Expression of CD34 and Platelet Glycoproteins During Human Megakaryocytic Differentiation

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Regulation of human megakaryocytopoiesis and platelet production is a complex phenomenon that comprises two different steps, i.e., a dividing compartment at early stages of differentiation and a compartment of nondividing cells that increases in size by polyploidization at later stages. This regulation is controlled by numerous cytokines, but it remains unknown whether these two mechanisms leading to platelet production (proliferation and endoreplication) are independently regulated or interrelated. To better understand this regulation, it is important to define the transitional stages of megakaryocyte (MK) differentiation in which MK cells switch from a mitotic to a nonmitotic phase of platelet amplification.

On one hand, several studies have shown that the primitive MK progenitor (burst-forming unit-MK (BFU-MK)) and a more differentiated MK progenitor (colony-forming unit-MK (CFU-MK)) express the CD34 antigen. On the other hand, it has been shown that the expression of platelet proteins, especially glycoprotein (GP) IIb/IIIa, occurs on an early cell in the MK lineage and precedes polyploidization. However, the presence of GPIIb/IIIa on dividing MK progenitors (CFU-MK) remains controversial.

The goals of this study were to define the cellular stages associated with the disappearance of the CD34 antigen and the acquisition of platelet GP because these steps may be associated with the transition from a proliferative to a nonproliferative phase. Because these cells are extremely rare in the normal marrow, we used a recently described liquid culture technique to selectively amplify the MK differentiation and to obtain MK precursors in much larger numbers than in the marrow.

Materials and Methods

Bone Marrow (BM) Cells

BM was obtained during hip surgery from normal adult donors after informed consent. Samples were obtained in accordance with institutional guidelines of the Committee on Human Investigation. BM low-density cells were separated by velocity centrifugation at 400g over a Ficoll-metrizoate gradient (d = 1.077 g/mL).

Antibodies

Three anti-CD34 monoclonal antibodies (MoAbs) were used: O8BEND 10 and My10 (IgG1 isotypes) were generous gifts from Drs M. Creaves (London, UK) and C. Civin (Baltimore, MD), respectively; TuK2 (IgG3 isotype) was purchased from Dako (Copenhagen, Denmark).

Three antiplatelet GP MoAbs of the IgG1 isotype, Y2-51, (anti-GPIIa, CD61), P11 64 (anti-GPIIb, CD41a), and 6D1 (anti-GP Ib, CD42a), were generous gifts from D. Mason (Oxford, UK).
Indirect Immunofluorescence Labeling

Isolation of CD34+ Cells by Immunomagnetic Beads

CD34+ cells were recovered from the marrow low-density cells or from liquid culture at day 6 by the immunomagnetic bead technique using the OBEOND 10 MoAb at a dilution of 1:2,500 followed by paramagnetic beads coupled to a goat antibody to mouse IgG (Dynabeads M-450; Dynal, Oslo, Norway) with a bead to target cell ratio of 5:1. CD34+ cells were isolated by magnetic separation and detached from the beads after chymopapain treatment (130 U/mL for 1 minute, repeated three times). The purity of this cell fraction was further assessed by relabeling them with My10. Purity was estimated to be greater than 90% (up to 97%) by immunofluorescence.

Cell Culture

Liquid culture. Low-density cells were cultured for 6 days at a concentration of 1 × 10^6 cells/mL in 75 cm^2 flasks to amplify MK precursors as described previously. Briefly, the culture medium was Iscove's modified Dulbecco's medium supplemented with 1% bovine serum albumin (Sigma Chemical, St Louis, MO) and 10% serum derived from platelet-poor plasma from patients with aplastic marrows (AS; thrombocytopenic patients after a BM transplantation [BMT]). To prepare AS, blood was collected an average of 12 days after BMT when the platelet count was less than 3 × 10^3/mm^3. AS was the only stimulant used in these cultures.

CD34+ cells purified from the marrow, “day 6 CD34+,” CD34+ platelet GP+, CD34+ platelet GP−, and CD34− platelet GP+ cells were grown at a concentration of 20,000 cells/mL in some experiments at limiting dilution. Cells were plated in 500-μL (24-well tissue plate), 100-μL (96-well tissue plate), or 25-μL (Terasaki plates; all tissue culture plates purchased from Costar, Cambridge, MA) of liquid culture containing 10% serum derived from platelet-poor plasma and 100 U/mL recombinant human interleukin-3 (rhIL-3; Immunex, Seattle, WA), 100 U/mL rhIL-6 (Genetics Institute, Cambridge, MA), and 50 ng/mL recombinant human stem cell factor (rhSCF; Amgen, Thousand Oaks, CA). In limiting dilution experiments, each well was scanned after plating and reexamined 4 and 7 days later.

Semisolid assays. Purified cells were cultured in the plasma clot technique to obtain colonies derived from CFU-MK. Stimulating factors were either a combination of 50 ng/mL rhSCF, 100 U/mL rhIL-3, and 100 U/mL rhIL-6 or AS. Cultures were incubated in a fully humidified atmosphere with 5% CO2 in air and studied 4, 7, and 12 days later. Colonies were quantified after an indirect immunofluorescence labeling by an anti-GPIIIa MoAb (Y2-51). Dishes were entirely scanned under a fluorescence microscope at a 400× magnification. Each isolated MK and clusters of two, three, 4 to 10 MK, and more than 10 MK were scored and ranged in five different classes.

Indirect Immunofluorescence Labeling

Cells were double-labeled with an anti-CD34 IgG3 MoAb (TiiK3) and an antiplatelet IgG1 MoAb (usually Y2-51) and then incubated with fluorochrome-conjugated isotype-specific antibodies (goat antimouse antibody [Southern Biotechnology, Birmingham, AL] anti-IgG3 and anti-IgG1 conjugated to R-phycoerythrin [R-PE] and FITC, respectively). In some experiments, either PH1 64 or 6D1, each used at a 1/100 dilution, were substituted for Y2-51.

Flow Cytometry

Cells were analyzed by an ATC 3000 flow cytometer (Bruker, Wissembourg, France) equipped with an argon ion laser tuned to deliver 400 mW at 488 nm and linear amplifiers. A pair of 90-μm nozzles was applied. In the two-color experiments, controls for setting the flow cytometer were labeled cells with (1) FITC or R-PE antibodies, (2) the platelet antibody (IgG1) alone followed by the two fluorochrome-conjugated isotype-specific antibodies, or (3) both an irrelevant IgG1 and TiiK3 followed by the two fluorochrome-conjugated secondary antibodies. A multiparameter gate was set using the wide-angle light scatter, the cell volume measured with an electrical impedance particle counter incorporated in the cell sorter and high green fluorescence to identify all MK. In all sorting experiments, after having determined the morphologic features of the MK population on 5,000 GPIIIa+ cells (Fig 1a), the gate was limited in volume to examine only the cells with a small and intermediate volume (lymphocyte and blast cells) (Fig 1a and b). This approach has the advantage of precluding MK with a ploidy of 8N or more.

Spill over of green fluorescence into the red fluorescence detector was electronically compensated using a single labeling with Y2-51 and the FITC-conjugated anti-IgG1 antibody. However, as a consequence of the intense FITC-labeling and limitation in the electronic components of the flow cytometer, this compensation (maximum 50%) could not reach the background level. Therefore, the FITC axis of indirect fluorescence experiments was not vertical and was determined by using cells stained in only FITC (Fig 1c).

A directly conjugated Y2-51 that gives a weaker FITC-labeling allowed a complete compensation (Fig 1e) and the use of orthogonal windows. Analytic results were essentially the same as in Fig 1d, therefore validating the indirect immunofluorescence-labeling approach.

Cells were steriley sorted at a flow rate of 1,500 cells per second into three populations (Fig 1d): CD34+/platelet GP+ (gate 1), CD34+/platelet GP− (gate 2), and CD34−/platelet GP− (gate 3). The purity of these sorted populations exceeded 90% as verified by deflecting droplets onto a slide and examination by fluorescence microscopy.

MK Ploidy

MK ploidy was measured after paraformaldehyde fixation and DNA staining by Hoechst 33342 (Sigma) (10 μmol/L; Hoechst 3342 for 2 hours at 37°C).

MK ploidy measurements were performed in one-, two-, or three-color experiments. Single staining was performed on aliquots of sorted cells that were subsequently reanalyzed for their ploidy. Dual-color staining (Y2-51 and Hoechst) was used after culture of purified MK precursors and was performed on 5,000 to 10,000 cells. Three-color staining was performed after having double-labeled cultured cells by the anti-CD34 (TiiK3) and an antiplatelet (Y2-51 or 6D1) MoAbs as described above. Cells were fixed and incubated with the Hoechst dye. Analysis was performed on the morphologic gate that includes all MK (Fig 1b). Cells were analyzed by the ATC 3000 equipped with two argon ion lasers tuned at 488 and 360 nm, respectively, and logarithmic amplifiers for the UV light.

Ultrastructural Studies

The different purified cells obtained either by cell sorting or by the immunomagnetic bead technique were examined by electron microscopy.
Fig 1. Correlation between the expression of CD34 and platelet GP\textsubscript{II}a in liquid marrow culture at day 6. (a) Dot plot analysis (volume versus wide-angle light scatter) of the GP\textsubscript{II}a\textsuperscript{+} cells. A gate was set on the volume to only select small MK (2N and 4N). (b) This gate is reported on the entire cell population. (c) Determination of the FITC axis in double-staining labeling. In indirect labeling the electronic compensation of the green fluorescence in the red fluorescence was not complete because staining by Y2-51 was extremely intense and did not allow an analysis according to the orthogonal axis. The FITC axis was precisely determined after cell labeling by Y2-51 (lgG1) followed by the two fluorochrome-conjugated isotype-specific antibodies. The R-PE anti-lgG3 antibody was only used to exclude some cross-reactivity between isotypes. This axis was determined on 5,000 FITC-labeled cells. (d) Correlation between the expression of GP\textsubscript{II}a and CD34 on cells gated as in (a) after indirect fluorescent labeling with the Y2-51 (IgG1) and TiiK3 (lgG3). Cells were sorted in three nonorthogonal electronic gates 1, 2, and 3 that correspond to CD34\textsuperscript{-} GP\textsubscript{II}a\textsuperscript{+} (1), CD34\textsuperscript{+} GP\textsubscript{II}a\textsuperscript{+} (2), and CD34\textsuperscript{+} GP\textsubscript{II}a\textsuperscript{-} (3) cells. (e) Same correlation using a directly FITC-conjugated anti-GP\textsubscript{II}a (Y2-51). A total electronic compensation was obtained and a classical orthogonal analysis can be performed that does not differ from the previous one.

microscopy (EM). They were washed twice in Hanks’ medium at 4°C, fixed by 1.25% glutaraldehyde in Gey’s buffer\textsuperscript{14} for 10 minutes, washed, and incubated in diaminobenzidine medium.\textsuperscript{19} Cells were then postfixed with osmium tetroxide, dehydrated, and embedded in epon. Thin sections were examined with a Philips CM 10 (Eindhoven, The Netherlands) electron microscope after lead citrate staining.

RESULTS

Culture of Purified CD34\textsuperscript{+} Cells in Liquid Culture

Marrow CD34\textsuperscript{+} cells were purified by the immunomagnetic bead technique with a purity exceeding 90% and cultured in liquid medium either in the presence of AS or in a combination of rh growth factors (SCF plus IL-3 plus IL-6). After 3 days in culture, mature MK were detected (Fig 2a), with their frequency varying from 0.5% to 3% (average, 1.2%; n = 5). Lysis of these mature MK occurred at day 7 to 8 of culture.

Mature MK obtained between 3 and 7 days in culture from CD34\textsuperscript{+} marrow cells did not arise from the survival of MK present in the initial purification because no morphologically identifiable MK were seen in this cell fraction. Neither did they arise from the differentiation of usual CFU-MK progenitors, which take 11 to 13 days.\textsuperscript{4,7} Therefore, this observation suggested that a MK precursor, intermediate between true MK progenitors and maturing MK, was present in the CD34\textsuperscript{+} cell fraction.

To characterize both the phenotype and the properties of these cells, which might be scarce among the marrow CD34\textsuperscript{+} cell fraction, we studied whether we could amplify this cell population in liquid culture. Low-density marrow cells were thus cultured in liquid medium in the presence of
Fig 2. Characteristics of MK precursors in culture. (a) Liquid culture of CD34+ marrow cells. Some large MK can be observed after 4 days of culture. (b) Liquid culture of "day 6 CD34+ cultured cells." A high number of MK are obtained after the purification of "day 6 CD34+ cultured cells." After 7 days of culture, a significant fraction of MK has begun to lyse. (c) Limiting dilution experiments with CD34+ GPIIIa+ cells. After 7 days of culture, aggregates of more than four cells can be observed, whereas only one cell was present at day 0 in this well. (d) A presumptive platelet shedding MK from a cultured CD34+ GPIIa+ cell.

As to stimulate MK production. After 5 to 7 days in culture, the CD34+ cells were purified with a cell recovery ranging from 0.2% to 2%. These “day 6 CD34+ cells” were subsequently grown in liquid culture and after 3 to 7 days a large number of MK (up to 50% of the total cell number) (Fig 2b) was obtained, showing that “day 6 CD34+ cells” were greatly enriched in MK precursors.

Phenotypic Analysis of MK Precursors

As shown in Fig 3a, b, and c, the morphology of the marrow and “day 6 CD34+ cells” greatly differ. On an average, “day 6 CD34+ cells” were larger and had a more basophil and often vacuolated cytoplasm. Rare cells (1% to 2%) were clearly immature MK with a large size and a polychromatophilic nucleus (Fig 3c). At the ultrastructural level, the majority of marrow CD34+ cells were a subtype of monocytic precursors identifiable by their indented nucleus and the presence of few small peroxidase-positive granules without peroxidase reaction in the endoplasmic reticulum (ER) (Fig 4). These cells are equivalent to the blasts of M0 leukemia (blasts with small peroxidase-positive granules identifiable by EM). No promegakaryoblasts were identified in this cell population. In contrast, large peroxidase-negative blasts were also present (Fig 5) and rare elongated cells could be detected that may represent stromal cells. In contrast, “day 6 CD34+ cells” were a totally different cell population (Fig 6). Promegakaryoblasts identifiable by the platelet peroxidase (PPO) were present in a significant proportion (10% to 30%, n = 4), although PPO was not always detected in these cells as it was previously observed.
Fig 4. A marrow CD34- sorted cell. Cell was reacted for peroxidase. The nucleus contains two large nucleolus (Nu). In the cytoplasm, the cisternae of endoplasmic reticulum are devoid of peroxidase (white arrows), while small vesicles and granules are peroxidase positive (arrows) (original magnification, x 14.040).

Most promegakaryoblasts had large multivesicular bodies (MVB), but no α-granules and demarcation membranes (DM).

CD34+ cells were subsequently analyzed by flow cytometry. A very low percentage of double-positive cells for CD34 and platelet GPIIIa was found in fresh marrow; less than 2% of the CD34+ cells expressed GPIIIa at a detectable level. Because the CD34+ cells expressing platelet GP were extremely rare in fresh marrows, we focused our study on day 6 cultured cells. In these cultures, the MK population was heterogeneous and contained a significant fraction of large mature MK (0.5% to 1.2%; average, 0.8%). Analysis of cultured cells in electronic gates for wide-angle light scatter and volume showed that all small MK (presumably immature), as well as CD34+ cells, exhibit low wide-angle light scatter properties (Fig 1a). In five repeated experiments, the frequency of GPIIIa+ small cells at day 6 ranged from 0.5% to 2.5%, 40% to 75% being in the gate of the small cells (Fig 1a). A small percentage of cells was CD34+ (1% to 2%), and approximately 80% of them were included in the gate. As shown in Fig 1d, three populations of cells could be determined: CD34- GPIIIa+ cells (gate 1), CD34+ GPIIIa+ cells (gate 2), and CD34+ cells with no detectable GPIIIa (gate 3). However, there was a clear continuum between these cells because an inverse relationship between GPIIIa and CD34 expression was found. In average, 40% of the “day 6 CD34+ cells” expressed GPIIIa, whereas 35% to 85% of the GPIIIa+ cells expressed CD34. No differences were observed in the volume of these two GPIIIa+ cell populations, whereas the CD34+ GPIIIa- cell population was slightly smaller.

When the antibody against GPIIIa was replaced by an antibody against GPIb, a slight difference in the percentage of labeled cells in the gate was observed (0.8% vs. 1.1% for GPIIIa). In contrast, marked differences were observed when the expression of GPIIIa and GPIb were compared. The expression of GPIb was present on fewer cells (2 to 6 times less) than GPIIIa and only 10% to 30% of them fall into the gate of small MK. Therefore, the total number of small MK labeled by the anti-GPIb MoAb represented 0.07% of the day 6 cultured cells. Among the population of small MK labeled by the anti-GPIb or GPIIIa MoAb, the frequency of CD34+ cells was in the same order of magnitude (63% and 85.3%, respectively). Thus, the percentage of CD34+ cells expressing GPIb was only 2% and 2.63% in the two experiments performed.

The CD34+ GPIIIa+ and the CD34- GPIIIa+ cells were sorted. They were blast cells with usually a high nucleo/cytoplasmic ratio (Fig 3d and e). Rare contaminant cells (approximately 5%) were present in both fractions. A large number of cells, particularly in the CD34+ GPIIIa+ cell fraction, exhibited numerous blebs (Fig 3d). In the double-positive population, about 5% of the cells were in mitosis (Fig 3e). More than 90% of the cells in each fraction were stained by an anti-vWF antibody, and most CD34+ GPIIIa+ cells had a labeling localized in the golgi area (Fig 3f).
Fig 5. Marrow CD34\(^*\)-sorted cells. Cells were reacted for peroxidase. (A) Two peroxidase-negative blasts of different size possess a majority of euchromatin; a large central nucleolus (Nu) is seen in the smaller blast. Note the numerous ribosomes and the rare endoplasmic reticulum cisternae. (Original magnification, \( \times 7,375 \).) (B) Another peroxidase-negative blast from the same fraction exhibits two nucleoli (Nu) that are not surrounded by heterochromatin, as in the cell illustrated in Fig 4. The nucleus is highly indented and the mitochondria are clustered; the short cisternae of endoplasmic reticulum (arrows) are more numerous that in the blasts shown in (A). (Original magnification, \( \times 12,600 \).)
These two cell populations were studied by ultrastructural techniques and corresponded in their majority to promegakaryoblasts at different stages of maturation. The CD34+ GPIIIa+ cells (Fig 7) differed from the marrow CD34+ blasts by the presence of several blebs and multivesicular bodies. In addition, some cells presented several vesicles with a dense core resembling immature α-granules in the golgi area. Rare larger cells had an indented nucleus, but no DM could be identified. The CD34+ GPIIIa+ cells had fewer ribosomes, but all cells exhibited numerous α-granules and clusters of DM (Fig 8).

These results indicate that the CD34+ GPIIIa+ cells are more immature than the CD34- GPIIIa+. We subsequently studied whether they differed in their proliferative status.

Proliferative Capacities of MK Precursors (CD34+ Platelet GP-, CD34+ Platelet GP+, and CD34- Platelet GP+)

In two experiments, CD34+ GPIIIa+ and CD34- GPIIIa+ were sorted and studied by limiting dilutions. Cells were grown up to 7 days in the presence of a combination of SCF, IL-3, and IL-6. Each well was controlled for the presence of cells after the plating and reexamined after different times in culture. Of about 200 wells (n = 2) that initially only contained one cell, 46% and 35% of the CD34+ GPIIIa+ and CD34- GPIIIa+ cells, respectively, were able to give at least one mitosis 4 days later. Most cells began to lyse later on in culture. However, about 5% of the CD34+ GPIIIa+ cells formed aggregates of up to 8 cells at day 7 (Fig 2c); no such cluster was observed with the CD34- GPIIIa+ cells.

Similar results were obtained when cells were plated in plasma clot in the presence of AS or in the association of IL-3 plus IL-6 plus SCF. In addition, this technique associated to a fluorescent labeling enabled us to show that all aggregates were composed of MK, whereas no other type of colony could be seen. More than 15% of the plated cells gave rise to clusters of two or more MK after 3 days of culture (Fig 9). Some clusters of up to 16 MK were seen in the CD34+ GPIIIa+ cell population. They were never
Fig 7. CD34+ GPIIa+ blasts sorted at day 6 of culture. (A) A general view of a blast that exhibits a nucleolus (Nu) in the irregular nucleus. The characteristic of such blast is the high frequency of cytoplasmic blebs (arrows). (B) Enlargement of the golgi zone from another blast. A cytoplasmic bleb located on the left is devoid of organelles. Numerous vesicles and small granules (arrows) at the trans-face of the golgi apparatus represent the immature α-granules. Numerous MVB are present, as usually seen in the cultured MK. (Original magnification, ×24,030.)
observed when the CD34<sup>-</sup> GPIIIa<sup>+</sup> cell population was plated (maximum size, 6 cells).

We next compared the properties of the CD34<sup>-</sup> GPIIIa<sup>+</sup> and CD34<sup>+</sup> GPIIIa<sup>-</sup> cells for their abilities to give rise to MK colonies. As shown in Fig 10, CD34<sup>-</sup> GPIIIa<sup>+</sup> gave clusters of MK with a maximum at day 4, but some true MK colonies composed of 3 to 16 cells were still present at days 7 and 11. The CD34<sup>+</sup> GPIIIa<sup>-</sup> cells gave larger MK colonies than the previous cell fraction, with a maximum between days 7 and 11 of culture.

We subsequently studied the endomitotic properties of these cells by measuring the ploidy of their progeny in liquid culture. The three cell populations were sorted. In the two GPIIIa<sup>+</sup> populations, the cell ploidy was essentially 2N and 4N because less than 0.1% of the sorted cells were 8N (data not shown). A significant fraction (32%) of CD34<sup>-</sup> GPIIIa<sup>+</sup> cells were in S and M phases (13% for the CD34<sup>-</sup> GPIIIa<sup>+</sup> cells) after flow cytometric analysis of the distribution of DNA content. Cells (10,000) of each fraction were cultured and 6 days later MK ploidy was measured by a
Fig 9. Comparison of the size of MK clusters grown from CD34+ GPIIia- and CD34+ GPIIia+ as a function of time. Data represent the average of two dishes in which 1,000 cells were plated from the cell-sorting experiment in plasma clot and grown in the presence of AS.

Fig 10. Comparison of the size of MK clusters grown from CD34+ GPIIia- and CD34+ GPIIia+ as a function of time. Data represent the average of two dishes in which 1,000 cells were plated from the cell-sorting experiment in plasma clot and grown in the presence of SCF plus IL-3 plus IL-6.

DISCUSSION

Several techniques have allowed for the description of different classes of MK progenitors and precursors. First, two types of progenitors were defined by semisolid culture assays, i.e., the BFU-MK and the CFU-MK.\(^1,3,21\) Second, ultrastructural techniques using PPO as a differentation marker have defined the promegakaryoblast as a small cell not recognizable morphologically that precedes terminal MK differentiation.\(^22\) Third, immunologic markers (platelet antigens and different CD) have shown that platelet proteins are already expressed on small MK precursors.\(^5,9\) By combining these immunologic markers with the two other techniques, it was shown that, on one hand, both BFU-MK and CFU-MK express the CD34 antigen,\(^3\) and, on the other hand, that the majority of promegakaryoblasts express the GPIIb/IIIa complex.\(^7\) These two cell types are 2N and 4N,\(^1\) but their precise relationships are still unclear because the presence of GPIIb/IIIa on CFU-MK remains controversial.\(^10,12,23\) It is generally considered that these GP are absent from the MK progenitor.\(^1,22\) In favor of this hypothesis, no GPIIb/IIIa was detected on marrow CD34+ cells.\(^24\) Therefore, it could be suggested that the CD34 antigen is expressed only on the stages of MK differentiation associated with cell division (progenitors), whereas the GPIIb/IIIa complex appears on transitional cells that correspond to immature MK having lost their proliferative capacities and entering the endomitotic process. However, some mitotic figures of GPIIb/IIIa+ cells have been observed in culture\(^7\) and, in the murine system, acetylcholinesterase (ACHE), a cytochemical marker showing a pattern of expression similar to the human GPIIb/IIIa, is expressed on small MK cells capable of limited cell division.\(^25,26\)

We have combined a liquid culture technique that
but this population was greatly enriched by our culture conditions in MK progenitors that gave rise to intermediate or large size MK colonies 11 days later (overall, 17 days of culture). The CD34+ GPIIIa+ and the CD34- GPIIIa+ cell fractions were nearly pure MK precursors. They contained less than 5% contaminant cells, which were randomly distributed, except for the presence of some nonhematopoietic cells, possibly stromal cell precursors.

Ultrastructural studies clearly showed that the CD34+ GPIIIa+ cells were the immediate precursor of the CD34- GPIIIa+ cells. Indeed, the former cells had no DM and expressed α-granule precursors in the golgi zone, whereas the latter exhibited these two MK-specific organelles that were well developed in the cytoplasm. In conclusion, the first part of this study shows that GPIIIa increases during differentiation in parallel with a loss of CD34.

We subsequently tested the proliferative capacities of the two GPIIIa+ cell populations. One-third to one-half of these two cell types were capable of at least one cell division in limiting dilution experiments. However, the double-stained population differed from the other by (1) higher proliferative capacities (giving rise to MK colonies of up to 16 cells), (2) a higher percentage of cells in DNA cycle (32% in S and M phases), and (3) a capacity for producing a progeny of higher ploidy. This last difference may be explained by two mechanisms. First, some micromegakaryocytes, 2N mature cells that have no DNA synthesis capacity, were detected in the CD34+ GPIIIa+ cell population. Micromegakaryocytes have been previously detected in fetal liver, cord blood, and human malignancies; they are also frequently observed in adult marrow cell cultures. Second, the ploidy of a mature MK may depend on the stage of proliferation and differentiation of the MK progenitor from which it derives. These data suggest that, during MK differentiation, CD34 is lost on a 2N MK precursor with a limited division capacity and is turned off just before polyploidization. However, among “day 6 CD34+ cells,” we have observed some large immature cells having the appearance of megakaryoblasts that, at EM level, contained α-granule precursors. By three-color staining, we analyzed the ploidy of all (large and small) platelet GP+ cells present in the culture. We observed that some CD34+ GPIIIa+ (22%) were polyploid (>4N). These results were even more striking with GPlb, a later marker of differentiation than GPIIIa, because the modal ploidy of the CD34+ GPlb+ cells was 8N, with 55% polyploid cells.

Our study is consistent with the following views of MK differentiation. GPIIIa appears on a MK progenitor with low proliferative capacities as previously suggested by cytotoxicity. The in vitro characteristics of the GPIIIa+ MK progenitor are very close to the light-density megakaryocyte progenitor cell of the mouse and also to those reported for human MK progenitors cloned in agar. It is noteworthy that experiments showing that GPIIb/IIIa was expressed on CFU-MK were performed in this semisolid medium. CD34 is present on immature MK progenitors, but is definitively lost on a polyploid immature MK. GPlb is expressed later during differentiation and its appearance seems to correlate with the beginning of polyploidization. Expression of GPIIb seems to mimic that of GPIIIa;

permitted an amplification of the MK differentiation and multiparameter flow cytometry to analyze the properties of small human MK precursors and the early steps of MK differentiation. By appropriately gating the cell culture population for volume and wide-angle light scatter, we were able to restrict our study to a 2N and 4N cell population and to phenotypically identify three different MK precursor cell types whose flow cytometric characteristics were very similar to those reported for murine CFU-MK. The CD34+ GPIIIa- phenotype was not restricted to the MK lineage,
CD34 AND MEGAKARYOCYTIC PROGENITORS

Fig 12. Ploidy distribution of CD34+/platelet GP⁺ and CD34⁻/platelet GP⁺ MK at day 6 of culture. (a, b, c, and d) Three-color staining using Tük3 (anti-CD34), Y2-51 (anti-GPⅢa MoAb), and the Hoechst dye. (e, f, g, and h) Three-color staining using Tük3, 6D1 (anti-GPⅠbα MoAb), and the Hoechst dye. (a and e) Correlation between the expression of CD34 and GPⅢa (a) or GPⅠb (e) on the entire MK population (small and large cell) (linear amplification for FITC or RPE staining). CD34⁺/platelet GP⁻, CD34⁺/platelet GP⁺, and CD34⁻/platelet GP⁻ were included in gates 1, 2, and 3, respectively, and analyzed in UV light. Auto-fluorescent cells are present in area 4. (b and f) Ploidy distribution of the CD34⁻/platelet GP⁺ cells (b, GPⅢa; f, GPⅠb) (logarithmic amplification for the UV). (c and g) Ploidy distribution of the CD34⁺/platelet GP⁺ cells (c, GPⅢa; g, GPⅠb) (logarithmic amplification). (d and h) Ploidy distribution of the CD34⁻/platelet GP⁻ cells (d, GPⅢa; h, GPⅠb) (logarithmic amplification). A second repeated experiment gave exactly the same results.

however, slight differences were found among the immature MK that will require further investigations. Synthesis of vWF begins quite early because it is detected in CD34⁺ GPⅢa⁺ cells, as previously suggested by the presence of α-granules or transforming growth factor-β in MK cells synthesizing DNA.³⁴,³⁵

Further studies are needed to understand the precise relationships between the CD34⁺ HLA-DR⁺ MK progeni-
tors and those expressing GPIIIa during MK development. However, two points suggest that these two populations overlap: (1) HLA-DR is detected on GPIIb/IIIa+ MK cells, and (2) colonies derived from CD34+ HLA-DR+ CFU-MK are composed in average of 5 to 12 cells, depending on the culture conditions. All these observations indicate that MK progenitor cells are a continuum of cells from high to low proliferative capacities associated with changes in their surface phenotype.

The characterization of this transition (from a dividing to a nondividing compartment) should help in understanding this key step in megakaryopoiesis and the stages at which the different MK regulators act.

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Expression of CD34 and platelet glycoproteins during human megakaryocytic differentiation

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