyzed on a Hewlett-Packard 310 using the FACScan research software Gates were set on forward (FSC) and sideward light scatter (SSC), to exclude

erythro-cytes, platelets, and non-cellular particles, and on fluorescence 2 vs. 3, to exclude propidium iodide positive, i.e., dead cells. The difference in relativefluorescence intensity in channels (difference in mean of relative fluorescence: Am) was calculated between CD34"/CDX" cells and $CD34^+$ cells in the use of each second antibody.

Flow Cytometry Equipment

In some experiments, after purification by MACS, the CD34 * cell-enriched populations were sorted in a FACStar-f either to obtain a homogeneous population of $CD34^r$ cells, as described in detail later in Figure 3, or to isolate cells expressing high or intermediate amounts of CD34. The sorted cells were cytocentri-fuged and stained with May-Grim wald-Giemsa for microscopic inspection.

For MACS and FACS experiments, the enrichment rate E and depletion rate 1/E were defined as

$$E = \frac{\%\,\text{positivecells after sort}/\%\,\text{negative cells after sort}}{\%\,\text{positivecells before sorU}\%\,\text{negative cells before sort}}$$

HEMATOPOIETIC COLONY FORMING ASSAYS

The frequency of CFU-mix, BFU-E, and CFU-GM,hematopoietic progenitor cells was evaluated by clonal assays as previously described (11). $CD34^+$ cell-enriched, depleted, and unfractionated PBMC were cultured in Iscove's Modified Dulbecco's Medium (IMDM),0.8% methylcellulose supplemented with 20% fetal calf serum (FCS)1% BSAneutralizedwith. $NaHCO_5$ m tfce preaeiwe or absence of 2 U/ml

ISOLATION OF HEMATOPOIETIC STEM CELLS

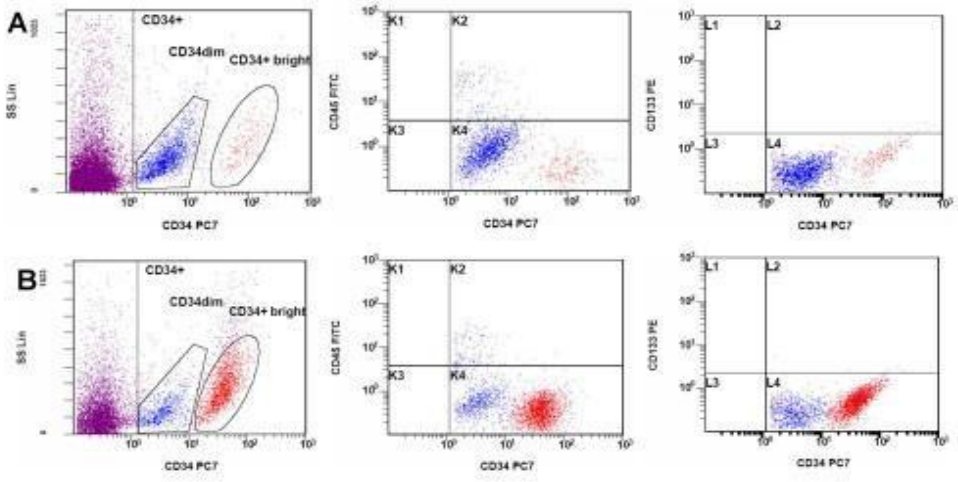
Recombinant human erythropoietin10 ng/ml recombinant human G-CSF and/or 100 U/ml re-combinant human GM-CSFCultures were incubated at 37°C with 5% CO_2. To obtain countable hematopoietic colonies, cells were cultured at very low concentrations (10^3—10" cells/ml). After 7 and 14 days of incubation, colonies consisting of more than 50 cells were examined and counted using phase contrast microscopy. To confirm typing of colonies, each colony was lifted from semisolid medium,

cytocentrifuged for 8 min at 800 rpm in PBS containing *1%* BSA and stained with May-Gninwaid-Giemsa for morphologic analysis.

Conclusion

Phenotypic Characterization of MACS-Enriched PB-CD34^{+} Cells

From leucine-methylester-treated PBMC, PB- CD34' cells, stained with the monoclonal antibody IOM34, were purified by high-gradient MACS. From an initial frequency of 0.18 ± 0.052% (n = 24), CD34" cells were purified to a frequency of 38.6-87.1% (54.5 ± 12.3%, n = 20)CD34~ cells in the CD34-enriched population contained 37.6 ± 10.3% CD2" T-cells and 35.0 ± 12.2% CD20^{+} B-cells.



The phenotype of MACS-enriched PB-CD34 $^{+}$ cells was analyzed by counterstaining the CD34-PE-stained cells with FITC-conjugated monoclonal antibodies against a variety of other lineage and differentiation markers. For analysis, gates were set on FSC and SSC, as indicated in Figure 1, to exclude debris and dead cells. For the exclusion of dead cells according to pro-pidium iodide (PI) uptake, additional gating was performed on fluorescence 2 vs. 3. Due to the different fluorescence spectra of PE and PI, the F2/F3 ratio (570 nm/630 nm) is specific for each dye, irrespective of the intensity of staining (Fig. 1), and allows clearcut distinction between PE-CD34- and Pi-stained dead cells.

The immunophenotype of PB-CD34" cells is shown in Figure 1. As is evident from the two-parameter dot plots in Figure 1, small numbers of CD34 ^{r} cells stain for CD7, CD10, CD19, CD20, CD33, and CD45RA and brightly for CD71. Very few if any CD34^{+} cells are CD2, CDS, CDS, CD14, CD15, CD16, and CD45RO positive. In the CD34/CD45RA double positive population, we could detect a CD45RA intermediately stained sub-population.

Most CD34 + cells are positive for CD38 (Am: 152) and also CD71 (110), as is indicated by the

shift in mean fluorescence. Small shifts in mean fluorescence of doubtful significance are observed for the whole population of $CD34^+$ cells with respect to CD2 (Am: 42), CDS (18), CD13 (39), and CD45RO(9).

According to expression of CD34 itself, we could detect two subpopulations, one of which had not been reported previously: CD34 "high" and "intermediate" positive cells (Figs, 1-3). In various blood samples (n = 20), the frequency of CD34 highly positive cell

PURIFICATION OF PB-CD34+ CELLS BY MACS AND FACS

By MACS, we could enrich CD34* cells from an initial frequency of 0.18 ± 0.052% among PBMC to a frequency of 38.6-87.1% (54.5 ± 12.3%) with recovery rates of 39.0-72.0%. The MACS-enriched cells, when eluted from the column, were immediately ready for further cellsorting by FACS, offering the advantage of multiparameter analysis and gating to obtain a highly purified population of viable $CD34"^1$ cells and to isolate subpopulations of $CD34^+$ cells for further microscopic and CFU analysis. Shows 532-fold enrichment of $CD34^+$ cells from PBMC by MACS and a further 54.9-fold enrichment by FACS, resulting in an enrichment rate of 29,227-fold by MACS and FACS combined, whereas enrichment rates in experiment 2 were 425- and 116,860-fold, respectively. In terms of frequency, the $CD34"^1"$ cells were enriched from 0.17 to 24.0% by MACS and from 42.0 to 99.5% by FACS. Recovery was 48.4% after MACS and 13.4% after MACS and FACS in experiment 2. In 20 experiments, the enrichment rates by MACS ranged from 319- to 3,339-fold.

Colony Formation by $CD34^+$ Cells Enriched by MACS

The proliferative and differentiative capacity of $CD34^+$ cells was evaluated in tissue culture in the presence of various cytokines. The result of the colony-forming assay from two experiments on PB-$CD34^+$ cells enriched by MACS. CFU could be readily detected in the MACS-enriched $CD34^+$ population, containing about 40% $CD34^+$ cells, and were nearly absent in the MACS-depleted population, which contained less than 0.1% CD34+ cells. On day 14 of culture in the presence of erythropoietin, G-CSF and GM-CSF in experiment 1, from 10^4 $CD34^+$-enriched cells, containing 3.98 x 10^3 $CD34^+$ cells, 264 CFU-GM, 211 reticulocyte colonies (BFU-E), and 51 colonies with cells of mixed phenotype were obtained, corresponding to a total of 526 colonies; i.e., a colony was derived from 13.2% of the $CD34^+$ cells. Besides BFU-E, CFU-GM, and CFU-mix, pure macrophage colonies and megakayocyte colonies could be observed (data not shown). In cultures of 10^3 MACS-enriched cells, containing less than 400 CD34' cells, 43 colonies were detected. In comparison, the frequency of colony formation in unfractionated and CD34-depleted populations, con-tainingO.17% and 0.07% of$CD34^+$ cells, was very low. Only in the presence of erythropoietin, G-CSF, and GM-CSF, a total of 5 colonies could be detected in CD34-depleted population seeded at 10^5 cells per plate. Essentially the same results were obtained in an independent experiment.

DISCUSSION

In previous studies, the enrichment of CD34+ cells from PB by negative or positive selection using immunoadherence (panning) or analysis by multiparameter receptor (CD71) by PB-CD34* cells, with a small sub-population of high-expressing cells, suggests heterogeneity with respect to potential of erythroid maturation or proliferation. In contrast, all PB-CD34 cells stained homogeneously for CD38, an antigen, which is expressed by a fraction of myeloblasts, promyelocytes, large granular lymphocytes, activated lymphocytes, and plasma cells (24).

Enrichment of $PB\text{-}CD34^{1}"$ cells also allows the detection of a small subpopulation, expressing high levels of CD34, which comprise 1.20 ± 0.21% (n = 20) of the PB-CD34 * cells. Most of these cells probably

belong to the B-cell lineage because they express not only CD34 and CD38, but also CD19 and CD20. It should be noted that the CD34 highly positive cells express little if any CD71, suggesting that this subpopulation is not actively proliferating. According to light scatter and microscopy of sorted cells, the cells have small lymphoid and lymphoblastoid morphology. Further cellular and molecular analysis, e.g., of their immunoglobulin genes, will reveal the differentiation stage of these cells.

Peripheral blood stem cell transplantation (PBSCT) is an alternative to autologous bone marrow transplantation (A-BMT), especially in patients with malignant cells in the BM (12,13,32). It is known that multipotent stem cells are circulating in the blood in numbers sufficient to sustain muitilineage engraftment after transplantation (3,12). CKflical trials of PBSCT have already been performed with several hundred patients with various malignancies (7,10,12,13,16,26). There are several compelling reasons to use stem cells from PB rather than from BM <2,<10,15,32). First, it has been argued that contamination with malignant cells may be less of a problem with PB stem cells (32), but still the low frequency of malignant cells in PB causes problems for conventioaal use of PB-stem cells, occurring also at low frequency. It even is a problem to detect the low frequency malignant eel Is by conventional morpho-logical or flow cytameiiric techniques.

Techniques to select the desired rare stem cells positively rather than to deplete the majority of unwanted other cells are a relatively recent 3evel0pment. Although multiparameter flow cell sorting is now well established, this superior technology has several fundamental drawbacks for the isolation of K^e cells, like PB-stem cells. The most severe of these drawbacks are the difficulty to discriminate between rare cells and cells of the major population due to the variation of immunofluorescence (% CV), leading to a population overlap, and serial analysis at relatively low flow rates, required for acceptable resolution, but leading to long sorting times. The sorting of PB-$CD34^+$ cells by MACS as described here is a rapid and efficient procedure to enrich $CD34^+$ cells and at the same time to deplete CD34~ malignant cells from PB. Because it is a parallel sorting method, it takes only 60-90 min to sort $CD34^+$ cells from 5 x 10* or more of PBMC. From 39.0 to 74.0% of the CD34 * cells are recovered, which is much more than the re- covery rate of the FACS, which was about 20% for MACS-enriched cells (approximately 50% positive}, in the experiments described here. Furthermore, the rate of depletion of CD34" cells (1/E) ranged from 2.99 x 10^{-4} to 3.13 x 10^{-3} after MACS, sterility was maintained with minimum effort, and the cells were functionally intact at least in vitro. Thus, for PBSCT, efficient enrichment of CD34"'" cells and concomitant depletion of malignant cells from PB can be achieved by a brief MACS sort.MACS-enriched PB-$CD34^+$ cells still can be induced to proliferate and differentiate in vitro.

The relationship between colony-forming efficiency and number of cells plated was almost linear in two independent experiments. Colony-forming efficiencies that we achieved in these experiments (8.82% and 13.4%) are almost comparable to those of previous reports, using almost the same culture conditions (5.27: 3.6-20.6%). In addition, we observed not only CFU-GM, BFU-E, and CPU-mix, but also pure macrophage colonies and megakaryocyte colonies, showing that a considerable variety of hematopoietic lineages could be derived in vitro from MACS-enriched $CD34^+$ cells.A single leukapheresis of a healthy individual will yield 0.3 x 10^4 CFU-GM/kg, but a dose of 30 x 10^4 CFU-GM/kg is required for successful engraftment (10). However, use of the recovery phase after moderately intensive chemotherapy and/or use of hematopoietic jfrowth factors before collection can increase the frequency of PS-stem cells to allow collection of sufficient numbers of stem cells by about four leukaphere-ses (8,10,24). Although there are variations in the CFU-GM count between laboratories (24), and although it remains to be proven whether $CD34^+$ cells are actually capable of complete permanent hematopoietic

reconstitution (9), our present study, which shows the effective enrichment of CD34 $^{+}$ cells and deletion of CD34~ cells by MACS, and the ability of sorted CD34 $^{+}$ cells to proliferate and differentiate into a variety of mature hematopoietic cells, provides important information on hematopoietic progenitors from PB already now and allows a more detailed analysis of these cells in future, for clinical use of defined cell populations in PBSCT. In addition, successful autografting by means of highly enriched CD34^{+} cells from human BM has been recently reported (2). Here again, the use of the MACS will greatly facilitate cell sorting.

It is clear from recent studies, including the present one, that CD34^{+} cells in PB have different characteristics from those in BM, and for a variety of reasons it is important to analyze CD34^{+} cells from both sources and compare their differentiation potential. One such reason is the recent development in gene therapy for the treatment of a variety of inherited diseases using hematopoietic stem cells (30). Isolation of the infrequent stem cells from blood or BM by MACS adds a powerful technology for genetic manipulation of the hematopoietic system.

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