The Mpl Receptor Is Expressed in the Megakaryocytic Lineage From Late Progenitors to Platelets

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The Mpl receptor (Mpl-R) is a cytokine receptor belonging to the hematopoietin receptor superfamily for which a ligand has been recently characterized. To study the lineage distribution of Mpl-R in normal hematopoietic cells, we developed a monoclonal antibody (designated M1 MoAb) by immunizing mice with a soluble form of the human Mpl-R protein. With few exceptions, Mpl-R was detected by indirect immunofluorescence analysis on all human leukemic hematopoietic cell lines with pluripotential and megakaryocytic phenotypes, but not on other cell lines. By immunoprecipitation and immunoblotting, M1 MoAb recognized a band at 82 to 84 kDa corresponding to the expected size of the glycosylated receptor. Among normal hematopoietic cells, M1 MoAb strongly stained megakaryocytes (MK) and Mpl-R was detected on platelets by indirect immunofluorescence staining or immunoblotting. On purified CD34+ cells, less than 2% of the population was stained, but the labeling was weak and just above the threshold of detection. However, dual-labeling with the M1 and antiplatelet glycoprotein MoAbs showed that most Mpl-R/CD34+ cells coexpressed CD41a, CD61, or CD42a, suggesting that cell surface appearance of Mpl-R and platelet glycoproteins could be coordinated. M1-positive and M1-negative subsets were sorted from purified CD34+ cell populations. Colony assays showed that the absolute number of hematopoietic progenitors was extremely low and no primitive progenitors were present in the CD34+/Mpl-R+ fraction. However, this cell fraction was significantly enriched in low proliferative colony-forming units-MK. When the CD34+/Mpl-R+ fraction was grown in liquid culture containing human aplastic serum and a combination of growth factors, mature MK were seen as early as day 4, whereas the predominant cell population was erythroblasts on day 8. Similar data were also obtained with the CD34+/Mpl-R- fraction with, however, a delay in the time of appearance of both MK and erythroblasts. In conclusion, Mpl-R is a cytokine receptor restricted to the MK cell lineage. Its expression is low on CD34+ cells and these cells mainly correspond to late MK progenitors and transitional cells. These data indicate that the action of the Mpl-R ligand might predominate during the late stages of human MK differentiation.

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MATERIALS AND METHODS

Cell lines culture. UT-7, Mo-7E, and TF-1 (factor-dependent pluripotent cell lines obtained from Dr Komatsu, Tochigi-ken, Japan, nucleotides significantly decreased c-mpl transcripts in CD34+ cells and resulted in a profound reduction of in vitro colony-forming unit-MK (CFU-MK) colony formation, whereas the development of erythroid (burst-forming unit-erythroid [BFU-E]) and granulocyte-macrophage (CFU-GM) colonies was not impaired.9 Biologic and molecular characterization of the Mpl ligand (Mpl-L) have just been performed.10-14

To further clarify the role of Mpl-R in human hematopoiesis, we developed a monoclonal antibody (M1 MoAb) directed to the extracellular portion of the human receptor. Here, we provide data showing that Mpl-R is a growth factor receptor restricted to the MK lineage of differentiation from late MK progenitors to platelets.

PRODUCTION OF blood cells is regulated by specific hematopoietic growth factors acting on survival, proliferation, and differentiation. These cytokines exert their biologic effects through the binding of specific receptors on the cell surface. Most cytokine receptors are members of a large family characterized by a common structural motif within the extracellular domain, containing four conserved cysteine residues and a short tryptophan-serine-X-tryptophan-serine (WSXWS) motif located proximal to the transmembrane region. These receptors do not contain consensus sequences for catalytic activities within the cytoplasmic domain.1

The murine myeloproliferative leukemia virus (MPLV) contains a truncated form (v-mpl) of the coding region of the c-mpl gene that is a member of the hematopoietin receptor superfamily.2 This mutant virus was isolated from a mouse originally infected at birth with a Friend helper virus.3 It was shown that the oncogene v-mpl transforms, in vivo and in vitro, murine multipotential and committed progenitors leading to the generation of various immortalized hematopoietic cell lines capable of spontaneous differentiation.2,4 cDNAs encoding the human and murine c-mpl proto-oncogenes were isolated and sequence analyses showed that c-mpl had strong homology to members of the cytokine receptor superfamily.5,6 Using chimeric receptor constructs, it was shown that the cytoplasmic portion of c-mpl contained the elements necessary to transduce a proliferative signal in murine hematopoietic cells.5,8

Several lines of evidence strongly suggested that the c-mpl product was the receptor of a cytokine specifically involved in the regulation of the megakaryocytic lineage. c-mpl transcripts were detected by reverse transcription-polymerase chain reaction (RT-PCR) analyses in human leukemic cell lines with a megakaryocytic phenotype, in megakaryocytes (MK) and platelets, as well as in CD34+ cells.7 Addition of c-mpl synthetic antisense oligodeoxy-

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The recombinant Flag-Mpl protein was expressed in yeast and purified by the addition of a Flag epitope tag24 and at the C-terminus by the addition of a termination codon. The mpl domain cDNA was inserted into a yeast expression vector under the control of the ADH-2 promoter as a fusion to the recombinant c-mpl-encoded protein under the same conditions (unpublished data).

Generation of a human anti-Mpl-R polyclonal antibody. A recombinant protein was obtained by subcloning a Spl-HindIII fragment encompassing amino-acids 84-231 of the extracellular domain of the human c-mpl cDNA2 into the pGEX2T vector. The recombinant protein was expressed in bacteria. The purified fusion protein (100 µg) was injected subcutaneously into New Zealand rabbits that were boosted twice intramuscularly at 3-week intervals.

Generation of human anti-Mpl-R MoAbs. The N-terminal hematopoietin receptor domain of the human c-mpl cDNA, from amino acids 26-284, was amplified by PCR and modified at the N-terminus of human c-mpl sequence used to amplify c-mpl and P2-microglobulin transcripts and internal oligonucleotide probes were described.23 Isolation of bone marrow cell populations. Bone marrow or blood mononuclear cells (PBL) from normal informed individuals were separated through a Ficol-Hypaque metrizate gradient (1,077 g/L; Eurobio, Paris, France). CD34+ cells were recovered from mouse light-density cells (usually 2 x 10^6 cells) by either the immunomagnetic bead or immunopanning techniques. For the immunomagnetic bead technique, cells were first incubated at 4°C for 30 minutes with the QBEND 10 MoAb (CD34; Immunotech, Luminy, France) at a dilution of 1/2,500 (3 µg/mL) and then with paramagnetic beads coupled to a goat antibody to mouse IgG (Dynabeads M-450; Dynal, Oslo, Norway) at a bead to target cell ratio of 5 to 1. CD34+ cells were isolated by magnetic separation and detached from the beads by chymopapain treatment (Sigma; 130 µM for 10 minutes). The CD34 epitope recognized by QBEND 10 is cleaved by chymopapain. This procedure allows the collection of CD34+ cells free from beads and an immediate labeling of the cells with MoAbs recognizing chymopapain-resistant epitopes on the CD34 molecule. Procedures for flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated anti-Leu-7 (4G7, anti-CD19) and anti-Leu-M3 (T16, anti-CD71), IgG, and anti-CD38, Dako IIA (Y2-5, anti-CD61), and anti-CD15 were purchased from Becton Dickinson Monoclonal Center (Mountain View, CA), Immunotech (Luminy, France), and Dako (Glostrup, Denmark), respectively. Phycoerythrin (R-PE)-conjugated anti-Leu-12 (4G7, anti-CD19) and anti-Leu-M3 BSA-negative cells followed by the isolation of CD34+ cells from the SBA-negative population.

Platelets were isolated from the blood by differential centrifugation and contaminating leukocytes were eliminated by filtration. To obtain a highly enriched population of human megakaryocytes, total bone marrow cells were cultured in liquid suspension cultures containing Iscover's Modified Dulbecco's Medium (IMDM, Gibco BRL), 10% serum from human aplastic patients (AP), and 1% deionized bovine serum albumin (BSA; fraction V, Sigma), as previously described.11 Cells were washed and usually serum-depleted before being analyzed. In addition, in a patient with a M7 leukemia, blood samples containing more than 75% CD41+ blast cells were collected and deep frozen for subsequent biochemical analysis.

Antibodies and flow cytometric analysis. Several MoAbs were used for flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated HPCA 2 (SG12, anti-CD34), Be1 (anti-CD42a), IOA71 (YD11, anti-CD11b), IIB6 (T16, anti-CD38), Dako IIA (Y2-5, anti-CD61), and anti-CD15 were purchased from Becton Dickinson Monoclonal Center (Mountain View, CA), Immunotech (Luminy, France), and Dako (Glostrup, Denmark), respectively. Phycoerythrin (R-PE)-conjugated anti-Leu-12 (4G7, anti-CD19) and anti-Leu-M3
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(anti-CD14) were purchased from Becton Dickinson. Control isotype antibodies (IgG1, IgG2a, FITC-IgG1, and PE-IgG1) were obtained from Dako. FITC- or PE-labeled sheep antimouse antiserum or IgG (anti-CD14) were purchased from Becton Dickinson. Control isotype and Dako, respectively.

Briefly, cells were incubated with M1 MoAb (purified IgG1, 236 μg/mL, 1/100 dilution) in phosphate-buffered saline (PBS; pH 7.4) supplemented with 0.1% BSA for 30 minutes at 4°C. Cells were washed in PBS and then incubated with 1/100 dilution of FITC- or PE-conjugated sheep antimouse IgG F(ab)2 fragments for 30 minutes at 4°C. The negative control for M1 MoAb was an unrelated mouse IgG1. Cells were subsequently incubated for 10 minutes at 4°C with mouse serum and washed. Cells were then incubated with a directly conjugated MoAb for 30 minutes at 4°C and washed. 7 Amino-actinomycin-D (1 pg/mL; Sigma) was added just before analysis with a FACSort (Becton Dickinson) to eliminate dead cells.

Sorting. Cells were sorted on an ODAM, ATC 3000 cell sorter (ODAM/Brucker, Wissembourg, France) equipped with an INNOVA 70-4 argon ion laser (Coherent Radiation, Palo Alto, CA) tuned at 488 nm and operating at 500 mW or on a FACsort. For both cytometry, a "morphologic" gate that included 80% of the events and all the CD34+ cells was determined on two-parameter histograms by side scatter (SSC) versus either electric measurement of the cell volume or forward scatter (FSC). Compensation for two-color labeled samples was set up with singly labeled samples. Among the CD34+ population, M1 MoAb positivity or negativity were determined by using cells labeled with the R-PE HPCA 2 and an FITC-labeled irrelevant IgG1 MoAb. Because the expression of Mpl-R was quite low in comparison to the IgG1 control, the M1-positive (Mpl-R+) fraction was contaminated by about 15% of negative cells.

Colonies assays and liquid cultures. For colony assays, cells were plated in the fibrin clot culture system as previously described. Culture medium was composed of IMDM containing 10% preseparated from an aplastic patient, 1% denitized BSA, L-asparagine (20 μg/mL), CaCl2 (28 μg/mL), 10% bovine citrated plasma (BCP; GIBCO BRL), recombinant human interleukin-3 (r-hu IL-3; 100 U/mL), r-hu GM-CSF (2.5 ng/mL), r-hu G-CSF (20 ng/mL), and recombinant human erythropoietin (r-hu Epo; 1 U/mL). Colonies were counted under an inverted microscope after 12 days of incubation. 217 (data not shown). These results are in partial agreement with the threshold of detection to a clear labeling, whereas mRNA expression was at a similar level to Dami (Fig 2). The intensity of staining on Mo-7E, UT-7, AP217, and TF-1 was also variable from one experiment to another. This variability was not dependent on the culture conditions (plastic or exponentially growing cells) or on the presence of serum or growth factors (data not shown). In K 562 cells, a labeling just over the control IgG1 could be detected in some experiments, whereas no transcript could ever be detected by RT-PCR.

Immunobiochemical characterization of Mpl-R from human leukemic cell lines. Expression of Mpl-R on human leukemic cell lines was further examined by immunoprecipitation from metabolically labeled Dami cells or by immunoprecipitation followed by immunoblotting from unlabeled cells. A rabbit polyclonal antiserum (J2) raised against a portion of the Mpl-R extracellular domain was used for immunoprecipitation and M1 MoAb for immunoblotting. As shown in Fig 3, Mpl-R appeared as a protein of approximately 82 to 84 kD that was recognized by both the polyclonal and monoclonal anti-Mpl-R antibodies in lysates from metabolically labeled (lanes 1 and 2) or unlabeled cells (lanes 3 and 4). When analyzing various cell lines, we observed that Dami cells expressed high amounts of Mpl-R protein. The amount of Mpl-R protein was weak in HEL, Mo-7E, and CHRF, low in UT-7, AP217, and TF-1 (data not shown). These results are in partial agreement with the data obtained from indirect immunofluorescence as exemplified by AP217 or TF-1 cells, which showed weak surface staining (Fig 1). It is noteworthy that, from cell lines to cell lines, the size of the Mpl-R protein identified by the M1 MoAb varied from 84 kD (Mo-7E and UT-7) to 70 kD (HEL) or appeared as a doublet at 84 kD and 74 kD in the CHRF cell line. These bands could correspond to multiple forms of Mpl-R such as those detected by the cloning data.

Mpl-R expression on normal hematopoietic cells. To examine the distribution of Mpl-R among bone marrow cells, light-density mononuclear cells were labeled with M1
MoAb. In the different scatter gates for granulocytic cells, blast cells, and lymphocytes, no staining distinguishable from the background level of the nonrelevant IgG1 control was observed, with the exception of very rare cells (<0.1%). Mpl-R staining of cells with high scatter properties, which could correspond to polyploid MK, was not investigated because these cells were too rare in marrow samples. No significant labeling was detectable on mononuclear cells from peripheral blood.

Because we have previously shown that c-mpl transcripts were detected by RT-PCR in CD34+ cell populations, MK, and platelets,8 we focused our studies on these three populations. To determine whether the Mpl-R protein could be seen at the cell surface of MK, bone marrow cells were cultured in liquid in the presence of human aplastic serum to obtain a culture highly enriched in MK (up to 10%). Cells were dually labeled with a PE-conjugated anti-CD61 MoAb and M1 MoAb indirectly labeled with FITC. Staining was analyzed by flow cytometry. The majority of CD61+ cells were stained by M1 (Fig 4). However, some CD61+ cells also appeared to be weakly stained with M1. This was caused by a nonspecific reactivity of M1 MoAb on dead cells, as shown by the addition of 7-amino-actinomycin D to the medium. Among MK, those with large forward and side scatter properties (which corresponded to MK with a high ploidy level) exhibited the strongest staining. Cells from these liquid cultures were also cytospun onto slides and smears were dually labeled with M1 MoAb and a polyclonal anti-vWF antibody. All MK were strongly stained with both antibodies, whereas no significant M1 labeling was seen on cells unlabeled with the anti-vWF antibody (data not shown).

Next, Mpl-R expression was investigated on freshly isolated platelets by both indirect immunofluorescence and immunoblotting. Figure 5 shows that M1 MoAb binds to the Mpl-R expressed on the platelet surface. Fluorescence was much weaker than that observed with an anti-CD61 MoAb, but clearly higher than the background level (Fig 5A). By immunoblotting, M1 MoAb recognized a protein of expected size (82 kD) in a platelet lysate (Fig 5B). The same band but much fainter was also found in lysate from M7 leukemia cells (Fig 5B, lane 3) but not from normal PBL (Fig 5B, lane 2). In addition, in some but not all experiments, a 150-
Mpl-R expression was next analyzed on subsets of CD34' cells. To determine whether Mpl-R expression on CD34' cells stained with M1 MoAb averaged 0.5%. Mpl-R expression was next analyzed on subsets of CD34' cells purified by immunomagnetic beads, because chymopapain treatment helps to eliminate most platelets or platelet fragments attached to CD34' cells. To determine whether CD34'/Mpl-R' cells coexpressed platelet glycoproteins, two-color flow cytometry analyses were performed using CD34' cells labeled with either FITC-conjugated CD41a (anti-GpIIb), CD42a (anti-GpIX), or CD61 (anti-GpIlla) and R-PE-labeled M1. Results showed that the vast majority (>80%) of the CD34'/M1' cells coexpressed the platelet glycoproteins CD41a (Fig 6) or CD61 (not shown). Nevertheless, a significant fraction of CD41a' or CD61' cells were not labeled with M1 MoAb. In contrast, all CD42a' cells were M1'. However, because the percentage of CD42a' cells in the CD34' population was very low, a large proportion of the M1' cells were in the CD42a' fraction. The data indicate that the expression of Mpl-R on CD34' cells appears to be coordinated with the appearance of GpIIb/IIIa. No labeling with M1 was observed on the CD34'/CD19' and CD34'/CD13' cell populations (data not shown).

Cell sorting. Several cell sorting experiments (n = 14) were performed using CD34' cell populations. Clonogenic assays and liquid cultures were used to determine the number and nature of progenitors contained in the CD34'/M1' or CD34'/M1' subpopulations. In the CD34'/M1' cell fraction, the absolute number of day-12 clonogenic progenitors (BFU-E, CFU-GM, and CFU-MK) was extremely low and reproducibly less than 2% of the total number of hematopoietic progenitors contained in the initial unseparated CD34' cell populations (Fig 7A). No primitive progenitors giving rise to colonies within 20 days in culture were present in this cellular fraction. When analyzed in detail, the number of CFU-MK--derived colonies containing 3 or more MK was twofold to threefold increased in the CD34'/M1' fraction as compared with the CD34'/M1' fraction (Fig 7B). However, the majority of these MK colonies was of small size (range, 3 to 10 MK/colony). In addition, in all experiments, we observed a marked enrichment in individual MK and clusters composed of only 2 or 3 MK. Notably, the ratio between BFU-E and CFU-GM appeared to be slightly inverted in the CD34'/M1' fraction when compared with the CD34'/M1' or unfractionated CD34' cells (Fig 7B). Because the CD34'/M1' fraction could be contaminated by M1' cells, the rare cells (<0.1% of the CD34') that were stained by the control nonrelevant IgG1 were sorted and plated in similar semisolid culture conditions. No isolated MK, MK clusters, or MK colonies were observed. The only contaminant progenitors were rare CFU-GM. As controls, cell sorting was also performed in parallel using CD34' cells labeled either with anti-CD41 a or anti-CD61 MoAbs. A positive staining was clearly seen on a subset (about 3%) of the cells. These positive cells were sorted and grown in culture. Within 4 to 8 days, they gave rise to a nearly pure population of MK clusters (2 to 3 MK) and MK colonies composed of up to 8 cells.

CD34'/M1' cell fractions were also cultured for 12 days in liquid culture containing aplastic plasma and a combination of growth factors (r-hu IL-3, 100 U/mL; r-hu GM-CSF, 2.5 ng/mL; r-hu SCF, 20 ng/mL, and r-hu Epo, 1 U/mL). At various times, cells were phenotyped by flow cytometry after being labeled with anti-CD41 a, anti-glycophorin A, anti-CD14, and anti-CD15 MoAbs. Mature MK were detected as soon as day 4 in these cultures. At later times, the number of erythroblasts increased greatly and became the predominant population (>90%) on day 8. Similar data were obtained with the CD34'/M1' fraction with, however, two main differences: MK appeared later (day 8) and erythroblasts were also the main population at the end of the cultures, but glycophorin A was detectable only after day 10. These results suggest that the CD34'/M1' cell fraction may have been slightly enriched in late erythroid progenitors.

**DISCUSSION**

In this study, we have used a novel MoAb called M1 directed to the extracellular domain of the human Mpl recep-
Fig 3. Immunoprecipitation of Mpl-R from a human leukemic cell line. Dam cells (2 × 10^6) were incubated in the presence (lanes 1 and 2) or the absence (lanes 3 and 4) of ^35S-methionine/^35S-cysteine for a few hours and lysed. Extracts were immunoprecipitated with a preimmune (lanes 1 and 3) or immune (lanes 2 and 4) anti-Mpl-R rabbit polyclonal antiserum. Immunoprecipitated Mpl-R was detected by autoradiography (lanes 1 and 2) or immunoblotting using the M1 MoAb (lanes 3 and 4) and shown by chemoluminescence.

tor (Mpl-R), the c-mpl product, to define the distribution of this growth factor receptor among hematopoietic cells. M1 MoAb recognizes the Mpl-R on leukemic and normal human hematopoietic cells committed to the MK lineage.

With a few exceptions, all studied human leukemic cell lines with an erythroid/megakaryocytic phenotype display the Mpl-R at their cell surface. In contrast, cell lines with erythroid, myeloid, or lymphoid phenotypes were negative with the exception of a KG1a clone, which, in some experiments, showed a low staining with M1 MoAb. It is noteworthy that this KG1a clone expresses a low level of GpIIb/IIIa. The Mpl-R protein was also detected on three pluripotent factor-dependent cell lines (UT-7, TF-I, and Mo-7E). These cell lines might be useful models to characterize the

Fig 4. Coexpression of GpIlb (CD61) and Mpl-R on cultured human MK. Marrow cells were cultured for 12 days in liquid cultures stimulated with a preselected human aplastic serum. Nonadherent cells were labeled simultaneously with a PE-conjugated anti-GpIlb MoAb (Y2-51) and either a nonrelevant control IgG1 (left panel) or the specific anti-Mpl-R M1 MoAb (right panel), followed by sheep anti-mouse FITC-conjugated IgG (F(ab)') fragments. The upper right panel indicates cells positive for both GpIlb and Mpl-R surface expression.
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Fig 5. Immunofluorescence staining and immunoblotting of Mpl-R on peripheral blood platelets. (A) Platelets, depleted in leukocytes, were labeled with the anti-Mpl M1 MoAb and indirectly stained with FITC-conjugated sheep antimouse IgG F(ab) fragments (black profile). Labeling with the control IgG1 is shown by the thick line. The thin line shows the staining with an anti-Gpllla MoAb. (B) Immunoblotting of Mpl-R in a total platelet lysate shown with M1 MoAb and chemoluminescence. The positions of prestained molecular mass markers are shown on the right. The arrow indicates the molecular mass of the Mpl-R. NR is a nonrelevant IgG1.

effects of the Mpl ligand and to study the transduction signals generated through activation of Mpl-R. Our data are in agreement with prior evaluation using RT-PCR.4 They indicate that distribution of this growth factor receptor is restricted within the hematopoietic hierarchy. However, we did not find a clear correlation between the cell surface expression of the Mpl-R protein and the amount of mRNA by Northern analysis. For example, CHRF and Dami cells had similar levels of c-mpl transcripts by Northern blot analysis. However, in contrast to Dami cells, binding of M1 MoAb to CHRF was low. Several hypotheses could explain this observation. The human Mpl-R molecule might be heterogeneous, possibly due to different alternate splicing as already described44 and observed for other hematopoietic receptors such as the Epo-R.19 Thus, the epitope recognized by M1 MoAb may not be present on all Mpl-R molecules. Alternatively, only a fraction of the Mpl-R molecules might be transported to the cell surface, with the remainder retained in the cytoplasm. Further experiments are required to determine the exact processing of Mpl-R and its intracellular trafficking.

The data show that, in normal unseparated hematopoietic cells, Mpl-R was detected by flow cytometric analyses on a very small number of cells (<0.1% of total bone marrow cells) that nearly all belonged to the MK lineage. Noteworthy, cells showing the highest fluorescence intensity in total bone marrow cells were polyploid MK, which were excluded from the gates. The ability to detect Mpl-R on MK was further shown by analysis on marrow cultures enriched in MK where M1 MoAb binding clearly increased in parallel to MK maturation. In agreement with this observation, Mpl-R was also detected on platelets both by immunolabeling and Western blotting. These results on Mpl-R protein expression extend our previous observations obtained at the mRNA level.

To further delineate the developmental and lineage expression of Mpl-R in normal hematopoietic progenitors, subsets of CD34+ cells were analyzed because c-mpl transcripts were previously detected in this cell population. Only a small fraction of CD34+ cells (average, 0.5%) reacted positively and unambiguously with M1 MoAb and their great majority coexpressed the platelet glycoproteins GpIIb/IIIa. No labeling was seen on other CD34+ subsets. However, because both the staining intensity obtained with M1 MoAb and the number of positive cells were low, it remains difficult to exclude that a weak staining might also exist on other CD34+ subsets. The weak labeling observed with M1 MoAb is likely due to the low number of receptor molecules expressed on the surface of CD34+ cells as reported for other cytokine receptors.40,41

To sort the CD34+ subsets expressing Mpl-R, we cautiously outlined the windows that gave the clearest separation between CD34+/Mpl-R- and CD34+/Mpl-R+ cells. This did not avoid a cross-contamination of about 15%, in comparison to the control IgG1. Nevertheless, colony assays showed that the CD34+/Mpl-R- fraction contained a number of BFU-E, CFU-GM, and CFU-MK progenitors similar to that found in unseparated CD34+ populations. In contrast, the CD34+/Mpl-R+ fraction was almost totally depleted in primitive progenitors (<2%) and clearly enriched in late MK progenitors and transitional cells. These MK progenitors had limited proliferative capabilities because they gave rise to MK clusters comprising 2 to 3 MK or individual MK within 5 to 7 days.
Fig 6. FACS analysis of surface expression of Mpl-R and its coexpression with platelet glycoproteins on purified CD34+ cells. CD34+ cells were purified by the immunomagnetic beads technique using the QBEND 10 MoAb, detached from the beads by chymopapain, immediately labeled with an R-PE-conjugated anti-Mpl-R MoAb (8G12), and analyzed on a FACsort. (A) The side scatter (SSC) versus forward scatter (FSC) of the cell population. (B) shows that more than 90% of the gated cells expressed the CD34 antigen. (C) and (D) show the fraction of CD34+ cells expressing Mpl-R. Cells were labeled with either a nonrelevant IgG1 (C) or the anti-Mpl-R M1 MoAb (D) and indirectly stained with R-PE-conjugated sheep antimouse F(ab)$ fragments. Cells positively stained with M1 MoAb are shown in the upper left rectangle in (D). (E) and (F) illustrate the results of two-color analyses of CD34+ cells dually stained with R-PE-labeled M1 MoAb (y axis) and FITC-conjugated antGpIIb (CD41a) or antGpIX (CD42a) MoAbs (x axis). Cells appearing in the upper right panels are those that coexpressed Mpl-R and platelet glycoproteins.

days in culture. Some true day-12 CFU-MK were also present and about twofold to threefold enriched in some experiments. As reported previously, although controversial,42,43 CFU-MK progenitors coexpress CD34 and GpIIb/IIIa. We thus examined the CFU-MK progenitor content in the CD34+/GpIIb/IIIa+ sorted fraction in comparison to that in the CD34+/Mpl-R+ subpopulation. A much higher proportion of day-12 CFU-MK was found in the CD34+/GpIIb/IIIa+ cell fraction. This difference may only be caused by the low level of cell surface Mpl-R expression as compared with GpIIb/IIIa, which likely introduces a skew in the cell sorting. Previously, we have reported that c-mpl transcripts were present in the CD34+/CD38− fraction that has been described to be a cellular fraction highly enriched in primitive progenitors.44,45 In more recent studies, we found that this fraction is also enriched in MK progenitors, including late CFU-MK.46 Thus, the presence of c-mpl mRNA in the CD34+/CD38−/low fraction might be more the reflection of an enrichment in MK progenitors than an expression in primitive hematopoietic cells.

It is noteworthy that, in all experiments, we observed an increase in the relative ratio between mature BFU-E and CFU-GM in the CD34+/Mpl-R+ fraction. This enrichment was obvious in liquid cultures because erythroblasts were nearly the only population found after 8 days. Presently, it is difficult to ascertain whether this finding reflects a specific labeling of mature BFU-E by M1 MoAb because only 2% of the total BFU-E number was present in this cellular fraction. Still, when the rare cells (<0.1%) labeled with the nonrelevant IgG1 were sorted and grown in semisolid cultures, almost no BFU-E-derived colonies were observed. Therefore, the data cannot exclude that a subset of BFU-E does express Mpl-R.

Together, our results indicate that expression of Mpl-R seems to follow the same sequence as GpIIb/IIIa during MK differentiation. Interestingly, recent studies on the human c-mpl gene have shown that the structure of its proximal promoter resembles that of GpIIb and other MK-specific genes with both the presence of GATA and ets binding sequences.35 This finding may explain the restricted expression of the Mpl-R protein to the MK lineage and its coexpression with the platelet glycoproteins IIb/IIIa.

Finally, it is tempting to compare the expression of the Mpl-R to that of the Epo receptor (Epo-R). Both are lineage-restricted receptors, but a promiscuity between the erythroid and megakaryocytic lineages of differentiation is supported by numerous observations. First, the existence of a bipotent EMK progenitor has been reported.46 Second, the Epo-R is expressed on cells from the MK lineage46 and we cannot formally exclude the possibility that the Mpl-R is transiently expressed on erythroid progenitors (this report). Third, production of red blood cells and platelets appears to be inversely related.50,52 Expression of both the Epo-R and Mpl-R predominates during the late stages of differentiation. In particular, it has been shown using fluorescent-labeled Epo that binding was only detected on glycophorin A+ cells but not on CD34+ cells.50 Using an MoAb recognizing the Epo-R, we were able to sort immature proerythroblasts and CFU-
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From the recent experiments that have functionally and molecularly characterized Mpl-L,\textsuperscript{10-14} it is clear that Mpl-L is a humoral growth factor restricted to the MK cell lineage that has both the properties of thrombopoietin and MK-CSF. Its biologic activity on the megakaryocytic lineage mimics that of Epo in the erythroid lineage. The present study further strengthens the assumption that the Mpl-R is restricted to the MK lineage and strongly suggests that the action of its ligand will predominate during late stages of MK differentiation.

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The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets

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