Development of Human Megakaryocytes: I. Hematopoietic Progenitors (CD34+ Bone Marrow Cells) Are Enriched With Megakaryocytes Expressing CD4

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CD34 is expressed by essentially all human hematopoietic progenitors including cells of the megakaryocyte (MK) lineage. We have previously reported CD4 expression by some human MK (Blood 81:2.664, 1993). To study the role of maturation on CD4 expression by MK, we examined CD34+ bone marrow cells for their expression of CD41 (GPIIb-GPIIIa) and CD4 with specific monoclonal antibody (MoAb)-fluorochrome conjugates and for DNA polyploidization with propidium iodide or 7-aminoactinomycin D (7-AAD). Surprisingly, MK were at least 20-fold more common in the CD34+ progenitor pool (≈10%) than in the more mature CD34- population (≈0.5%) of low-density bone marrow cells. CD4 expression correlated with markers of immaturity in that CD4 was enriched among CD34+ cells, and the proportion of CD4+ MK declined with increasing ploidy. Almost all CD34+ polyploid (≧8N) cells were CD4-. Despite these correlations with immaturity, CD34+CD4+ MK precursors were unable to produce MK colony-forming units (CFU-MK) when cultured under conditions that supported the growth of CFU-MK from CD34+CD4+ MK lineage cells. MK became polyploid before the loss of either CD34 or CD4 expression. The presence of CD4 on these cells correlates with the onset of endomitotic reduplication and is associated with the loss of the ability of these cells to undergo normal mitotic division. The role of CD4 on immature MK as a differentiation antigen and/or receptor for the human immunodeficiency virus (HIV)-1 virus remains to be determined.

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MATERIALS AND METHODS

Bone Marrow

Bone marrow specimens were obtained from discarded orthopedic surgical specimens. A total of 11 samples were examined, eight men and three women. The patients ranged in age from 52 to 76 years. Samples were resuspended in cold Ca++ and Mg++ free phosphate buffered saline (PBS), containing 13.6 mmol/L sodium citrate, 2.2 mmol/L prostaglandin E1 (PGE1), 1 mmol/L theophylline, 3% bovine serum albumin (BSA), 11 mmol/L glucose, adjusted to pH 7.3 (MK medium). The tissue was gently pipetted with a 12-mL plastic syringe through an 18-gauge needle until a single cell suspension was obtained. MK and their precursors were enriched by centrifugation over Ficoll-Hypaque (specific gravity, 1.077 g/mL) at 400g for 20 minutes at 20°C. The MK-rich fraction at the interface was isolated, washed, and resuspended in MK medium.

Antibodies

MK were identified with fluorescein-conjugated monoclonal antibodies (MoAb) with specificity for the GPIIb-GPIIIa complex (CD41) fluorescein isothiocyanate (FITC), clone P2, mouse IgG1, purchased from AMAC (Westbrook, ME). Other antibodies used included anti-CD34 coupled to phycoerythrin (PE) [clone QBEND 10, mouse IgG1] and anti-CD4 FITC [clone J3B8.2, mouse IgG1] purchased from AMAC as well as anti-CD4 [clone 5 3.5, IgG2a], conjugated with PE, TriColor (TC) or biotin, and anti-CD14 TC [clone MEM18,
from Gaithersburg, MD). Isotype controls employed were visualized with streptavidin coupled to Red613, purchased IgG1, (CALTAG; South San Francisco, CA). Biotinylated MoAbs of mouse nonimmune IgG1 and IgG2a labeled with the appropriate fluorochromes.

Bone marrow cells (10^7/mL in MK medium) were incubated with saturating concentrations of antibody for 30 minutes on ice with gentle mixing. In three-color experiments, we stained the cells with anti-CD41 FITC, anti-CD34 PE, and anti-CD4 TC MoAbs. TC is a tandem conjugate of PE and cyanine-5 (CY5). It can be excited at 488 nm and emits at 670 nm. After incubation, MK medium supplemented with 6% BSA was gently layered under the cell suspension and the cells centrifuged at 350g for 7 minutes. The pellet of stained cells was resuspended in 2% paraformaldehyde in MK medium and stored at 4°C until analyzed. Analysis of these samples showed a spuriously high proportion of CD41 positive cells caused by the binding of CD41 platelets to monocytes (Fig 1). The finding was also confirmed visually by sorting the double positive cells directly onto slides. To confirm that the CD14^+CD41^+ cells were not MK, the cells were stained first with anti-CD41 FITC and anti-CD14-biotin and Streptavidin Red613 (a tandem conjugate of PE and Texas Red; E[max] 613 ± 20 nm) and then with 7-aminoactinomycin D (7-AAD) to quantify their DNA content. All of the CD14^+ cells had a normal diploid DNA content. No polyploid (>4N) MK were detected among the CD14^+CD41^+ cells. To analyze developing MK and exclude monocytes, the cells were stained with saturating amounts of CD41 FITC (E[max] 525 ± 20 nm), CD34 PE (E[max] 575 ± 20 nm), CD4 TC (E[max] 670 ± 20 nm), and CD14 biotin. After washing, the cells were stained with Streptavidin Red613 and fixed in 2% paraformaldehyde. Monocytes (CD14^+) were eliminated by excluding all cells positive for Red613 and the remaining cells analyzed on the basis of their expression of the MK marker CD41.

DNA Staining

Propidium iodide (PI) and 7-AAD, (Sigma Chemicals, St Louis, MO) were used to quantify DNA. PI was used for two-color analysis with FITC while 7-AAD was used for three-color analyses in conjunction with FITC and PE. For the analysis of polyploid MK (>8N), a two-color analysis was used in which the cells were stained with anti-CD4 FITC and PI. CD41 staining could be omitted because, in bone marrow, only MK have a DNA content >8N. Cells were stained with anti-CD4 FITC MoAbs, washed as above, and resuspended in MK medium containing 0.5% Tween-20 for 30 minutes to permeabilize the cell membranes. The suspension was diluted with an equal volume of MK medium containing 0.5% Tween-20 and 2% paraformaldehyde. After 5 minutes at 4°C, the cells were pelleted and freshly prepared PI (50 μg/mL in 0.1% citrate saline buffer, containing 1% BSA) added. The suspension was stored overnight in the dark at 4°C. The following day 50 μg/mL of RNase type A (Boehringer Mannheim, Indianapolis IN) was added for 30 minutes at room temperature in the dark, and the cells then analyzed by flow cytometry. To detect CD4 and CD34 on polyploid MK, we used three-color staining (CD4 FITC, CD34 PE, and 7-AAD (25 μg/mL) for DNA. Staining with MoAbs and permeabilization were performed as described above. 7-AAD can be excited by the 488 nm emission of the argon laser and its red fluorescence detected in the third channel of the FACSscan cytometer. To distinguish between aggregates and polyploid cells, the DNA signal was acquired in width and area modes simultaneously. The measurement of the width of each signal is essentially a measurement of the time that the particle is in the laser beam and can be used to provide a measure of aggregation. All samples were filtered through a 70-μm nylon cell strainer before analysis on the flow cytometer.

Flow Cytometry

Two- and three-color experiments were performed on a Becton Dickinson FACScan cytometer equipped with a 15 mW argon laser.
emitting at 488 nm. Electronic compensation was used to eliminate spectral overlap between the various fluorochromes. Ten thousand CD41 positive cells were analyzed.

For the analysis of DNA content in high-ploidy classes, 10,000 to 15,000 cells with DNA content >8N were acquired and when analyzing progenitor cells similar numbers of CD34+ cells were acquired. In each case the stained cells were compared with an appropriate isotypic control.

Four-color experiments were performed on an Epics Elite Cell Sorter (Coulter, Hialeah, FL) equipped with an argon laser, excited at 488 nm. Electronic compensation for the spectral overlap between different fluorochromes was performed as described above.

**Cell Sorting**

Low-density bone marrow cells expressing CD34 were separated with a MACS Separation system from Miltenyi Biotec GmbH (Sunnyvale, CA). The cells are labeled with an anti-CD34 (Q Bend 10) and superparamagnetic particles coated with rat antimouse Ig. Cells labeled with these small (60 to 70 nm) magnetic beads have unaltered scatter properties and can be analyzed in the flow cytometer. Positive selection of CD34+ cells with this system produced populations of 85% to 95% purity, with 70% to 80% recovery of the input CD34+ cells. The CD34-enriched population was then stained with anti-CD41 FITC, anti-CD34 PE (clone HPCA-2, Becton Dickinson, San Jose CA), and anti-CD4-TC. These cells were first sorted on the basis of their expression of CD41 and CD34. Reanalysis of the sorted population gave CD41 purities of >99%. These cells were then sorted on the basis of their CD4 expression. After this second sort, the CD41 content was always more than 95%. The purity of the CD4- population was greater than 95%, but the purity of the CD4+ cells varied between 85% and 95%.

**CFU-MK Culture**

Sorted CD34+CD41-CD4+ and CD34+CD41-CD4- cells were cultured in plasma clots using a modification of the method of McLeod et al.7 The medium contained 10 mg/mL deionized BSA (Boehringer Mannheim), 25 ug/mL soybean lipids (Boehringer Mannheim), 7.8 ug/mL linoleic acid (Sigma Chemical Co), 5.6 ug/mL sodium pyruvate (Sigma Chemical Co), 1 mmol/L α-thioglycerol (Sigma Chemical Co), 300 ug/mL 30% iron saturated transferrin (Boehringer Mannheim), 20 ug/mL L-asparagine (Sigma Chemical Co), 33 ug/mL CaCl2 (Sigma Chemical Co), in Iacove’s modified Dulbecco’s medium (with L-glutamine), and 10% bovine plasma (Sigma Chemical Co). The medium was supplemented with the following growth factors: rhu interleukin (IL)-3 (a gift of the ImmuneX Co, Seattle, WA) 1 ng/mL: rhu IL-6 (PeproTech, Rocky Hill, NJ) 10 ng/mL; rhu SCF (PeproTech) 50 ng/mL, and Thrombopoietin (TPO) (a gift of Donald Foster of ZymoGenetics Corp, Seattle, WA), 300 U/mL. CD4+ and CD4- MK were plated in 100 ul of medium in the wells of 96-well microtiter plates at 100, 200, or 400 cells/well. After 7 days, CFU-MK were counted in each well. Clusters containing three or more MK were scored at a colony. Under these conditions, using CD4+ cells to initiate the cultures, all of the colonies observed consisted of cells committed to the MK lineage and as expected, all of the cells stained immunohistochemically with anti-CD41.

**Statistics**

Statistical significance was determined using Student’s two-tailed t test for paired samples.

**RESULTS**

**MK Are Enriched in the CD34+ Progenitor Population**

Megakaryocytes were identified and distinguished from other cells in bone marrow on the basis of their expression of CD41and by their DNA content. Progenitor cells were identified on the basis of their expression of CD34. In these experiments, cells with DNA contents equal or greater than 8N were considered to be mature MK. MK constitute ~0.5% of the low-density (<1.077 g/cm) bone marrow cells (mean = 0.61 ± 0.24; n = 11). Among the progenitor pool of CD34+ cells, CD41+ cells constituted ~10% of the population (mean = 8.8% ± 3.5%; n = 7) (Fig 2). In the reciprocal analysis, ~20% of CD41 positive cells (mean = 19.8% ± 6.7%; n = 7) were also CD34 positive (Fig 3). These results demonstrate that MK are 20-fold more common in the progenitor pool than in the mature, CD34 negative population, where less than 0.4% (mean = 0.38% ± 0.21%, n = 7) of the cells express MK markers.

**CD4+ MK Are Enriched in the CD34+ Progenitor Population**

A substantial fraction of human MK express CD4 (mean = 18.7% ± 9.3%; n = 6). A representative sample is shown in Fig 4. Figure 4A shows the total population of bone marrow cells stained with anti-CD4 MoAb. When immature (CD34+) MK were analyzed, CD4 antigen was detected on 35% ± 10% (n = 5) of the cells (Fig 4B). In contrast only 17.5% ± 8.3% (n = 5) of more mature CD34- MK expressed CD4 (Fig 4C).

**CD4 expression declines with increasing ploidy.** As MK mature they become increasingly polyploid. To determine if the proportion of CD4+ cells varied with increasing ploidy, a two-color analysis, correlating CD4 expression and DNA content was performed. The data were acquired in logarithmic mode to accommodate the wide range of DNA content. After identification of the 2N and 4N peaks, the data were gated on the cells with DNA content equal to or greater than 8N. Experiments on DNA content were performed with both total bone marrow and with a low-density population obtained by flotation Ficoll-Hypaque. The distribution of megakaryocytes in the different ploidy classes, as well as proportion of CD4 in each class, were identical. Figure 5 shows a typical pattern of DNA staining of the total bone marrow population. In this sample the cells were distributed as follows: 8N, 12%; 16N, 44%; 32N, 29%; >32N, 14%. To measure the proportion of CD4+ cells, the samples were gated separately on each ploidy class. The results of such an analysis on four patients is shown in Table 1. Although there is considerable variation among the samples, in each case CD4 expression declined with increasing ploidy. The largest decline took place as the cells progressed from 8 to 16 N. Differences between the proportion of CD4+ cells of the 8N and 16N classes and between the 16N and 32N classes were significant at P values of <.01. Despite the progressive decline, CD4 was detectable on some cells of all ploidy classes.

**Relationship between ploidy, CD34, and CD4 on MK.** The CD4 antigen is expressed by virtually all human hematopoietic progenitors and appears to be a reliable marker for immature cells.7,8 In the next set of experiments we examined CD4 expression and ploidy in the CD34+ population. In the sample illustrated, 18% of the cells were CD4+ (Fig 6A), and 2.1% had a DNA content >8N (Fig 6B). The CD34+ CD4+ and CD34+ CD4- populations were analyzed sepa-
Fig 3. CD34+ expression by the CD41+ MK population. Anti-CD41 FITC was used to detect progenitor cells. CD41+ and CD41- cells were analyzed separately using the electronic gates shown in the top panel. The lower left panel is a histogram of the staining by anti-CD41 FITC of the CD41+ cells. The right lower panel is a histogram of CD41 FITC of the CD41+ cells. The sample was also stained with anti-CD14 TC and gated to exclude monocytes.

Fig 2. CD41 expression by CD34+ bone marrow progenitor cells. Anti-CD34 PE was used to detect progenitor cells. CD34+ and CD34- cells were analyzed separately using the electronic gates shown in the top panel. The lower left panel is a histogram of the staining by anti-CD41 FITC of the CD34- cells. The right lower panel is a histogram of CD41 FITC of the CD34+ cells. The sample was also stained with anti-CD14 TC and gated to exclude monocytes.

CD41 (FITC) -->

CD34 NEGATIVE

CD34 POSITIVE

Number

FS -->

CD34 (PE) -->

CD34- 97.0%

CD34+ 2.4%

2.3%

18.5%

0.58%

11.43%
CD34+ BONE MARROW CELLS ENRICHED WITH CD4+ MK

Fig 4. Expression of CD4 by CD34+ and CD34- MK. A four-color analysis was employed using anti-CD14 biotin + streptavidin Red 613 to exclude monocytes, anti-CD4 TC, anti-CD41 FITC, and anti-CD34 PE. CD14+ cells were excluded from the analysis. (A) is a correlated two-color histogram of the staining of the total bone marrow with anti-CD41 and anti-CD4. In this sample, 21% of the CD41+ cells were also CD4+. (B) depicts the staining of the same cells gating only on the CD34+ cells. A total of 44% of the CD41+ CD34+ were also CD4+. (C) depicts the staining of the CD34- population. A total of 18% of the CD41- CD34- cells were also CD4+.

Fig 5. DNA content of CD41+ bone marrow cells. The marrow was stained with propidium iodide as described. Unshaded histogram shows the pattern obtained with the whole bone marrow sample. The prominent 2N peak is readily apparent. The acquisition of data for polyploid cells was performed using a gate that included only cells with a DNA content >4N. The shaded area shows the pattern obtained using this gate with prominent 8N, 16N, and 32N peaks. Logarithmic amplification was used to permit cells with a wide range of DNA content to be shown on the same histogram.

rately for ploidy. The vast majority of the polyploid (>8N) CD34+ cells were found in the CD34+CD4+ population. (Fig 6C and D).

Functional significance of CD4 expression by immature MK. The enrichment of CD4 expressing MK in the CD34+ population and the decline of CD4 expression with increasing ploidy suggested that the expression of this marker might play a significant role in the development of the MK lineage. CD4+ and CD4- CD34+ MK were separated by a combination of magnetic and fluorescence activated cell sorting. Representative histograms of the sorted populations are shown in Fig 7A and B. When these cells were placed in culture under conditions that support the growth of CFU-MK, striking differences were found in colony formation. CD4+ MK have almost no capacity for clonal growth in a plasma clot, whereas the corresponding CD4- population give abundant colonies (Table 2). This CD4+ population appears to contain all of the colony forming activity of the total CD34+ MK population.

DISCUSSION

The present data indicate that MK are markedly (~20 fold) enriched among the CD34+ progenitor population of the bone marrow. This CD34+ MK population is also enriched with cells expressing the CD4+ antigen. The proportion of MK expressing CD4 declined with increasing ploidy. These results suggest that CD4 expression in this lineage

<table>
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<th>Experiment No.</th>
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The low density BMC were stained with anti-CD4 FITC and PI as described. Data were collected in log mode and each of the polyploid classes (8N and greater) were analyzed separately. The results are reported in terms of percentage CD4+ in each class. Abbreviation: NA, not available.
is limited to early stages of development. The inability of immature CD34+ cells expressing CD4 to proliferate in culture suggests that expression of this antigen is associated with the onset of endomitotic replication.

Because we used CD41 antigen as an indicator of the MK lineage, we were unable to enumerate the earliest CD34+CD41- MK precursors. Despite this, we could show that CD41+ MK lineage cells are 20-fold enriched in the CD34+ progenitor compartment. This progenitor population (cells staining brightly with CD34) makes up a small (2% to 3%) fraction of the bone marrow. However ~10% of these cells express the CD41 marker compared with ~0.5% of CD34- cells. Indeed ~20% of MK, defined by the CD41 antigen, are CD34+. We believe that this enrichment of CD41+ cells is a consequence of their limited growth potential and is necessary to maintain normal MK levels in the marrow. Compared with other hematopoietic lineages, MK appear to undergo few cell divisions because the last 5 to 7 divisions of the lineage are lost to endomitotic reduplication. In a lineage with limited capacity for expansion, a large precursor population is required to maintain the final mature cell number. The striking enrichment of CD41+ cells among the CD34+ population has not been described previously.

When MK precursors (CD34+) and more mature megakaryocytes (CD34-) were analyzed for the expression of CD4 antigen, we found, that CD34+ MK have a higher proportion of CD4 positive cells (~40%) than the more mature CD34- cells (~20%). The conclusion that CD4 expression is related to maturation is further strengthened by the inverse correlation between DNA content and CD4 expression.

The presence of a significant CD34+CD41+CD4- population (Fig 6) suggests that MK lineage cells express CD41 (GPIIIb/IIIa) before they express CD4 and before they undergo endomitotic polyploidization. A CD34+CD41- MK precursor has been reported previously, suggesting that the order of development is CD34+CD41-CD41+CD41-CD4-. While it is possible that a CD34+CD41+CD4- cell committed to the MK lineage exists, no evidence for such a cell has been produced and a precursor with this phenotype would not have been detected in these studies. The results shown in Table 2 suggest that in the principal pathway of MK development, the expression of CD4 is correlated with the loss of the capacity to undergo normal mitotic division. Colony formation was tested in growth medium containing both IL-3 and TPO, so that both of the MK populations suggested by Kaushansky et al22 (TPO-dependent and TPO + IL-3-dependent) would be detected.

The functional significance of CD4 expression in the MK lineage is not clear. Superficially it appears that some MK express CD4, whereas others do not. It is possible that CD4+ MK represent a specialized subset with an as yet unknown function. If this were to be true, cells that become CD4+ would remain so throughout their lifetime. Conversely CD4- MK would be part of a subset that never expressed this antigen. The relative decline in the proportion of CD4+ cells with increasing ploidy could reflect differential survival of the two subsets rather than loss of expression. MK subsets have been demonstrated but their functional significance has not been established.24

The association of CD4 expression with a switch from mitotic to endomitotic replication suggests a likely alternative to the concept of separate lineages. This model suggests...
that all MK go through a developmental stage in which they express CD4. This model is illustrated in Fig. 8. Transient, developmentally-regulated expression of CD4 occurs in all CD8 T cells as they pass through a "double positive" stage and is also known to occur in the development of most, if not all, murine hematopoietic cells. 16,25 If this is the case in the MK lineage, then the differences between CD4+ and CD4- MK could be attributed to asynchronous loss of the antigen as the cells mature.

The role of CD4 in MK development remains unknown. The antigen could serve as a ligand for Class II major histocompatibility complex (MHC) antigens on stromal cells of the hematopoietic microenvironment. In this role CD4 would serve primarily as an adhesive molecule, increasing the strength of the bond between a stromal cell and a developing MK. This is analogous to the generally accepted function for this molecule on T cells as a coreceptor, interacting with Class II MHC antigens on antigen-presenting cells. Improved contact between the cells could facilitate the transfer of other signals. Sulfated polysaccharides surrounding stromal cells could also serve this function. The CD4 antigen contains a binding site for sulfated polysaccharides and thus could play a role in binding the developing MK to the surrounding matrix. 16,26-28 These matrices bind a variety of growth factors including those involved in the regulation of hematopoiesis. 29 Thus, engagement of MK CD4 by MHC or an as yet unidentified ligand could lead to the activation (or downregulation) of the progenitor.
We have shown that MK are enriched in the CD34 bone marrow cell compartment and that these CD34+ CD41+ cells preferentially express CD4. These cells are in an early stage of their development when they are undergoing both mitotic division and endomitotic DNA replication. This stage of maturation represents a possible developmental window where megakaryocytes could be infected by HIV-1. Future investigation will be required to test the hypothesis that direct infection of MK progenitors is possible and could be responsible for impaired platelet production in HIV-1 idiopathic thrombocytopenia patients as well as required to understand the role of CD4 in MK differentiation.

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