Identification and Comparison of CD34-Positive Cells and Their Subpopulations From Normal Peripheral Blood and Bone Marrow Using Multicolor Flow Cytometry

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Four-color flow cytometry was used with a cocktail of antibodies to identify and isolate CD34+ hematopoietic progenitors from normal human peripheral blood (PB) and bone marrow (BM). Mature cells that did not contain colony forming cells were resolved from immature cells using antibodies for T lymphocytes (CD3), B lymphocytes (CD20), monocytes (CD14), and granulocytes (CD11b). Immature cells were subdivided based on the expression of antigens found on hematopoietic progenitors (CD34, HLA-DR, CD33, CD19, CD45, CD71, CD10, and CD7). CD34+ cells were present in the circulation in about one-tenth the concentration of BM (0.2% ± 1.8%) and had a different spectrum of antigen expression. A higher proportion of PB-CD34+ cells expressed the CD33 myeloid antigen (84% ± 43%) and expressed higher levels of the pan leukocyte antigen CD45 than BM-CD34+ cells. Only a small fraction of PB-CD34+ cells expressed CD71 (transferrin receptors) (17%) while 94% of BM-CD34+ expressed CD71+. The proportion of PB-CD34+ cells expressing the B-cell antigens CD19 (10%) and CD10 (3%) was not significantly different from BM-CD34+ cells (14% and 17%, respectively). Few CD34+ cells in BM (2.7%) or PB (7%) expressed the T-cell antigen CD7. CD34+ cells were found to be predominantly HLA-DR+, with a wide range of intensity. These studies show that CD34+ cells and their subsets can be identified in normal PB and that the relative frequency of these cells and their subpopulations differs in PB versus BM.

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MATERIALS AND METHODS

Preparation of leukocytes. Human PB or BM was obtained from normal adult volunteers after informed consent. Approval was obtained from the Institutional Review Board for these studies. Volunteers were informed that blood or bone marrow samples were obtained for research purposes, and that their privacy will be protected. The cell suspension was underlayed with Histopaque 1077 (Sigma, St Louis, MO) and centrifuged at 300g for 20 minutes. The interface was removed and washed with phosphate-buffered saline containing 0.1% sodium azide and 0.5% bovine serum albumin (PAB). All procedures were performed at 0°C on melting ice.

Cells (10^7:50 μL PAB) were incubated for 10 minutes with 10 μg of purified human Ig (Hyland, Duarte, CA) to block Fc receptor binding. Without washing, HPCA-1 (Becton Dickinson [BD], Mountain View, CA) or an IgG1 isotype control antibody (MOPC21; Sigma) was added followed by 15 minutes of incubation. After washing by centrifugation in PAB, the cells were
incubated for 15 minutes with allophycocyanin (APC) goat antimouse IgG (Calbiochem, San Diego, CA). Another wash was followed by addition of 10 μg mouse IgG (Sigma) and 10 μg human Ig and 10 minutes of incubation. This step is required to block any free binding sites on the goat antimouse IgG. Without washing, the following antibodies were added: biotinylated anti–HLA-DR (BD); either fluoresceinated (FITC)-anti-CD33 (MY9; Coulter, Hialeah, FL), FITC-anti-CD45 (HLe-1; BD), FITC-anti-CD19 (Leu.12; BD), FITC-anti-CD7 (Leu@ BD), FITC-anti-CD71 (anti-HTR, BD), or FITC-anti-CD10 (anti-CALL& BD); and phycoerythrin (PE)-labeled mature marker antibodies against CD3 (Leu4), CD14 (LeuM3), CD20 (Leu16), and CD11b (Leu15; BD). After 15 minutes of incubation the cells were washed and counterstained with Texas red (TR)-labeled avidin (Molecular Probes, Eugene, OR), and analyzed by flow cytometry. In some experiments, samples were stained with HPCA-1 and counterstained with PE-GAM (Fab b x 2) (Caltag, San Francisco, CA). After blocking the free combining sites with mIg, the samples were stained with FITC-labeled antibodies to CD3 (Leu4), CD14 (LeuM3), CD11b (Mo-1; Coulter), and CD20 (Leu16), and analyzed.

Flow cytometry. The flow cytometers used in these studies included a Becton Dickinson two-laser FACStar Plus and a FACScan. Fluorescence attributable to FITC- and PE-labeled antibodies was determined using excitation by an argon laser operating at 488 nm and adjusted to 0.3 W. Emission from fluorescein and PE was measured using short band pass filters of 530 ± 15 nm and 575 ± 15 nm, respectively. TR and APC were excited with a dye laser circulating rhodamine 6G and operating at 630 ± 22 nm bandpass filter for TR and 660 ± 20 nm bandpass filter for APC.

Compensation levels were set by gating on the lymphocyte population identified by forward versus side scatter and aligning mean channels of the single-stained positive populations for each color with the corresponding unstained control. This method was used because these cells stained the brightest and as such were the most sensitive to small changes in compensation levels. Compensation levels were usually set at 1% PE from FITC subtraction, 16% FITC from PE subtraction, 39% TR from APC subtraction, and 5.5% APC from TR subtraction. PMT voltages were usually set at 580 V for FITC, 610 V for PE, and 680 V for APC and TR. Samples containing only one antibody were prepared for comparison with results obtained using multiple antibodies. Nonspecific fluorescence was assessed by omitting the MoAbs and using only the second antibody. Forward and side scatter measurements were made using linear amplification and all fluorescence measurements were made with logarithmic amplification. Data was acquired in list mode using the FACStar Research software. A minimum of 30,000 events was analyzed on each sample. In samples in which the frequency of the cells of interest was low, gated files were collected of the regions of interest and compared with ungated files.

RESULTS

Presence of CD34 + cells in BM and PB in the Lin - population. A mixture of 4 PE-labeled antibodies was used to define mature cells in BM preparations. These cells included antibodies to T lymphocytes (CD3), monocytes (CD14), B lymphocytes (CD20), and the CR3 receptor (CD11b) found on mature granulocytes and monocytes. This combination of antibodies identified an average of 69% ± 11% (n = 10) cells in BM preparations and were designated Lin + cells, while cells that did not stain with these antibodies were designated Lin - cells. Phenotype analysis and sorting studies of the Lin - population in BM showed this population to be enriched for immature cells (not shown). A Lin - CD34 + population was resolved (6.25% ± 2.97% of total, mean ± 1 SD, n = 9) that, when sorted and examined morphologically, included myeloblasts, promyelocytes, and myelocytes as well as a Lin - CD71 + population (17.61% ± 6.68% of total, n = 7) that included erythroblasts and other erythroid precursors. In addition to identifying populations of immature cells in BM, the combination of PE-labeled antibodies recognizing mature cell antigens provided a unique way to resolve CD34 + cells in BM and PB. As shown in Fig 1, CD34 + cells in BM (upper left panel) were completely resolved in the Lin - region when compared with an identical staining protocol with an isotype control antibody (MOPC21) instead of the CD34 (MY10) antibody. Analysis of cell preparations stained for CD34 (MY10) and isotype control (MOPC21) stained preparations indicated that little or no apparent specific staining was observed in the Lin - CD34 + region, providing evidence that CD34 + cells in BM do not coexpress these mature cell antigens (CD3, CD11b, CD14, CD20).

Analysis of PB mononuclear cells (Fig 1, right panels) indicated this combination of PE-labeled antibodies stained 98% ± 1.2% (mean ± 1 SD, n = 9) of the cells (Lin +) present in PB. In other studies (not shown), sorts of Lin - and Lin + cells showed that all of the CFCs were present in the Lin + region. Although the Lin 'CD34 + population was discrete with a Gaussian distribution and could be resolved from the negative cells on the basis of CD34 staining, there was not always complete resolution of the cells in the axis of PE fluorescence. More nonspecific binding of the APC-

FIG 1. Identification of CD34 + cells from BM and PB in the Lin + region. Shown are bivariate dot plots of FITC-fluorescence attributed to staining of CD34 (MY10) (upper panels) or an isotype control antibody (MOPC21) (lower panels) compared with PE-fluorescence from combined staining with antibodies to CD3, CD11b, CD14, and CD20. Days are from the analysis of representative preparations of BM (left panels) or blood leukocytes (right panels) and 20,000 or 30,000 events are displayed, respectively. Dotted lines represent threshold levels delineating positive events in stained preparations when compared with unstained control samples.
CHARACTERIZATION OF CD34+ CELLS

GAM to PB cells than to BM was observed (upper right quadrants in top panels of Fig 1). The measurement of CD34+ cells in PB preparations was also validated using a two-color staining protocol in which the mature cell antigen staining was defined by FITC-labeled antibodies and CD34 antigen was measured with PE-GAM (Fab') (not shown). The CD34+ population was better resolved using the PE-GAM and the quantitation was equivalent to that using APC-GAM. Analysis of light scatter characteristics of CD34+ cells in PB (not shown) showed them to be similar to a subpopulation of BM CD34+ cells that have higher forward and side scatter characteristics. Comparison of PB and BM mononuclear cell preparations from normal adults indicated the level of CD34+ cells in the circulation (0.19% ± 0.1%, mean ± 1 SD, n = 10) is about one-tenth of that in BM (1.84% ± 0.9%, n = 16).

Antigen expression on CD34+ cells present in BM and PB. As CD34+ cells were adequately resolved using PE-labeled markers to antigens on mature cells and APC-labeled antibody to CD34, the expression of other antigens on CD34+ cells was determined by analyzing bivariate plots of TR–HLA-DR versus FITC-labeled antibodies to other antigens of interest. All the PE-labeled mature cells (Lin+) were gated out in this analysis. Only gated populations of Lin- cells with the granulocyte population removed from the analysis of BM samples by light scatter were studied and compared with staining with isotype control antibodies. Shown in Fig 2 and summarized in Fig 3 are comparisons of antigen expression on CD34+ cells from PB and BM.

There are clear differences between CD34+ cells in PB and BM. There is a higher proportion of CD33+CD34+ cells in PB (mean ± 1 SD of CD34+ cells = 82.5% ± 14.4%, n = 8) (Fig 2, upper left panel) than in BM (43.6% ± 19%, n = 16) (Fig 2, lower left panel). CD33+CD34+ cells in BM had lower forward light scatter than CD33+CD34+ cells, indicating a smaller size (not shown). CD33+CD34+ cells in PB on average expressed higher levels of CD34 than most CD33+CD34+ cells. In all of the experiments CD34+ cells identified by these methods were found to be predominantly positive for HLA-DR with variation in the level of DR expression. As shown in Fig 2, CD33+CD34+ cells in PB and BM expressed less DR than did the CD33+CD34+ cells (left panels).

CD34+ cells in BM predominantly expressed low levels of CD45 antigen (92% ± 4%, n = 4) (Fig 2, lower middle panel), while two populations of CD34+CD45+ cells were observed in PB: a larger population that expressed high levels of CD45 (69.4% ± 8%, n = 3) and a smaller population expressing CD45 equivalent to that observed in BM (30.1% ± 9%, n = 3) (Fig 2, upper middle panel). In PB, CD34+ cells expressing high levels of CD45 also expressed higher levels of DR than the CD45 dim population (Fig 2, upper middle panel).

The majority (94%) of CD34+ cells in BM expressed transferrin receptors (CD71) that were resolved into two populations almost equal in proportion (Fig 2, lower right panel). In contrast, only a small fraction of CD34+ cells in PB expressed lower levels of CD71 (16.9% ± 7%, n = 3) (Fig 2, upper right panel). This result suggests that CD34+ cells in PB are probably not proliferating. CD34+CD71+ cells in PB expressed low levels of DR while the CD34+CD71+ dim cells in BM expressed high levels of DR and the CD71+ bright cells expressed low levels of DR (Fig 2, right panel).

Although populations of CD34+ cells in PB that expressed CD10 and CD19 were observed, they were in a much lower proportion than those observed in BM (Fig 3). As in BM very few CD34+CD7+ cells were observed in PB. Interestingly, a population of Lin-CD34+CD7+ cells was consistently observed in PB and may represent CD7+CD3- cells from the circulation.

DISCUSSION

Although previous studies have described the presence of CD34+ cells in the PB of patients following induction protocols,12 we describe here the identification and quantitation of CD34+ cells from the PB of “normal adults” using a cocktail of antibodies and multiparameter flow cytometry. Furthermore, these studies demonstrate that both PB and BM CD34-positive cell populations can be subdivided based on the expression of a variety of other antigens and
that the distribution of CD34 subsets differs between normal PB and BM.

A major limitation to the detection of rare cells in PB and BM is the inability to adequately resolve small numbers of positive cells from a large population of negative cells. Others have shown that detection of rare malignant cells in PB can be significantly improved by using multiple flow cytometric parameters. One of the problems in identifying CD34+ cells in BM is the relatively dim expression of CD34. This problem is intensified by the nonspecific binding of antibodies and the inherent autofluorescence of cell populations such as monocytes and granulocytes. One solution has been to use the light scatter to define a “blast region” to remove undesired populations. This approach can detect populations as small as 0.5%. Another technique has been to perform a series of fractionations to enrich the CD34+ cells in an effort to deplete interfering cell types. However, this strategy makes quantitation of the original cell population difficult. We used multicolor analysis to exclude the predominant mature cells from our analysis and resolve and quantitate CD34+ cells and their subsets present in normal PB and BM. CD34+ cells are present in the circulation in about one-tenth the concentration of BM and have a different spectrum of antigen expression. The sensitivity of this method, estimated by its ability to resolve a positive fluorescent population of cells from the negative nonfluorescent cells, indicates that CD34+ cells present in concentrations of 0.5% can be accurately and reproducibly quantitated.

The markers used here to identify mature cells (CD3, CD11b, CD14, CD20) were initially chosen on the basis of other reports indicating these antigens were not expressed on progenitors or not coexpressed with CD34. Falkenburg et al reported that some colony-forming unit-erythroid (CFU-E) but not CFU-GM express CD11b; however, studies by Sieff et al describe opposite results. Our studies of PB indicated that all of the CFCs were found in the fraction of cells that fails to bind antibodies specific for these mature cell antigens (not shown). The phenotyping data presented here does not rule out the possibility of cells that coexpress these antigens and CD34. In a previous study, Siena et al reported CD34+ cells that express CD11b in the circulation of patients following recovery from chemotherapy. It should be noted that although the CD34 population in BM has been shown to contain progenitors for all hematopoietic cells, CD34+ progenitor cells cultured in interleukin-3 have been reported to lose the CD34 antigen. In addition, Kurtzberg et al have also reported CD34-negative myeloid progenitors in the thymus, suggesting that the CD34 antigen may be modulated on the surface of progenitor cells under certain conditions. Whether such cells exist in normal PB and BM is unknown, but if they do, they are likely in extremely low numbers given the failure of CD34-negative cells isolated from these sources to form colonies or cause engraftment.

In general, the studies presented here of BM-CD34+ cells are consistent with other studies of BM. The proportion of CD34+CD33+ cells observed in BM was similar to that observed by others. CD45 expression on BM-CD34+ cells was equivalent to that reported by Shah et al. In contrast to BM-CD34+ cells, a population of PB-CD34+ cells was also observed that expressed higher levels of CD45 and HLA-DR. The studies presented here also examined the expression of lymphoid antigens on CD34+ cells from BM and PB. We were unable to resolve a population of cells in BM or PB that express CD34 and CD7 but not CD3. This finding is consistent with other studies that reported that cells coexpressing CD7 and CD34 are not present in BM. This phenotype, which may represent pre-thymic T cells, has been reported to be present in the thymus and to contain a population that expresses CD34 and is capable of producing myeloid colonies. It is possible that this phenotype is very infrequent in PB and BM and is below the level of detection using this method, or that the gating strategy used here eliminated these cells from our analysis because they may be present among the “mature” cells. Further examination of lymphoid antigens indicated that only a small fraction of PB-CD34+ cells express the CD19 antigen found on pre-B lymphocytes or the CD7 antigen found on T lymphocytes and their precursors.

The expression of transferrin receptors (CD71) has been described during the maturation of erythroid cells and is present on proliferating cells and erythroid progenitors. The studies presented here define CD71+ cells in BM to be present in the Lin− region, indicating this antigen is only found on a very small number of Lin+ cells that may represent activated T or B cells. In addition, a large proportion of BM-CD34+ cells express high or low levels of CD71. This CD34+CD71+ population could be further characterized by the low level of DR expression on the CD71 bright population and the higher level of DR expression on the CD71 dim population. In contrast to BM, only a
CHARACTERIZATION OF CD34' CELLS

small fraction of PB-CD34' cells expressed low levels of transferrin receptors. This low level of transferrin receptor expression suggests that PB-CD34' cells are not actively proliferating, whereas BM-CD34 cells may be in a highly proliferative state.

Although very small numbers of cells were observed to be CD34'DR', no clear population of this phenotype was resolved. It is possible that the CD34'DR' cells as described by Brandt et al32 or DR' cells described by Moore et al33 that give rise to blast cell colonies or reconstituting long-term cultures are so infrequent that they are below the detection limit of this method. However, recent studies by Sutherland et al34 report that the cells reconstituting long-term cultures are CD34' and express low levels of DR, indicating the classification of DR' versus DR' cells may depend on the sensitivity of the assay system.

The demonstration of CD34' cells in PB and their similarities to subsets found in BM provides data supporting the use of PB cells as an alternative source of stem cells for autotransplantation. Satisfactory hematopoietic reconstitution has been demonstrated using PB cells collected during steady phase hematopoiesis.3 Although the use of blood progenitor cells collected during the time of hematopoietic recovery has gained some support because of the more rapid neutrophil and platelet reconstitution seen,4,11 whether the phenotypic profiles of CD34 cells isolated by these different procedures are similar remains to be seen. However, it is clear from the current studies that differences in CD34 cell subpopulations exist in normal PB and BM and that these may be important in understanding both the differentiation of CD34 cells to mature cell lineages as well as the relationship of CD34 cell transplants to full hematopoietic reconstitution.

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