Evidence for Malignant Transformation in Acute Myeloid Leukemia at the Level of Early Hematopoietic Stem Cells by Cytogenetic Analysis of CD34+ Subpopulations

By Detlef Haase, Michaela Feuring-Buske, Stefan Könenmann, Christa Fonatsch, Carmen Troff, Walter Verbeek, Arnulf Pekrun, Wolfgang Hiddemann, and Bernhard Wörmann

Acute myeloid leukemia (AML) is a heterogenous disease according to morphology, immunophenotype, and genetics. The retained capacity of differentiation is the basis for the phenotypic classification of the bulk population of leukemic blasts and the identification of distinct subpopulations. Within the hierarchy of hematopoietic development and differentiation it is still unknown at which stage the malignant transformation occurs. It was our aim to analyze the potential involvement of cells with the immunophenotype of pluripotent stem cells in the leukemic process by the use of cytogenetic and cell sorting techniques. Cytogenetic analyses of bone marrow aspirates were performed in 13 patients with AML (11 de novo and 2 secondary) and showed karyotype abnormalities in 10 cases (2q+, +4, 6p, t(6;9), 7, +8 in 1 patient each and inv(16) in 4 patients each). Aliquots of the samples were fractionated by fluorescence-activated cell sorting of CD34+ cells. Two subpopulations, CD34+/CD38- (early hematopoietic stem cells) and CD34+/CD38+ (more mature progenitor cells), were screened for karyotype aberrations as a marker for leukemic cells. Clonal abnormalities and evaluable metaphases were found in 8 highly purified CD34+/CD38- populations and in 9 of the CD34+/CD38+ specimens, respectively. In the majority of cases (CD34+/CD38+, 6 of 8 informative samples; CD34+/CD38-, 5 of 9 informative samples), the highly purified CD34+ specimens also contained cytogenetically normal cells. Secondary, progression-associated chromosomal changes (+8, 12) were identified in the CD34+/CD38- cells of 2 patients. We conclude that clonal karyotypic abnormalities are frequently found in the stem cell-like (CD34+/CD38-) and more mature (CD34+/CD38+) populations of patients with AML, irrespective of the phenotype of the bulk population of leukemic blasts and of the primary or secondary character of the leukemia. Our data suggest that, in AML, malignant transformation as well as disease progression may occur at the level of CD34+/CD38- cells with multilineage potential.

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In chronic myelogenous leukemia (CML) and myelodysplastic syndromes (MDS), it is supposed that the transformation occurs at the level of pluripotent stem cells. However, in most cases of acute myeloid leukemia (AML), especially in those with a differentiated phenotype, the leukemogenic genetic defect is believed to affect progenitor cells with myeloid differentiation.

AML is a heterogenous disease on the genetic, phenotypic, and clinical level. The heterogeneity results from predisposing factors, the transforming and progression-associated event(s), and the maturational status of the transformed progenitor cell. Human hematopoietic progenitor cells can be identified by function and by immunophenotype. They express a characteristic cell surface antigen, classified as CD34, and can be further subdivided by the expression of CD38, HLA-DR, CD45RO, CD117 (stem cell factor receptor, c-kit), and CDw90 (Thy 1). CD34+/CD38-/HLA-DR+ cells give rise to myeloid and B-lymphoid cells. Maturation and differentiation into functional cells are accompanied by acquisition of lineage-specific or lineage-associated cell surface molecules. Classification of acute leukemias is based on the comparison of the leukemic cells with their presumed normal hematopoietic counterparts. Fifty percent to 80% of newly diagnosed AML are CD34+. The antigenic profile of the early hematopoietic maturational pathway, characterized by the expression of CD34, CD38, and HLA-DR, is well conserved on myeloid leukemic blasts. Functional studies using the colony assay have shown a higher cloning efficiency for CD34+ blasts than for CD34- leukemic blasts. Recently, CD34+/CD38- blasts from a patient with AML M1 were injected into SCID mice and induced the clinical picture of AML. We described a patient with AML M4 and trisomy 4 occurring in the CD34+/CD38- stem cell-like compartment. These data suggest that the leukemogenic potential resides in the compartment of the CD34+/CD38- blast population and hence a population with the potential of multilineage development. However, it has not been clarified whether this functional behaviour differs between AML subtypes, especially in cases with no or only a minor population of CD34-alone positive blasts. Characterization, discrimination, and isolation of normal and leukemic stem or progenitor cells is the basis for improvements in myeloablative chemotherapy with subsequent autologous retransfusion, for targeted gene therapy, and for monitoring of minimal residual disease.

Fifty percent to 80% of all patients with AML display clonal chromosome abnormalities that can be delineated by classical cytogenetic techniques in bone marrow, peripheral blood, and other tissue specimens. Quantification of numerical and selected structural aberrations is facilitated by fluorescence in situ hybridization. The majority of chromosomal abnormalities are leukemia-specific. Whereas primary...
chromosomal changes are considered to be initiating factors, secondary abnormalities are involved in disease progression. We used cytogenetics as a parameter for the identification of leukemic cells in subpopulations of CD34+ cells from bone marrow aspirates of patients with newly diagnosed AML whose bulk populations of leukemic blasts were CD34-. It was our aim to determine the level of malignant transformation in AML within the hematopoietic hierarchy.

MATERIALS AND METHODS

Patients. Bone marrow aspirates performed for diagnostic purpose from 60 patients with AML were selected for sorting procedures according to their immunophenotype. In 13 patients (11 with newly diagnosed and 2 patients with relapsed leukemia), the metaphase yield in the sorted cellular subpopulations was sufficient for karyotyping. Patients were admitted to the Departments of Internal Medicine or Pediatrics at the University of Göttingen (Göttingen, Germany) or to the Department of Internal Medicine at the University of Münster (Münster, Germany). Diagnosis and classification of AML were based on light microscopy of Pappenheim stained slides, and on cytochemical reaction with periodic acid Schiff (PAS), myeloperoxidase, and esterase. Slides were reviewed by two independent hematologists according to the criteria of the French-American-British (FAB) classification.\textsuperscript{4,5}

Immunophenotyping and cell sorting. Immunophenotyping was performed at diagnosis by multiparameter flow cytometry using a whole blood lysis method and a set of monoclonal antibodies against myeloid and lymphoid lineage-associated antigens.\textsuperscript{6} The early maturation pathway was characterized with monoclonal antibodies against CD34 (HPCA-2, directly conjugated with fluoresceinisothiocyanate; Becton Dickinson Immunocytometry Systems [BDIS], San José, CA), CD38 (Leu17 conjugated with phycoerythrin; BDIS), and HLA-DR (conjugated with PerCP; BDIS). Directly conjugated isotype antibodies were used as negative controls.

For isolation of subpopulations by cell sorting, fresh bone marrow aspirates were used in 11 patients and cryopreserved aspirates were used in 2 patients (patients no. 10 and 11). Mononuclear cells were isolated by Ficoll gradient centrifugation, washed twice in RPMI 1640 (GIBCO, Karlsruhe, Germany), and counted. Cells (2 x 10\(^7\)) were double-stained with anti-CD34 and anti-CD38 monoclonal antibodies. All steps were performed under sterile conditions. Analysis and isolation were performed on a fluorescence-activated cell sorter (FACS Vantage; BDIS) equipped with a 488 nm laser using phosphate-buffered saline as sheath fluid. A minimum of 4,500 cells of each subpopulation were sorted with a purity between 96% and 98%.

In vitro culturing and cytogenetics. The FACS-isolated cells as well as one unsorted bone marrow specimen were incubated for 24 to 72 hours in RPMI 1640 supplemented with 20% fetal calf serum (FCS; HyClone, Logan, UT), 100 U/mL recombinant human (rh) granulocyte colony-stimulating factor (Amgen, Thousand Oaks, CA), 100 U/mL glycosylated rh granulocyte-macrophage colony-stimulating factor (Behringwerke, Marburg, Germany), 100 U/mL rh interleukin-3 (Behringwerke), 1 U/mL rh erythropoietin (Boehringer, Mannheim, Germany), and 50 ng/mL rh stem cell factor (Genzyme, Boston, MA). Cells were grown at 37°C. Details of chromosome preparation and staining by a modified GAG-banding technique have been described previously.\textsuperscript{7,24,25} The karyotypes were classified according to the International System of Chromosome Nomenclature (ISCN).\textsuperscript{22} Chromosome analysis of highly purified cell populations after cell sorting was performed by the same procedure except that the incubation time with 0.3 μg/mL colcemide (GIBCO) was increased from 2 hours for unsorted bone marrow to 12 hours for the sorted specimens and the unsorted control.

Fluorescence in situ hybridization (FISH) of one aspirate with trisomy 8 was performed as follows. Target DNA was denaturated for 5 minutes in a 74°C denaturant bath (70% formamide and 2× SSC, pH 7.0 to 8.0) followed by dehydronation in an ethanol series (70%, 80%, and 100%). The centromeric probe for chromosome 8 (spectrum green; Imagegenes, Stuttgart, Germany) was denaturated in a hybridization buffer at 74°C for 5 minutes and subsequently cooled on ice. For hybridization, the slides were prewarmed at 45°C and 10 μL of the probe mix was added. Coverslips were then mounted and sealed with rubber cement. For hybridization, the slides were incubated in a humidity chamber for 16 hours at 42°C. After hybridization, the slides were washed three times. Slides were dried in darkness. Propidiumiodide and DAPI counterstains were applied. Hybridization signals were counted using an appropriate filter set (Zeiss, Göttingen, Germany) at the same day. Internal threshold for trisomy was 3% cells showing 3 signals.

Mitotic indices were determined in 8 patients by counting the mitotic figures within 1,000 cells.

RESULTS

Morphology and immunophenotype. Patients were classified as M0 (1 patient), M1 (1), M2 (3), M4 (4), and M4Eo (4). Patients' characteristics are listed in Tables 1 and 2. One patient (patient no. 7) had been diagnosed with paroxysmal nocturnal hemoglobinuria (PNH) in 1991, based on loss of glycosylphosphatidylinositol (GPI)-anchored proteins.\textsuperscript{37} Recently, one patient (patient no. 8) has been examined intensively and previously published as a case report.\textsuperscript{25} Bone marrow aspirates from all patients were immunophenotypically characterized by the expression of CD34 and CD38. Based on the light scatter profile and the expression of CD34 and CD38, four subpopulations are distinguishable in the order of maturation (Fig 1): CD34+/CD38-, CD34+/CD38+, CD34-/CD38-, and CD34-/CD38+. The relative percentage of CD34+/CD38- cells ranged from 0.05% to 12.14% and of CD34-/CD38- cells from 0.13% to 66.7% (Table 1). The number of sorted cells ranged from 4.5 x 10\(^3\) to 2 x 10\(^6\). In 2 patients (patients no. 3 and 10), only CD34+/CD38- cells were available for chromosome analysis. In patient no. 5, metaphase cells only were found in the CD34+/CD38- population.

Cytogenetics of unsorted bone marrow. The number of completely analyzed metaphases in the unsorted samples ranged between 1 to 34 metaphases, with a median number of 18. In 10 of 13 patients, clonal chromosome abnormalities were identified. Seven patients displayed a mosaic of normal and abnormal cells. In a child with AML M2 (patient no. 3), no unsorted material was available, but cytogenetic analysis at initial diagnosis performed in another cytogenetic laboratory had showed monosomy 7. The most frequent karyotypic abnormality was a pericentric inversion of chromosome 16, inv(16)(p13q22), occurring in all 4 patients with AML M4Eo. Trisomy 8 was found in 1 patient with AML M2 and MDS-prephase (patient no. 4) as primary and sole abnormality. Patient no. 5 with AML M2 and atypical basophilia displayed a translocation t(6;9)(p23;q34) and trisomy 8 as secondary abnormality in her bone marrow cells at the time of relapse. An initial cytogenetic examination at the time of first diagnosis had shown translocation t(6;9) without additional abnormalities. A derivative chromosome 2 with unidentifiable additional material attached to chromosome...
Table 1. Yield of Cellular Subpopulations After Sorting Procedure

<table>
<thead>
<tr>
<th>Patient No./Age/Sex</th>
<th>FAB-type</th>
<th>No. of Cells After the Sort (x10^3)</th>
<th>Relative Frequency</th>
<th>CD34+/CD38</th>
<th>CD34'/CD38</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CD34+/CD38</td>
<td>CD34'/CD38</td>
</tr>
<tr>
<td>1/32/M</td>
<td>M0</td>
<td>4.5</td>
<td>1.18</td>
<td>0.42</td>
<td>0.26</td>
</tr>
<tr>
<td>2/46/M</td>
<td>M1</td>
<td>55</td>
<td>12.14</td>
<td>6.02</td>
<td>1.64</td>
</tr>
<tr>
<td>3/8/M</td>
<td>M2</td>
<td>12</td>
<td>0.15</td>
<td>1.12</td>
<td>0.03</td>
</tr>
<tr>
<td>4/60/M</td>
<td>M2</td>
<td>30</td>
<td>0.05</td>
<td>6.76</td>
<td>1.42</td>
</tr>
<tr>
<td>5/75/F</td>
<td>M2Baso</td>
<td>42</td>
<td>1.88</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>6/71/F</td>
<td>M4</td>
<td>100</td>
<td>1.21</td>
<td>17.72</td>
<td></td>
</tr>
<tr>
<td>7/73/M</td>
<td>M4</td>
<td>5</td>
<td>0.14</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>8/60/F</td>
<td>M4</td>
<td>45</td>
<td>1.01</td>
<td>15.97</td>
<td></td>
</tr>
<tr>
<td>9/58/F</td>
<td>M4</td>
<td>45</td>
<td>6.76</td>
<td>53.91</td>
<td></td>
</tr>
<tr>
<td>10/51/M</td>
<td>M4 Eo</td>
<td>20</td>
<td>6.86</td>
<td>56.58</td>
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<tr>
<td>11/34/F</td>
<td>M4 Eo</td>
<td>30</td>
<td>1.13</td>
<td>35.56</td>
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<tr>
<td>12/30/F</td>
<td>M4 Eo</td>
<td>600</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>13/81/M</td>
<td>M4 Eo</td>
<td>70</td>
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Cytogenetics of the CD34+/CD38- population. In comparison to the identically stimulated unsorted control population, the CD34+ cells displayed an extremely high mitotic index (Table 2).

Metaphase cells from the most immature population of CD34+/CD38- cells were available for cytogenetic analysis in 10 patients. Two to 36 metaphases (median, 11) were studied. Two patients displayed a normal karyotype corre-

Table 2. Cytogenetic Findings and Mitotic Activity in Sorted Cellular Subpopulations

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis (FAB)</th>
<th>Karyotype</th>
<th>No. of Analyzed Metaphases (% abnormal cells)</th>
<th>Mitotic Index (related to 1,000 cells)</th>
<th>Unsorted</th>
<th>CD34+/CD38</th>
<th>CD34'/CD38</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M0</td>
<td>Normal</td>
<td>2</td>
<td>&lt;1</td>
<td>50</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M1</td>
<td>Normal</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M2, relapse</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M2/MDS</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M2Baso, relapse</td>
<td>t(6;9)(p23;q34),+8</td>
<td>1</td>
<td>16</td>
<td>1</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M4</td>
<td>Normal</td>
<td>21</td>
<td>&lt;1</td>
<td>8</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M4/PNH</td>
<td>Normal</td>
<td>16</td>
<td>1</td>
<td>224</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M4</td>
<td>Normal</td>
<td>16</td>
<td>1</td>
<td>54</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M4</td>
<td>Normal</td>
<td>26</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M4 Eo</td>
<td>Normal</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>M4 Eo</td>
<td>Normal</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
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<tr>
<td>12</td>
<td>M4 Eo</td>
<td>Normal</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
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<tr>
<td>13</td>
<td>M4 Eo</td>
<td>Normal</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: FISH, results obtained by fluorescence in situ hybridization; ND, not done; -, no metaphases available; 0, metaphases available, but not in this cell population with the respective karyotype.

* After colony assay.
Fig 1. Four-dimensional flow cytometric analysis of the bone marrow from a patient (patient no. 13) with AML M4Eo. Ten thousand cells were acquired in listmode on a FACScan with four gates (R1 through R4) showing the four different maturational subpopulations (R1, CD34+/CD38-; R2, CD34+/CD38+; R3, CD34-/CD38+; R4, CD34-/CD38-) that were sorted in concordance with their light scatter characteristics (R5 and R6).

Corresponding to the findings in the unsorted bone marrow. In 7 of 8 cases with clonal abnormalities, changes identical to those in the unsorted specimens were identified in the sorted cell populations using classical cytogenetics (Table 2). In patient no. 4 with AML M2 and trisomy 8, no analyzable metaphases were obtained in the CD34+/CD38- fraction. FISH with an alphoid centromeric probe for chromosome 8 was performed in this case for quantification of leukemic cells by interphase cytogenetics and showed that 32 of 42 CD34+/CD38- cells contained three signals, indicating trisomy 8. The application of classical cytogenetics showed a mosaic of normal and abnormal metaphases in the CD34+/CD38- cells in 5 cases. In 1 additional case (patient no. 4), a mosaic karyotype was confirmed by FISH. The remaining 2 patients displayed only abnormal cells; however, in both patients only two metaphases were analyzable. Additional cytogenetic abnormalities (+8/+8, -12) that occurred in 2 patients in the unsorted bone marrow (patients no. 5 and 13) were also observed in the immature sorted cell compartment.

Cytogenetics of the CD34+/CD38+ population. Metaphase cells from the more mature population of CD34+/CD38+ cells were obtained in all but 1 patient; the number ranged from 1 to 36 (median, 13). In 9 of 10 cases with clonal chromosomal abnormalities, these were found in the sorted CD34+/CD38+ cells. A mosaic with normal metaphases occurred in 4 cases. In the patient with inv(16) and additional abnormalities (patient no. 13), trisomy 8 was observed in all metaphases, whereas monosomy 12 was not seen. FISH in patient no. 4 showed trisomy 8 in 14 of 16 cells in the CD34+/CD38+ compartment. In patient no. 5, no metaphase cells were detectable in this subpopulation.

DISCUSSION

The data of the current study strongly suggest that, in AML, malignant transformation as well as disease progression may occur at the level of immature stem cells, independent from the phenotype of the individual leukemia and from the de novo or secondary character of the disease.

This conclusion supports the concept of the pluripotent origin of the leukemic clone in AML and is based on the following findings. Clonal chromosomal changes were observed in the stem cell compartment (CD34+/CD38+) of 8 informative cases. Although these findings could have been expected in the patient (patient no. 7) with secondary AML after PNH or in the patient (patient no. 8) with trisomy 4, they were surprising in the patients with AML M4Eo and inv(16). A methodologic argument against this conclusion is a contamination of the sorted cell population with other leukemic cells. Considering the purity of FACS sorting of 96% to 98%, four observations argue against this assumption. First, we regularly used clearly separated, nonoverlapping gates (Fig 1). Second, we never were able to find metaphase cells in the mature subpopulations (CD34+/CD38+). Third, a contamination of 2% to 4% would not explain the high portion of abnormal cells in the CD34+ subpopulations (mean, 84% in the CD34+/CD38+ compartment and 77% in the CD34+/CD38+ compartment), especially considering the very high mitotic index of the CD34+ subpopulations (Table 1), which was significantly lower in the unsorted bone marrow grown under the same conditions. Finally, in parallel experiments on bone marrow samples from patients with C-ALL, we isolated CD3+ cells and did not find the leukemia-associated T-cell receptor δ gene re-
The proliferation of pluripotent hematopoietic stem cells and their differentiation into the myeloid, lymphoid, erythroid, and megakaryocytic cell lineages is a strictly regulated process. Transforming events lead to deregulation. The phenotype of the transformed cell lineage is often conserved, which has led to the classifications for leukemias and lymphomas in analogy to the phenotype of normal hematopoiesis. CML and MDS are considered to be stem cell diseases. However, those inferences supporting a stem cell involvement in myeloid malignancies were indirect and not based on the examination of stem cells themselves. The delineation of the stem cell character of the malignant transformed progenitor cells was based on the detection of leuke-mia-specific or -associated markers in different cell lineages and has recently benefited from the combined use of immunophenotyping and karyotyping using conventional cytogenetics or FISH.

Knuutila et al examined the cell lineage involvement in 10 cases of AML and other hematologic malignancies. The karyotypes of immunostained cells were analyzed either by conventional cytogenetics, by chromosome painting, and/or by interphase cytogenetics. Nine patients showed chromosome abnormalities in CD13+ myeloid cells and 1 in the CD64+ monocytic population. Three patients additionally displayed involvement of the erythroid lineage by translocation t(3;3) in 2 patients and by translocation t(8;12) and 12p− in another case. An involvement of the megakaryocytic lineage was seen in 1 case with t(3;3). In a patient with AML M0, complex karyotypic anomalies were shown in CD34+ cells by chromosomal painting. Suciu et al have examined material from 11 patients with AML. They observed an affection of the granulocytic/monocytic lineage in all patients. The erythroid population displayed abnormal karyotypes in 7 patients and the megakaryocytic lineage in 5 cases. Summarizing, trisomy 8, trisomy 11, and complex abnormalities were present in all three lineages examined. Trisomy 4 in AML M4 has previously been described in myeloid and lymphoid cells. Using combined immunophenotyping and FISH, Baurman et al detected monosomy 7 in myelomonocytic and erythroid lineages as well as in a part of CD34+ precursor cells in 1 case of AML. In an investigation combining morphology and FISH, van Lom et al showed monosomy 7 in blasts, promyelocytes, myelocytes, metamyelocytes, monocytes, and erythroblasts in the bone marrow of a patient with AML M7. It can be concluded from the published data that, in AML, t(3;3), +4, −7, +8, +11, chromosome 12p-anomalies, and complex cytogenetic changes frequently involving chromosomes 5 and/or 7 occur at the level of multipotent progenitors. However, all of these karyotypic changes are associated with secondary AML, which is believed to be derived from a stem cell transformation.

Very recently, Turhan et al published results on clonality and the presence of the PML-RARA fusion gene in CD34+ CD38− and CD34+/CD38+ sorted subpopulations in 3 patients with acute promyelocytic leukemia (AML M3). It was concluded that in AML M3 the primitive hematopoietic stem cells are not involved in the leukemogenic process.

For therapeutic purposes, it is most important to identify the leukemic progenitor or stem cell directly by use of visual and/or functional methods that allow their detection and isolation in leukemic and regenerative bone marrow. We report here the cytogenetic characteristics of highly purified stem cells by polymerase chain reaction (F. Griesinger et al, manuscript submitted).
cell populations in AML using fluorescence-activated cell sorting, chromosome banding, and FISH in 1 case. The advantages of this procedure are the availability of direct information about the genetic constitution of individual hematopoietic stem cells, the independence from the availability of numerical and selected structural (FISH-suitable) abnormalities, no interference of chromosome quality with immunostaining procedures, and the option to study very infrequent hematopoietic populations, even different subpopulations of CD34+ cells. Methodologic problems in the establishment of this method are the maintenance of a high viability of progenitors throughout the preparation and sorting process. The most significant negative factors are time after aspiration, freezing, and thawing and the use of cytotoxic substances in washing solutions.

Together with the published data showing frequent clonal chromosomal abnormalities in different cell lineages, our data suggest that leukemia-initiating as well as progression-associated genetic events might regularly occur at the stem cell level, irrespective of the phenotypic makeup of the bulk population of leukemic blasts. However, the biology of hematopoietic progenitors in acute promyelocytic leukemia may be different.4 One explanation for our findings could be that the genetic defect itself determines the differentiation program of the affected cell clone, which contrasts with the opinion that the leukemic phenotype is a reflection of the level of the hematopoietic hierarchy at which the genetic defect occurs. The high mitotic index shows that CD34+ cells can be stimulated to intensive proliferation. This confirms the functional data of the leukemogenicity of sorted cells from a patient with AML M1 in SCID mice.58 Transformation at the level of committed myeloid progenitors may be the exception rather than the rule. These findings have implications for the actual studies on monitoring of residual disease by multiparameter flow cytometry. Immunophenotypically normal stem cells may represent a reservoir for relapse-inducing leukemic daughter cells. The data also affect strategies for isolation of pluripotent progenitors as part of intensified, myeloablative postremission therapy. The clinical data of autologous bone marrow or peripheral blood progenitor cell retransfusion were not indicative for a significant improvement over conventional chemotherapy.68 Gene marker studies by Brenner et al69 identified residual leukemic cells in the retransfused stem cell product as one source for relapse, but did not show the immunophenotype of the residual blasts. Identification and isolation of the transformed progenitor cell is a prerequisite for targeted gene therapy. We have not identified the immunophenotype of the leukemic stem cell, but have closed the circle around the niche in which it hides.

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