Residual normal, highly proliferative progenitors can be isolated from the CD34+/33− fraction of AML with a more differentiated phenotype (CD33+)

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Since in AML differentiation is abnormal but not absent, a hierarchy of stem cells, progenitor cells and more differentiated cells is postulated. The leukemic stem cell might also be characterized by the expression of CD34 and the absence of differentiation markers. Bone marrow samples of 33 AML patients, including 10 patients both at presentation and after relapse, were double labeled for CD34 and CD33. In 14/33 AML less than 1% of the labeled cells were found in the CD34+/33− fraction. After relapse a certain shift towards a more primitive phenotype was observed, but in 4/5 relapsed AML the CD34+/33− fraction remained below 1%. Single cells from the different subfractions were cultured and showed heterogeneous colony and colony growth in both the CD34−/33− and CD34+/33− fraction. More colonies were observed in the CD34+/33− fraction. In AML with a more ‘mature’ phenotype (low number of CD34+/CD33− cells), highly proliferative myeloid, erythroid and mixed colonies could be cloned exclusively from this small CD34+/33− fraction. In five patients with numerical chromosomal abnormalities all these highly proliferative colonies appeared disomic using in situ hybridization (ISH) with centromeric probes. Based on these data we conclude that the CD34+/33− cell fraction in AML with a more mature immunophenotype (small fraction of cells CD34+/33−) comprise residual normal progenitors, while no primitive leukemic progenitors could be identified.

Keywords: AML; CPU-GM; CD34; CD33; ISH

Introduction

In normal hematopoiesis the CD34+ population comprises the clonogenic cells, ranging from stem cells to lineage committed progenitors. Primitive hematopoietic cells express CD34 and no lineage markers like CD33.¹ The existence of a very primitive stem cell, with both hematopoietic potential and the possibility of stromal cell formation has been described to be a cell that phenotypically expresses CD34 and no lineage markers.² During lineage commitment markers of the different pathways are co-expressed with CD34, while during further hematopoietic development both the proliferative capacity and CD34 expression are gradually lost.

AML is a clonal disease, which originates in either a pluripotent stem cell or a lineage committed progenitor cell, for example a progenitor mainly restricted to the granulocytic pathway.³ This heterogeneity with respect to the pattern of differentiative expression suggests that at least in part of the AML the leukemic stem cell has features of a more differentiated cell. Since the leukemic population regenerates, the cells must have acquired self-renewal capacity.

In normal bone marrow myeloid differentiation is characterized by the appearance of CD33. In AML CD33 expression is observed in about 90% of the patients.⁴ However, since in AML hierarchy in development might be partially retained, the more primitive progenitor cells might also lack CD33 expression. To answer this question CD34 and CD33 expression in 33 AML patients was evaluated. Mononuclear cells were double stained with CD34-FITC and CD33-PE and single cells were sorted and cloned from the three different subfractions.

The single cell sorting method allows analysis of the offspring of individual colonies. Potential inhibitory effects of accessory cells could be excluded. Furthermore, it appeared that cells could be sorted from very small subpopulations (less than 1 : 1000) in leukemias that showed almost exclusively CD33+ cells. Weekly evaluation of the single wells gave information on the clonogenic capacity and the duration of proliferation. In patients with a numerical chromosomal aberration (trisomy or monosomy) the individual colonies were checked for their origin by in situ hybridization (ISH).

Sorting and cloning from the different subpopulations revealed that in AML with a more mature immunophenotype, as referred to the hierarchy in normal hematopoiesis, the leukemic clonogenic cells were observed in both the CD34−/33+ and somewhat more frequently in the CD34+/33+ population. In the cells sorted from the small CD34+/33− subpopulation residual normal colonies were found. This became apparent in the presence of SCF, which had a limited effect on leukemic growth but resulted in large colonies (5000–10000 cells) derived from highly proliferative progenitors with multilineage potential. The normal origin of the colonies was supported by ISH in a number of AML with numerical chromosomal aberrations.

Patients and methods

AML bone marrow samples

Bone marrow aspirates were obtained from 33 newly diagnosed and untreated patients. From 10 of these patients bone marrow was also collected after relapse and before retreatment. Aspirates were diluted in buffered acid-citrate dextrose (pH 7.0). Nucleated cells were isolated from the aspirate by Ficoll (Sigma, St Louis, MO, USA) 1.077 g/ml density centrifugation. After washing twice with glucose-phosphate-buffered saline the interphase cells were resuspended in Iscove’s medium (Flow Laboratories, Irvine, UK) with 10% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 15 U/ml preservative-free heparin at a cell concentration of about 40 × 10⁶ cells/ml. This cell suspension was diluted 1 : 1 with medium containing 20% DMSO (v/v) on ice. The cells were cryopreserved (Kryo 10; PlanerBiomed, Sunbury, UK) in 2-ml freezing tubes and stored at −198°C.

Thawing and labeling of bone marrow cells

The vials were thawed rapidly in a 37°C waterbath, and diluted in FBS, containing 0.02 mg/ml DNAsε (deoxy-
Flow cytometric single cell sorting

The labeled cells were sorted by a Coulter Epics Elite Flow cytometer (Coulter, Hialeah, FL, USA) equipped with an auto­
clone unit. Cells were excited with a single 488 nm Argon­
cytometer (Coulter, Hialeah, FL, USA) equipped with an auto­
ble green and red fluorescent beads (Calibrite; Becton
Dickinson, San Jose, CA, USA) were used.

Culturing conditions of the single cells in 96-well plates

Single cells were sorted in round-bottom 96-well plates (Costar 3799, Cambridge, MA, USA), prefilled with 74 µl liquid medium, consisting of Iscove's, penicillin 50 U/ml, strepto­
icin 50 µg/ml (Flow Laboratories) with 20% FBS, 5% deion­
ized bovine serum albumin (BSA), 0.3 mg/ml human trans­
lacein and 5 x 10^{-3} M 2-β-mercaptoethanol. Recombinant growth factors were added, G-CSF (20 ng/ml) and SCF
(25 ng/ml) (a gift from Amgen, Thousand Oaks, CA, USA), IL-3
(50 ng/ml), GM-CSF (20 ng/ml) and erythropoietin (1.5 U/ml)
(Eprex, Cilag BV, Belgium). The plates were placed in an incub­
bator, 37°C, 5% CO₂, in a fully humidified atmosphere.

Evaluation of growth and differentiation in the single wells

The cells in each individual well were counted under an inverted microscope. Due to the roundbottom configuration of the wells, the cells grew in the center of the well. One day after sorting single cells were found in about 90% of the wells. Counting was started at day 3 and repeated weekly until the colonies were fully differentiated, stopped growing or became pycnotic. Apart from the number of cells, the configuration of the cells while differentiating was noted as granulocytic, monocytic, erythroid and mixtures of these. To check for dif­
ferentiation the colonies from single wells were split and lab­
elled with Leucogate (CD45-FITC/CD14-PE)(BD) or CD15­
FITC to differentiate monocytes from myelocytes and glyco­
phorin to identify erythrocytes. Also colonies were cytocen­
fuged on slides and stained with May-Grunwald-Giemsa to check differentiation.

ISH for detection of numerical chromosome aberrations

Cells picked from single wells were cytocentrifuged on
organosilane coated slides. Interphase cytogenetics on single
cell colonies was applied in seven AML patients with known
numerical chromosome aberrations to check for the leukemic
origin of the colonies.6-7

Fixation: The slides were heated for 30 min at 80°C. A
proteolytic digestion with pepsin (P-7000; Sigma) was done at
an optimized concentration of 100 µg/ml in 0.01 M HCI for
15 min at 37°C. Next, the slides were washed twice in 0.01 M
HCl, dehydrated with increasing ethanol concentrations (70–
90–100%) and postfixed in 1% formaldehyde in PBS for 5
min. Then the slides were washed in PBS (five times) and H₂O₂ (five times) and finally dehydrated again with increasing etha­
ol concentrations.

DNA probes and labeling: The different probes used in
this study were DBZZ for detection of chromosome 8,
pHUB9 for chromosome 9, pD10.1 for chromosome 106 and
pH17B for chromosome 17.11 The DNA probes were
hybridized to the cell preparations as described before9 in
60% formamide, 2 x SSC and 10% dextran sulphate at a probe concentration of 1 ng/µl hybridization mixture. Ten
microliters of the hybridization mixture were applied to the
slides under a coverslip (18 X 18 mm). Denaturation of probe and
target DNA was carried out simultaneously by heating the
slides in a moist chamber to 70°C for 3 min. Hybridization
was then performed overnight at 37°C in a moist chamber.
The coverslips were removed by immersing the slides in 60%
formamide, 2 x SSC, pH 7.0. Next the slides were washed
three times for 5 min in the same buffer at 42°C and sub­
sequently three times for 3 min in 2 x SSC, pH 7.0 at 42°C.
The hybridized biotinylated probe was detected by horse­
radish peroxidase (HRP)-conjugated avidin (Dakopatts,
Glostrup, Denmark) in 4 x SSC, 0.05% Tween 20 with 0.5%
blocking milk (Boehringer Mannheim) and, if necessary,
amplified with biotin-labeled goat-anti-avidin (Vector, Bur­
ingame, CA, USA), followed by a second layer of HRP-conju­
gated avidin. All immunocytochemical steps were performed
for 30 min at 37°C. Finally the DNA probe was visualized with
0.5 mg/ml 3,3-diaminobenzidintetrahydrochloride (DAB; Sigma), 0.65% imidazole (Merck, Darmstadt, FRG), 0.015%
H₂O₂ (Merck), at pH 7.8 in PBS. Finally the cells were stained
with mayers hematein.

Evaluation of ISH results: Using the described ISH pro­
cedure at least 90% of the interphase nuclei showed one, two
or three distinct ISH signals in all preparations. Evaluation cri­
teria were as described before.12 If possible at least 100 nuclei
per slide were counted. The sensitivity of interphase cytogen­
etics in the detection of the targeted chromosomal abnor­
malities in case of monosomy varied by 5–10%, depending on
the efficiency of the ISH procedure or the co-localization of
two ISH signals. The detection of three ISH signals in normal
cells is less than 1%.

Results

Expression of CD34 and CD33 in AML

Cells of 33 untreated AML patients were double-labeled
with CD34-FITC and CD33-PE. In the samples the percentage of
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blast cells, detected morphologically on May–Gruenwald–
Giemsa stained cytospin slides, varied between 30 and 95%.
Overall, 25% of the cells expressed CD34 (range <0.05 to
92%) and 58% CD33 (range 0.6 to 96%).

The distribution of labeled cells between the subfractions
CD34+/33+, CD34+/33+ and CD34+/33–, for all individual
patients is presented in Figure 1. The individual samples
are sorted for the frequency of CD34+/33– and as second
parameter CD34+/33+. The last bar represents the mean dis-
tribution found in normal bone marrow mononuclear cells
(BM) (Ficoll 1.077 g/ml, n = 4): 76% (±4.2%) of the cells
were CD34-/33+, 13% (±3.3%) CD34+/33+ and 11%  
(±0.6%) CD34+/33–.

Trends in the distribution of CD34 and CD33 were rec-
ognized in AML for different FAB types. Representative plots
are presented in Figure 2a (untreated). In the only patient
tested with AML-M1 and in all AML-M2 cases a high percent-
age of CD34+ cells were found, an average of 72%. The most
primitive phenotype (if related to normal hematopoiesis)
CD34+/33–, was observed in 16% of the cells. In AML-M3
a comparable distribution was observed, 69% CD34+ and
20% CD34+/33+. A more ‘differentiated’ phenotype was
seen in FAB-M4, 88% of the cells were CD34-/33+. Furth-
erremore, the cells that expressed CD34 were mainly
CD34+/33+. This distribution was very consistently found in
all M4 samples tested. In AML-M4 57% and AML-M5 61%
of the cells were CD34+/33+, a substantial part was
CD34+/33+, while as in AML-M4 almost no CD34+/33–
cells were detected.

Changes in CD34 and CD33 expression during
relapse

In 10 patients bone marrow aspirates were tested both before
treatment and after relapse, to see whether relapse resulted in
a higher percentage of cells with a more primitive phenotype
(CD34+/33–, CD34+/33+) (Figure 3). The samples are
sorted for the frequency of CD34-/33+ and CD34+/33+
cells in the untreated patients. This series included patients
with early and late relapses. The overall percentage of CD34+
cells increased from 50 to 59%. In 8/10 patients the relative
number of CD34+ cells increased after relapse, but in 4/8 the
increase was less than 10%. In two cases of AML a decrease
of CD34+ cells of 14 and 17% was observed. Examples of
fluorescence plots before and after relapse in some cases
showed a clear shift to a more primitive immunophenotype
(Figure 2a: untreated; 2b relapse).

In vitro growth of single-sorted cells from the different
subfractions

Sorted single cells were cloned in 96-well plates, one plate for
each fraction. To analyze in vitro growth recombinant growth
factors IL-3, GM-CSF, G-CSF, erythropoietin and SCF were
added. Both the plating efficiency (number of wells showing
growth) and the size of the aggregates (number of cells/well)
was highly variable, similar to observations of AML growth in
conventional semi-solid cultures. To quantify growth charac-
teristics between the different phenotypic subfractions, the
number of AML samples showing cluster growth (2-50
cells/well) and colony growth (≥50 cells/well) for each
subfraction is given in Figure 4a and b, respectively. The
actual plating efficiency (number of wells showing growth) in
the different subfractions is presented in Table 1. Mean values,
minimum and maximum numbers are given. As expected a
large variation was observed.

In contrast to normal bone marrow growth was also seen
in the CD34-/33+ fraction. In 75% of the AML samples clus-
ters and in 33% colonies could be grown. In the CD34+/33+
fraction the frequency of cluster and colony growth was some-
what higher, 91 and 44%, respectively. Most cluster and col-
yony growth was observed in the CD34+/33– fraction, 96 and
75%, respectively. The mean number of clusters and colonies
showed a trend to increase in the more primitive phenotypic
subfractions, 10.5 clusters and 3 colonies in the CD34+/33+
plate, 21.9 and 4.7 in the CD34+/33+ fraction and 19.6 and
6.6 in the CD34+/33– fraction. This trend was observed also
in normal bone marrow, 24 clusters and 15 colonies in the
CD34+/33+ fraction and 27 and 22 in the CD34+/33–
fraction.

Figure 1  Distribution of the CD34 and CD33 positively labeled AML cells among the different fractions. Sorting of the samples for increasing
size of the CD34+/33– and secondly CD34+/33+ fraction. The right hand bar represents the distribution in normal bone marrow (BM) (n = 4
different donors)
Figure 2 Representative examples of CD34 and CD33 distribution in the different FAB subtypes at presentation (untreated) and after relapse. Although some shift can be observed, in the samples with a small CD34+/33- fraction, this fraction remains small after relapse.

Apart from clusters and colonies, aggregates of >500 cells were counted separately. Overall, these larger colonies were observed more frequently in the more primitive subfractions, 43% in the CD34+/33- fraction, 25% in the CD34+/33+ and only 8% in the CD34-/33+ fraction (Figure 4c). In some AML with a substantial CD34+/33- fraction, colonies between 500 and 5000 cells were observed. In AML with a small CD34+/33- fraction (<5% of the cells in the CD34+/33- fraction), large colonies of more than 5000 cells were cultured (10/28 samples tested). Since these colonies included mixed type and erythroid colonies it appeared suggestive that these colonies originate from residual normal progenitor cells.

**Effect of SCF on colony growth**

The effect of addition of SCF to the combination of the recombinant growth factors IL-3, GM-CSF, G-CSF and erythropoietin was studied in 20 AML samples. In the absence of SCF, colonies were observed in 12 of the 20 samples, increasing to 16/20 after addition of SCF. The number of colonies increased from an average of 2.4 (0-10) to 6 (0-36) (Table 2). Even more obvious was the effect on colonies of >5000 cells/well. Without SCF, in only two patients was such a large colony observed, in the presence of SCF in 10 patients, an average of 1.1 (0-7) colonies of >5000 cells was found.

**ISH on colonies derived from single cells in patients with numerical chromosomal aberrations**

The large colonies of >5000 cells found in the CD34+/33- cell fraction included mixed colonies, both monocytic and myeloid as well as erythroid and myelomonocytic. Differentially cells of the myeloid and monocytic lineage were detected microscopically on slides derived from single colonies by cytocentrifugation and stained with May-Grunwald-Giemsa. The different patterns of differentiation recognized in the unstained colonies were checked in a number of representative colonies using flow cytometry after staining with leucogate (CD45-FITC/CD14-PE) or CD15-FITC in combination with glycophorin-A-FITC and CD14-PE. Thus, monocytic (CD14+/CD15-, erythroid (glycophorin+) and different myeloid colonies could be distinguished.

To provide further support for the normal origin of the large-sized colonies of mixed morphology ISH was performed in five patients with known numerical chromosomal aberrations. The five patients included three patients with a trisomy 8 (all AML-M4), one with a trisomy 9 (AML-M5), and one showing a monosomy 17 (AML-M3). Data on the distribution of the cells in the CD34 and CD33-labeled fractions and growth in single cell assay are presented in Table 3. All five patients had a small subfraction of CD34+/33- cells, varying between 0.1 and 2.3%. As shown in Table 4 classical cytogenetics showed normal metaphases in 0-38% of the evaluated cases. With ISH, performed on non-selected but viable cells, the percent of cells with an aberrant phenotype was between 14 and 90%.

As summarized in Table 4, the large (>5000 cells) single cell derived colonies grown from the CD34+/33- fraction were all disomic. In 3/5 of the patients a sufficient number of cells could be sorted from the CD34+/33- fraction for ISH. In comparison with the overall population this fraction was enriched for disomic cells, varying between 70 and 100% (Table 4). Additionally, single cell derived colonies and clusters from two patients with numerical chromosomal aberrations, a patient with an AML-M7 and a monosomy 7 and a patient with an AML-M2 and a trisomy 10 were tested. The CD34+/33- fraction in these two patients was much larger, 20 and 28%, respectively. In the patient with the monosomy 7 colonies up to 200 cells were cultured, showing monosomy, in the patient with the trisomy 10 only clusters were found, showing trisomic cells.

**Discussion**

The presence of primitive and more differentiated progenitor cells was studied in 33 AML patients by double labeling with
CD34 and CD33. CD34 and CD33 was expressed on all tested samples, but the percentage of labeled cells and the distribution between the different subfractions was highly variable; this in contrast with normal bone marrow which showed a rather consistent distribution.

A certain correlation between FAB subtype and the distribution of CD34 and CD33 was found. In AML M1, M2 and part of the M3 a substantial percentage of the cells were CD34+/33−, the most primitive phenotype if compared with normal bone marrow. In AML M4, M5 and 3S M3 samples the majority of the cells were CD33+. In these AML samples only a small fraction of the cells showed the most primitive CD34+/33− phenotype. Ten patients were also studied at relapse, to see whether a substantial shift towards a more primitive phenotype had developed. According to literature data the percentage of CD34+ cells should be higher after relapse. Thomas et al. concluded that AML tended to relapse with a less differentiated phenotype. An increase in CD34+ cells was observed in 8/10, but in only 4/8 samples was the increase more than 10%. In some individual samples a substantial shift to the more primitive phenotype was observed, for example in the patient with AML M1 (see Figure 2). However, in AML patients with a small CD34+/33− fraction (less than 1%) no increase was observed after relapse. These observations and data from the literature suggest that an only limited hierarchy in immunophenotype is present in AML.

The next question to address was whether this hierarchy also implicated that cells from the more primitive phenotypic subfractions were progenitors with a higher proliferative capacity and possibly self-renewal capacity. The results of single cell sorting and cloning from the different phenotypic subfractions showed that in general the cloning efficiency was highly variable for each individual AML sample. Cluster growth predominated, which is also observed in conventional semi-solid cultures. Cluster and colony growth was more or less comparable in the CD34+/33− and CD34+/33+ fraction. This is in contrast to normal bone marrow showing no growth in the CD34−/33+ fraction. In the most primitive CD34+/33+ fraction both the number and the frequency of colony (not cluster) growth was higher. The colonies in this fraction often were very large colonies of 5000–10,000 cells and of myeloid, erythroid and mixed morphology. Interestingly, such highly proliferative colonies were found exclusively in AML that showed a very small CD34+/33− subfraction. The growth of these large colonies was sustained for up to 4–6 weeks. In contrast the leukemic clusters and colonies stopped proliferating and became pycnotic after 1–2 weeks. To further analyze the origin of these single-cell derived highly proliferative colonies, ISH was applied in five patients showing numerical chromosomal aberrations in their leukemic cells. All these highly proliferative colonies were disomic. The size, duration of proliferation, mixed morphology and the disomy in the offspring support the idea that these colonies are residual normal colonies. In two AML patients with numerical chromosomal aberrations and a larger CD34+/33− subfraction (20 and 28%, corresponding to a more primitive immunophenotype), both the clusters and colonies did show the numerical aberration. In these samples the colony size did not exceed 200 cells, the colonies were monomorphic in their differentiation and stopped proliferating after 7–10 days.

The high proliferative capacity of the progenitors cultured in more mature AML from the CD34+/33− fraction became apparent after the addition of SCF to the culture medium with IL-3, GM-CSF, G-CSF and erythropoietin. Using this combination of growth factors the colonies reached a size of 5000–10,000 cells. It has to be emphasized that SCF also increased the leukemic colonies and clusters in about half the AML samples. Thus the potentiating effect of SCF is not specific for the normal cell population.

The presence of residual normal colonies in AML bone marrow and especially the progenitors that showed a high proliferative capacity in the presence of SCF has implications for studying the effect of SCF in vitro. For example, Ikeda et al described the effect of SCF on AML bone marrow samples cultured in liquid. They describe that SCF promoted prolonged growth of AML and differentiation into various cell lineages. They concluded that SCF changes phenotypic commitment. We postulate that their cultures contained residual normal cells that were quantitatively low, but after addition of SCF started to proliferate substantially. Since in our study a single cell culturing system was used, inhibitory effects of the AML cells on the growth of residual normal progenitors could be excluded. Such effects have been described, for example in AML producing TNFα.

Residual normal colonies have been observed in full blown AML bone marrow samples before. Bernstein et al found in
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3/4 AML patients immature and non-clonal (using G6PD as a marker) cells in the CD34+/33− fraction after culturing in a long-term culturing system. The cells of the colonies in the fourth patient were clonal. Their findings are confirmed in our study. The single cell system with prolonged follow-up for 4–6 weeks showed similar results as their long-term culturing system. In addition, we showed the finding of residual normal progenitors to be correlated with the immunophenotype of the leukemia. In AML with a more primitive immunophenotype no highly proliferative colonies were cultured in the presence of SCF. Residual normal progenitors in the CD34+/33− fraction do not exclude the presence of leukemic (clonogenic) cells in that fraction. For that reason ISH was performed also on directly sorted cells from the CD34+/33− population in patients with a more differentiated phenotype. In 2/5 the fraction was too small to sort enough cells for ISH. In one patient all cells from the CD34+/33− fraction were disomic, while in two patients cells with the numerical aberration were also detected. Further study with more sensitive and better discriminating AML markers such as specific translocations (eg t(15;17), t(8;21)) will probably demonstrate the purity of the subfraction. An explanation for admixture of the CD34+/33− fraction with leukemic cells can be a technical limitation, the low frequency (1 : 1000–10 000) of sorted cells increases the change of admixture with cells not in the sorting window. Furthermore, a negative selection for the CD33 marker holds the risk of admixture of incompletely labeled cells.

The data from this study cannot exclude leukemic stem cells to be present at a low frequency in the CD34+/33− fraction even in AML with a more differentiated phenotype. It may be

Figure 4 The percentage of AML showing growth of clusters (a) and colonies (b) in the phenotypic subtractions: CD34−/33+, CD34+/33+ and CD34+/33−. (c) Percentage of AML that showed large colonies (>500 cells) and colonies that are considered to be highly proliferative, residual normal colonies.
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Table 1 Growth in the phenotypic subfractions in single cell assay in 32 AML patients and normal bone marrow

<table>
<thead>
<tr>
<th>Cells/Well</th>
<th>CD34—/33+</th>
<th>CD34+/33+</th>
<th>CD34+/33—</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;2 Cells/Well</td>
<td>&gt;50 Cells/Well</td>
<td>&gt;500 Cells/Well</td>
<td>&gt;2 Cells/Well</td>
</tr>
<tr>
<td>AML M2</td>
<td>10.2 (0-69)</td>
<td>4.5 (0-43)</td>
<td>2.8 (0-28)</td>
</tr>
<tr>
<td>AML M3</td>
<td>8 (0-16)</td>
<td>2 (0-4)</td>
<td>0</td>
</tr>
<tr>
<td>AML M4</td>
<td>14 (0-55)</td>
<td>2.1 (0-17)</td>
<td>0</td>
</tr>
<tr>
<td>AML M4e</td>
<td>8 (0-23)</td>
<td>3 (0-9)</td>
<td>0.3 (0-1)</td>
</tr>
<tr>
<td>AML M5</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>AML overall</td>
<td>10.5 (0-69)</td>
<td>3 (0-43)</td>
<td>1.1 (0-28)</td>
</tr>
<tr>
<td>BM (n = 4)</td>
<td>0 (0-1)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Cells from the phenotypic subfractions after labeling with CD33 and CD34 were single cell sorted and cloned. The mean number of wells (minimum and maximum) that contain clusters (>2 cells/well), colonies (>50 cells/well) and large colonies (>500 cells/well) are shown.

Table 2 Effect of the addition of SCF to single cell cultures from AML patients

<table>
<thead>
<tr>
<th>Cells/Well</th>
<th>&gt;2 Cells/Well</th>
<th>&gt;10 Cells/Well</th>
<th>&gt;50 Cells/Well</th>
<th>&gt;500 Cells/Well</th>
<th>&gt;5000 Cells/Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>No SCF</td>
<td>11.8 (0-41)</td>
<td>8.2 (0-35)</td>
<td>2.4 (0-10)</td>
<td>0.7 (0-4)</td>
<td>0.1 (0-1)</td>
</tr>
<tr>
<td>SCF</td>
<td>18.9 (0-53)</td>
<td>12.6 (0-48)</td>
<td>6 (0-36)</td>
<td>3.1 (0-23)</td>
<td>1.1 (0-7)</td>
</tr>
</tbody>
</table>

Mean number of wells showing clusters and colonies (between brackets minimum and maximum number observed) after stimulation with GM-CSF + G-CSF + IL-3 + Epo and after addition of SCF to this combination. Results of the CD34+/33— fraction that included residual normal colonies.

Table 3 Distribution of the CD34 and CD33 labeled cells (size of the fractions) and growth in single cells assay (percentage of wells with >10/50/500 cells) in five patients with known numerical chromosomal aberrations, informative for ISH analysis

<table>
<thead>
<tr>
<th>CD34—/33+ fraction</th>
<th>CD34+/33+ fraction</th>
<th>CD34+/33— fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size %</td>
<td>Growth (% of wells)</td>
<td>Size %</td>
</tr>
<tr>
<td>&gt;10</td>
<td>&gt;50</td>
<td>&gt;10</td>
</tr>
<tr>
<td>1</td>
<td>59</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>99.5</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done

that these leukemic stem cells do not proliferate under the given in vitro circumstances. This is also suggested by Lapidot et al who transplanted cells from the phenotypically most primitive fraction (CD34+/38—) into SCID mice and found initiation of human AML almost exclusively if cells from this fraction were used. Human AML were transplanted to SCID mice from different, also more differentiated AML. However, the CD34+/38— cells were used only in an AML-M1, an example of a leukemia with a more primitive phenotype. Transplantation of CD34+/38— cells from an AML with a more differentiated phenotype has not been described. The practical implication is the usefulness of antiCD33 in AML with a more mature immunophenotype to purge leukemic cells. This approach has been applied in vitro in transplants for autologous reinfusion17 and also in vivo using 131I-labeled anti-CD33.18 It is too early to prove the value of this approach.

In conclusion, this study shows that in AML a limited hierarchy in phenotypic make-up exists. Leukemia cluster and colony growth was observed in all phenotypic subfractions, also in the CD34—/33— fraction showing no colony growth in normal bone marrow. Most colony growth was seen in the CD34+/33— population. However, the large colonies cultured from the very small CD34+/33— cell fraction in AML with a more mature immunophenotype most probably are residual normal colonies. In the presence of SCF and after prolonged in vitro culturing these highly proliferative colonies...
became apparent. All the highly proliferative and prolonged proliferating colonies were disomic in patients with numerical chromosomal aberrations in their AML. No definitive answer could be given on the purity of the CD34+/33− cells as residual normal progenitors or the presence of leukemic stem cells, that may be non-clonogenic in the culturing system used. Sorting and cloning in SCID mice of cells from this CD34+/33− fraction in CD33+ AML has to be performed to answer this question.

References

18 Jurcic JG et al. 131-I-labeled anti-CD33 (131-I-M195) may prolong disease-free survival (DFS) in relapsed acute promyelocytic leukemia (APL) after remission induction with all-transretinoic acid (RA). Blood 1993; 82: 193a (abstr.).