Myeloperoxidase Expression in CD34+ Normal Human Hematopoietic Cells

By Herbert Strobl, Masafumi Takimoto, Otto Majdic, Gerhard Fritsch, Clemens Scheinecker, Paul Höcker, and Walter Knapp

Bone marrow (BM), adult peripheral blood (aPB), and umbilical cord blood (CB) samples contain small proportions of CD34+ cells that include virtually all hematopoietic progenitor cells. Myeloperoxidase (MPO) is considered to be selectively expressed in cells committed to granulocytic differentiation. Using flow cytometry and an antibody against MPO, we studied at which stage of normal hematopoietic differentiation CD34+ cells begin to express MPO. We consistently observed a characteristic MPO/CD34 staining pattern and found that 35% ± 9% of CD34+ BM cells express MPO. The MPO+CD34+ subset and the CD33+CD34+ subset were of similar size and overlapped considerably. MPO+CD34+ cells expressed high levels of HL-A2 molecules, whereas weakly CD71/transferrin receptor positive to negative, were CD45RA+ and lacked the CD45RO isoform of the leukocyte common antigen. Additionally, MPO+CD34+ cells were on average larger in size than MPO−CD34+ cells. Virtually identical phenotypic features have previously been described for in vitro colony-forming granulomonocytic progenitor cells. In vitro clonogenic assays performed with MPO-enriched and MPO-depleted fractions of CD34+ BM cells performed by us also suggest, but do not formally prove, that at least a portion of MPO+CD34+ cells have in vitro cluster (10 to 50 cells/colony) or colony-forming unit granulocyte-macrophage (≥50 cells/colony) forming capacity. CD34+ cells from CB and aPB resembled CD34+ BM cells in that considerable proportions of them coexpressed CD33. However, in contrast to BM, CD34+ cells from CB and aPB samples lacked significant MPO expression and, in line with this, the majority of them (CB, 59% ± 7%; aPB, 66% ± 5%) coexpressed CD45RO.

We have addressed this question using double and triple staining immunofluorescence technology and monoclonal antibodies (MoAbs) to MPO, CD34, and additional informative surface molecules. MPO expression could be detected in a substantial proportion of CD34+ cells and an attempt was made to assess the stage of differentiation at which MPO protein first becomes detectable.

MATERIALS AND METHODS

MoAbs

The characteristics and sources of the MoAbs used in this study are listed in Table 1.

Cells

Human BM was obtained with informed consent from donors undergoing allogeneic or autologous BM transplant harvests. CB was collected during normal full-term deliveries and PB was obtained from normal adult volunteers after informed consent. MNC were isolated using Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Interphase cells were removed, washed in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.1% sodium azide (PBS-BSA), and then resuspended at 10⁶ cells/mL for further staining.

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normal BMMC. Cells were fixed, permeabilized, and double-stained with anti-MPO (FITC) plus anti-LF (PE). Arrows indicate described by Schmid et al. using commercially available fixation reagents (An der Grub Fix&Perm Kit; Scandic GmbH, Vienna, Austria). Washed membrane-stained or unlyzed or submitted to intracellular staining.

Immunofluorescence Staining Procedures

Membrane staining. For membrane staining, 50 µL of isolated MNC (10^7/mL) was incubated for 20 minutes at 0°C to 4°C with 20 µL of conjugated MoAb. Afterwards, cells were washed and analyzed or submitted to intracellular staining.

Suspension stainings of intracellular antigens were performed as described by Schmid et al. using commercially available fixation and permeabilization reagents (An der Grub Fix&Perm Kit; Scandic GmbH, Vienna, Austria). Washed membrane-stained or unstained cells were fixed for 15 minutes at room temperature with formaldehyde-containing fixation medium. Afterwards, cells were washed twice and incubated with Lysis I1 (Becton Dickinson) to remove membrane-bound reagents, and then fixed with 20 µL of detergent-containing permeabilization medium. After a further washing step, cells were stained with anti-MPO (FITC) plus anti-LF (PE).

Flow Cytometry

Flow cytometric analyses were performed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with an air-cooled argon laser emitting at 488 nm. Data acquisition was performed with FACScan Research Software. Forward light scattering, orthogonal light scattering, and the two or three fluorescence signals were determined for each cell and stored in listmode data files. Analysis of the five-dimensional data was performed with Lysis II software (Becton Dickinson).

Progenitor Cell Enrichment

To facilitate further analysis of subsets of CD34+ BM cells, we enriched for immature progenitors by one to two rounds of immunomagnetic depletion of more mature (CD34-) MNC, as previously described. For this purpose, MNC (1 x 10^7/mL) were incubated for 40 minutes at 4°C with a cocktail of MoAbs containing the specificities CD3, CD11b, CD14, CD16, CD20, and anti-Glycophorin-A. Afterwards, cells were washed twice and incubated with immunomagnetic beads (2 x 10^7 per 10^7 cells; Dynal, Hamburg, Germany). Magnetic beads, together with bound cells, were then removed with a magnet and the remaining cells were washed with PBS-BSA. Two rounds of immunomagnetic depletion were usually performed. These steps led to an enrichment of CD34+ cells in BM and CB samples from 1.3% ± 0.8% to 11% ± 2% and 1.2% ± 0.6% to 16% ± 7%, respectively. The total numbers of MNC were depleted by ≥86%. Recovery of CD34+ cells ranged from 31% to 66% (mean, 53%) of CD34+ cells present in the initial MNC fractions. To exclude a possible selective bias by inadvertent cell loss, we performed double stainings of unseparated and negatively depleted MNC fractions and could show comparable proportions of CD34+ cells coexpressing MPO or the CD45RA isoform before and after depleting the more mature MNC (data not shown). In the experiments with adult PB, depletion of mature cells resulted in an enrichment of CD34+ cells from ≤0.2% in MNC to 2% to 5% in the separated fractions.

Fluorescence-Activated Cell Sorting

BMMNC enriched for immature progenitor cells as described above, but including also the CD19 antibody HD37 to remove CD19+ early B cells, were double stained with HPCA2 (phycoer-

Table 1. Antibodies Used in This Study

<table>
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<th>Antibody Name</th>
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<td>4C5</td>
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* Scandic (Vienna, Austria).

Table 2. MPO Expression in CD34+ Normal BM Progenitor Cells

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<tr>
<th>BM Donor No.</th>
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<th>Percentage of CD34+ Cells MPO</th>
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PBS plus 100 µL fixation medium). Afterwards, cells were washed again, resuspended in 50 µL PBS, and incubated for 15 minutes at room temperature with 20 µL fluorochrome-labeled antibody plus 100 µL detergent-containing permeabilization medium. After further washing with PBS, cells were analyzed as described below.

Fig 1. Two-color FACS analysis of MPO and LF expression in normal BMMC. Cells were fixed, permeabilized, and double-stained with anti-MPO (FITC) plus anti-LF (PE). Arrows indicate increasing fluorescence intensity displayed in log scale.
When evaluating MPO expression in normal Ficoll/Hy-paque isolated BMMNC by staining with the FITC-labeled

Colony Assays

Sorted cells were cultured in methylcellulose-based semisolid culture medium as described previously. One milliliter of culture medium contained 2.5 U of recombinant human erythropoietin (rhEpo; Cilag, Schaffhausen, Switzerland), 100 U of rh granulocyte-macrophage colony-stimulating factor (GM-CSF; Genzyme, Bos-
anti-MPO antibody H-43-5,24 wide ranges of levels of MPO expression were observed. This is best demonstrated in Fig 1, which shows a staining of BMMNC for MPO and lactoferrin (LF), a bacterizidal protein present only in the maturation compartment of the granulocytic lineage starting from the myelocyte stage.25 As can be seen, the combined intracellular staining for MPO and LF clearly divides the large MPO+ population of normal BMMNC into the granulocytic maturation compartment (MPO+LF+) with intermediate MPO expression, and the MPO+LF− subset that can again be divided into strongly MPO+ cells and weakly MPO+ cells. From previous experiments we know that promyelocytes reside in the strongly MPO+LF− fraction, whereas monocytes and (myelo)blasts are enriched in the weakly MPO+LF− fraction.

In the following, we combined intracellular MPO staining with the staining for surface antigens and extended our analysis to the small population of cells expressing the CD34 molecule.

Expression of MPO in a Subset of CD34+ BM Cells

Double staining experiments for MPO and CD34 were performed on Ficoll/Hypaque-separated BMMNC from eight normal BM samples. As shown in Table 2, MPO was detected in a subset of 35% ± 9% (23% to 50%) of CD34+ progenitor cells.

The relationship between MPO and CD34 expression was similar in all BM samples tested. A typical CD34/MPO double staining is shown in Fig 2. MPO antigen expression was low in cells expressing medium to high levels of CD34 antigen. With decreasing CD34 density, stronger MPO expression and larger proportions of MPO+ cells were observed.

Light Scatter Characteristics of MPO+ and MPO− BM Cells

Typical examples of light scatter profiles of MPO+CD34+ and of MPO−CD34+ cells are shown in Fig 3. Virtually identical patterns were observed in three additional Ficoll/Hypaque-separated BMMNC preparations. They all showed that, compared with MPO−CD34+ cells, MPO+CD34+ cells have on average considerably higher forward as well as slightly higher orthogonal light scattering properties.

Relationship Between MPO and CD33 Expression of CD34+ BM Cells

Using complement-dependent lysis the CD34+ BM cell population has previously been subdivided in a CD33+CD34+ subset comprising most in vitro colony-forming cells, including CFU-GM, BFU-E, BFU-Meg, and CFU-mix, as well as in a CD33−CD34+ fraction containing the more immature long-term culture-initiating cells and...
MPO EXPRESSION IN CD34+ CELLS

Fig 5. Bivariate plots of MPO versus HLA-D, CD71, CD45R0, and CD45RA expression by CD34+ BM cells. BM samples were triple-stained with anti-MPO (FITC), HLA-D, CD71, CD45R0, or CD45RA (Tricolor), and CD34 (PE) antibodies. The dot plots show gated CD34+ cells analyzed for expression of two additional molecules. Quadrants were set according to isotype-matched negative control stainings. The lower and upper right quadrants represent MPO+ cells. The upper left and upper right quadrants represent cells positive for (A) HLA-D, (B) CD45R0, (C) CD71, and (D) CD45RA, respectively. Arrows indicate increasing fluorescence intensity displayed in log scale.

In four experiments we found 26% to 48% (mean, 36%) of CD34+ cells to coexpress CD33 and 21% to 40% (mean, 33%) of CD34+ cells to coexpress MPO. Thus, the mean percentages of MPO+ or CD33+ cells are rather similar. However, as similarly observed by other investigators,3,8,11,26 we found considerable variations in the CD33 antigen expression patterns of individual BM samples. The histograms of all six experiments presented in Fig 4A illustrate these individual variations. As can be seen in most cases, no clearcut bimodal distribution can be detected. Whereas in some experiments the majority of CD34+ cells were homogeneously CD33 low to negative, in others, three populations (CD33+, CD33++, and CD33−) were distinguishable. Therefore, cells were considered as CD33+ if they showed higher CD33 fluorescence intensity than 99% of cells stained with the isotype control antibody. On the other hand, no such interindividual variations were observed for MPO expression in CD34+ cells (data not shown).

Direct Correlation of MPO Expression With the Expression of CD33

To directly correlate MPO expression with CD33 expression we performed triple stainings of BMMNC enriched for progenitor cells by immunomagnetic depletion of mature cells, gated on CD34+ cells, and analyzed MPO versus CD33 expression. As can be seen from the staining patterns shown in Fig 4B, the majority of but not all CD34+ BM cells that express MPO also coexpress CD33. In three BM samples analyzed, we found 70%, 64%, and 80% of MPO+ CD34+ BM cells to coexpress CD33. Within the CD33+CD34+ progenitor cell subset, 48%, 81%, and 49% of the cells were found to coexpress MPO. Most of the CD34+ cells expressing high levels of CD33 molecule were also MPO+. In all three experiments, MPO-coexpressing cells exhibited a significantly stronger CD33 expression than the MPO− subset.

Correlation With HLA-D and CD71 Expression

Major histocompatibility complex (MHC) class II (HLA-D) antigens and transferrin receptor molecules (CD71) are expressed on CD34+ progenitor cells in various densities and these differences in expression density have previously been shown to correlate with maturation and lineage commitment.4,5,9 Therefore, we double-stained BMMNC enriched for progenitor cells by immunomagnetic depletion of mature cells with the respective antibody conjugates and we compared MPO expression with the expression of HLA-D and CD71, respectively.

As can be seen from Fig 5, MPO+CD34+ cells clearly represent the subset of CD34+ cells with the highest density of HLA-D expression and most HLA-D+CD34+ cells coexpress MPO. Virtually the same MPO/HLA-D staining patterns were found in four additional BM samples.
In contrast to BM, only a small proportion (3% to 8%, n = 6) of CD34+ cells in CB samples expressed MPO, and only at low densities. CD34+ cells from aPB samples (n = 5) lacked (<3%) detectable MPO expression. Typical MPO staining patterns of CD34+ cells from CB and aPB samples are presented in Fig 6.

Because of the observed positive correlations between the expression of MPO and CD33 on CD34+ BM cells, we also studied CD33 expression on CD34+ cells from CB and aPB. Similar proportions of CD34+ cells from CB (19% to 41%, n = 4) and BM (23% to 50%, n = 6) expressed CD33. On average, higher percentages of CD33 coexpressing cells (52% to 65%, n = 4) were found in aPB samples. As can be seen from the representative double stainings in Fig 6, CD33 expression on CD34+ cells appeared to be more homogenous in CB and aPB than in BM samples (shown in Fig 4).

Comparison of CD45RO and CD45RA Expression on Enriched CD34+ Cells From CB, aPB, and BM

Because of the observed lack of MPO expression but not of CD33 on circulating CD34+ cells from CB and aPB, we...
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Fig 8. Sort window settings used for the fractionation of CD34+ cells. BM cells preenriched for progenitors by immunomagnetic depletion of mature cells and CD19+ early B cells were double-stained with HLA-D (FITC) and CD34 (PE) and FACS sorted into four equally sized fractions (I-IV) with increasing HLA-D expression levels (HLA-D+ to +++*). Shown as an example are the four sort windows used in experiment 2 (Table 3). Each of the windows contains 25% of the CD34+ cells, and is combined with a separate light scatter window (not shown) that excludes strongly granulated cells (high orthogonal light scatter) and debris (low forward light scatter). Arrows indicate increasing fluorescence intensity displayed in log scale.

In Vitro Growth Characteristics of CD34+ BM Cells Enriched or Depleted for MPO Expression

The restricted expression of MPO in CD34+ BM cells with the phenotypic characteristics previously described for CFU-GM suggests but does not prove that MPO+CD34+ cells have the capacity for in vitro colony and/or cluster formation. Therefore, we made an attempt to examine the in vitro colony and cluster-forming capacity of MPO+CD34+ cells. Because the detection of intracellular MPO in CD34+ cells requires fixation and precludes direct sorting, an alternative strategy had to be followed for an enrichment or depletion of viable MPO+CD34+ cells. The best way appeared to be FACS sorting of CD34+ cells into four fractions on the basis of their HLA-D expression density, because our phenotypic studies (Fig 5) had shown that MPO+CD34+ cells express high levels of HLA-D. Fig 8 shows an example of the sort window settings used in these experiments. Triple stainings for MPO, CD34, and HLA-D expression performed in parallel showed that MPO+ cells were clearly enriched in fraction IV (HLA-D+++) preparations (67%, 60%, and 73%) and depleted in all other fractions (Table 3).

The MPO+ enriched fraction IV contained in all three experiments the highest numbers of cluster-forming cells and in two of three experiments also the highest numbers of dispersed CFU-GM. CFU-mix and, in two experiments, also BFU-E were depleted in the MPO-enriched fraction IV. No positive or negative correlation with the proportion of MPO+ cells was observed for compact-type CFU-GM. Total cloning efficiencies, including colony and cluster formation, were constant in all four fractions in experiment 1 and increased with increasing HLA-D density in experiments 2 and 3.

DISCUSSION

MPO is considered to be selectively expressed in cells committed to the granulomonocytic lineage of hematopoietic cells. Antibodies against MPO have been shown to be specific and sensitive early markers for the detection of undifferentiated leukemia cells, and analysis of MPO expression by immunofluorescence has been proposed as a reliable adjunct for leukemia classification.38,39 However, so far, only very limited information is available about the earliest stages of myeloid differentiation in normal BM and blood. Using a recently developed flow cytometric method for the analysis of MPO+ cells,39 we analyzed the small CD34+ population of normal BM and PB samples obtained from newborn and healthy adults for MPO expression, and correlated this with the known phenotypic features of myeloid progenitor cells.

We found that MPO is expressed by a substantial subset of 23% to 50% (mean, 35%) of CD34+ cells from BM, whereas circulating CD34+ populations from umbilical CB and aPB lacked significant MPO expression. MPO+CD34+ BM cells clearly have features previously described as being characteristic for granulomonocyte-committed colony-forming cells.3,4,27 First, they are, on average, larger in size than MPO-CD34+ cells, as shown by light scatter characteristics. Second, they express, on average, lower levels of CD45RA than observed in BM (mean, 63%; 47% to 76%; n = 2). As can be seen from Fig 7, the patterns of CD45RA and CD45RA expression on CD34+ cells are somewhat different in BM, CB, and aPB. In aPB, similar to BM, two major populations of positive and negative cells can be resolved, whereas no such clearcut sub divisions are found in CB.

In contrast, immature blast...
granulocytes and/or monocytes. This is particularly interesting, because Lansdorp et al. observed a negative correlation between surface HLA-D expression and MPO content, and separating the MPO+CD34+ BM cells observed by us from the erythroid-committed colony-forming cells reported previously by others. Despite that, on average, similar proportions of CD34+ cells with the phenotype of granulomonocyte-committed colony-forming cells (CFU-blast) and long-term culture-initiating cells (LTC-IC) segregate within the CD33-CD34' population.

Because the demonstration of intracellular antigens such as MPO requires fixation, it was not possible to directly sort MPO+CD34+ cells for further functional analysis. Therefore, we used the observed correlation between the intensity of surface HLA-D expression and MPO content, and separated the CD34+ cell population on the basis of HLA-D expression density into four fractions (fraction I-IV corresponding to HLA-D- to HLA-D+++ see Table 3 and Fig 8). This led in all three experiments to a significant enrichment of MPO+ cells in fraction IV. Semisolid cultures of these FACS-sorted fractions showed clearcut shifts in the types and numbers of clonogenic cells in individual fractions. Clusters with 10 to 50 cells/colony and dispersed (late) CFU-GM with ≥50 cells/colony originated primarily from the MPO-enriched fraction IV, CFU-mix, and BFU-E primarily from MPO-depleted fractions I-III (Table 3). Highly committed progenitors of the granulocyte and monocyte lineage giving rise to dispersed CFU-GM and to small cell clusters with 10% to 50% cells/colony thus seem to be enriched in fraction IV.

Because it was not possible with our indirect approach to purify MPO+CD34+ cells to homogeneity and because the proportion of cells giving rise to colony or cluster formation in fraction IV and all other fractions never exceeded 37%, this finding does not directly prove that MPO+CD34+ cells can give rise to colony or cluster formation. Theoretically, all observed colony- or cluster-forming cells in the MPO-enriched fractions IV could have originated from contaminating MPO+CD34+ cells. However, in this instance, one would have to postulate a colony- or cluster-forming efficiency for MPO+CD34+ cells in fraction IV of approximately 60% in experiments 1 and 2, and virtually 100% in experiment 3 (Table 3). Such high values would be surprising and are in sharp contrast to the observed total cloning efficiencies of all other cell fractions tested, including the HLA-D++CD34+ cells in fraction III. This fraction contained 85% to 90% MPO- cells, which expressed only slightly lower numbers of HLA-D molecules than fraction IV cells. Fraction III cells still had total cloning efficiencies in our system of only 16% to 21%. It seems to us much more

### Table 3. In Vitro Growth Characteristics of MPO-Enriched and MPO-Depleted Fractions of CD34+ BM Cells

<table>
<thead>
<tr>
<th>Fraction Number*</th>
<th>HLA-D Expression Density</th>
<th>% MPO+ Cells</th>
<th>Numbers of Colonies or Clusters Formed/1,000 Cells</th>
<th>Total No. of Colonies + Clusters per 1,000 Cells</th>
<th>Total No. of Late G and M Progenitors* per 1,000 Cells</th>
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* CD34+ cells were sorted into four fractions (II-IV) based on HLA-D fluorescence intensity. For gate settings see Fig 8.
† Percentage of MPO expressing cells as determined in parallel stainings.
‡ Values obtained from duplicate dishes plated with 325 to 2,100 cells/mL.
§ Small colonies with 10 to 50 cells/colony.
‖ Total number of dispersed (late) CFU-GM (>50 cells/colony) and clusters (10 to 50 cells/colony) per 1,000 plated cells.

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likely that at least a portion of MPO+CD34+ cells has prolif-erative progenitor cell activity and can give rise to mature granulocytes or monocytes. Most likely, these MPO+CD34+ progenitor cells are highly committed and represent end-stage progenitors with maintained capacity for in vitro cluster- or dispersed-type CFU-GM colony formation.

In contrast to BM, the circulating CD34+ progenitor population from normal aPB completely lacked MPO and in umbilical CB only low proportions of CD34+ progenitor cells (3% to 8%) were MPO+. In line with this lack of MPO expression, the majority of CD34+ cells from aPB or CB expressed the leukocyte common antigen CD45RO on their surface, but failed to express CD45RA. On BM cells, CD45RO molecules have previously been shown to be selectively expressed by BFU-E-forming cells and immature LTC-IC but not by granulomyocyte-committed CFU-GM cells. Also in agreement with the absence of MPO expression by CD34+ cells from normal aPB is the observation that, in contrast to BM, CD34+ cells from blood were unable to form colonies on stimulation with the later-acting cytokines G-CSF and M-CSF, and higher proportions of PB colony-forming cells were found to grow as erythroid colonies. On the other hand, although lacking MPO, considerable proportions of circulating CD34+ cells coexpressed CD33, and thus do not seem to resemble CD33+CD34+ BM cells.

Therefore, one can assume that CD33+CD34+ cells in BM and aPB that lack MPO are either more immature than the majority of CD33+CD34+ BM cells or are not committed to granulomonocytic differentiation.

ACKNOWLEDGMENT

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