General Introduction

Part of it has been adapted from:
New Approaches for the Detection of Minimal Residual Disease in Acute Myeloid Leukemia

A.v. Rhenen *
B. Moshaver *
G.J. Ossenkoppele
G.J. Schuurhuis

* Both authors (A.v. Rhenen and B. Moshaver) contributed equally to this manuscript.
1. GENERAL: HEMATOPOIESIS AND LEUKEMIA

1.1 Normal hematopoiesis and hematopoietic stem cells

Blood cell formation occurs in the yolk sac in the first weeks of gestation. From 6 weeks until the 7th month of fetal life, hematopoiesis takes place in the liver and spleen, after which hematopoiesis is transferred to the bone marrow, which is the only source of blood cells during adult life under healthy conditions. All blood cells are derived from hematopoietic stem cells (HSC), being defined by its ability to reconstitute hematopoiesis in serial bone marrow transplantations in mice who underwent myeloablative therapy. HSCs maintain hematopoiesis by the capacity of both self-renewal and of differentiation into multilineage committed progenitor cells, either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP) (1, 2). These cells then give rise to more differentiated progenitors and ultimately, they will mature towards unilineage committed progenitors for monocytes, granulocytes, erythrocytes, platelets and natural killer cells, B- and T-lymphocytes (3, 4). The process of proliferation and differentiation of HSCs is strictly regulated and balanced in the bone marrow environment, consisting of bone marrow stromal cells (BMSCs), such as macrophages, fibroblasts, adipocytes and endothelial cells, cytokines and the extracellular matrix. The relation of hematopoietic cells with their environment is highly dynamic allowing preservation of stem cells in the so-called “stem cell niche” as well as expansion and differentiation on demand (5, 6). Although HSCs were previously thought to be resting cells within the stem cell niche, recent evidence from mouse experiments show that 8-10% of HSC randomly enter the cell cycle every day, with all HSCs entering the cell cycle in 1-3 months (7).

2. ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a clonal expansion of immature hematopoietic progenitor cells, being caused by somatic mutations causing a disturbance in differentiation. In general, only multiple genetic changes will result in the development of AML. The incidence of AML is 3.7 and 2-3 per 100,000 men and women per year in the United States and Western Europe, respectively (8-10). The disease affects both children and adults.
2.1 Diagnosis
Diagnosis of AML in BM and PB samples of patients is based on morphologic, immunophenotypic, and cytogenetic features. Thereby, AML can be classified according to the French-American-British (FAB) and the World Health Organization (WHO) classifications.

2.1.1 Morphology
May-Grunwald-Giemsa (MGG) staining is being used to visualize the morphology of the different type cells in patients specimens. AML is usually characterized by hypercellularity with an accumulation of myeloid blasts in BM or PB with displacement of normal erythropoietic and myeloid progenitor cells as well as disappearance of megakaryocytes (11). In the FAB classification, AML is subdivided in 8 types on bases of morphological and cytochemical criteria: AML-M0 to AML-M7 (12, 13) (table 1).

2.1.2 Immunophenotyping
Immunophenotype analysis has a central role in distinguishing between minimally differentiated AML and acute lymphoblastic leukemia (ALL). Immunophenotyping performed by flow cytometry concerns the protein expression on the surface and in the cytoplasm of the blast population. Myeloid immature cells are characterized by low side-scatter (SSC), low expression of CD45 (leukocyte marker), expression of CD34 and/or CD117, and the expression of lineage specific markers CD13, CD33 and myeloperoxidase (MPO). Multiple markers analysis helps to identify phenotypes associated with particular cytogenetic aberrations, or may help to identify immunophenotypic abnormalities that are useful in monitoring patients for the presence of minimal residual disease (MRD) (14, 15).

2.1.3 Cytogenetic aberrations
Cytogenetic studies are important for the sub-classification of hematopoietic malignancies. Approximately 50% of patients with de novo AML show chromosomal aberrancies. Some of these rearrangements coincide largely or completely with certain FAB classes: t(8;21) (q22;q22) for FAB M2 (AML1/ETO), t(15;17)(q22;q11-12) [PML/RARα] for FAB M3 and variants, t(16;16)(p13;q22) [CBFβ/MYH11] for FAB M4, and t(9;11)(p22;q12) [MLLT3-MLL] for M4eo. However, most of the chromosomal abnormalities add to the FAB-classification. Therefore, the WHO has recently developed a new classification of AML in which information on chromosome analysis is now being incorporated (table 1). In addition, information on chromosomal aberrancies provides information on the prognosis of these patients, thereby allowing patient tailored therapy (18).

Table 1  The WHO 2008 classification system for AML

<table>
<thead>
<tr>
<th>WHO classification ≥20% blasts</th>
<th>FAB classification ≥30% blasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML with recurrent cytogenetic translocations</td>
<td>Particularly in M2</td>
</tr>
<tr>
<td>AML t(8;21)(q22;q22) [AML1/ETO]</td>
<td>M3 and variants</td>
</tr>
<tr>
<td>AML t(15;17)(q22;q11-12) [PML/RARα]</td>
<td>M4eo</td>
</tr>
<tr>
<td>AML inv(16)(p13;q22) or t(16;16)(p13;q22) [CBFβ/MYH11]</td>
<td>M7</td>
</tr>
<tr>
<td>AML t(9;11)(p22;q12) [MLLT3-MLL]</td>
<td></td>
</tr>
<tr>
<td>AML t(6;9)(p23;q34) [DEK-NUP214]</td>
<td></td>
</tr>
<tr>
<td>AML inv(3)(q21q26.2) or t(3;3)(q21;q26.2) [RPN1-EVI1]</td>
<td></td>
</tr>
<tr>
<td>AML (megakaryoblastic) t(1;22)(p13;q13) [RMB15-MKL1]</td>
<td></td>
</tr>
<tr>
<td>AML with mutated NPM1</td>
<td></td>
</tr>
<tr>
<td>AML with mutated CEBPA</td>
<td></td>
</tr>
<tr>
<td>AML with MDS-related changes</td>
<td></td>
</tr>
<tr>
<td>Therapy-related myeloid neoplasms</td>
<td></td>
</tr>
<tr>
<td>AML not otherwise specified</td>
<td></td>
</tr>
<tr>
<td>AML with minimally differentiation</td>
<td>M0</td>
</tr>
<tr>
<td>AML without maturation</td>
<td>M1</td>
</tr>
<tr>
<td>AML with maturation</td>
<td>M2</td>
</tr>
<tr>
<td>AML myelomonocytic</td>
<td>M4</td>
</tr>
<tr>
<td>AML monocytic and monocytic</td>
<td>M5</td>
</tr>
<tr>
<td>AML erythroid</td>
<td>M6</td>
</tr>
<tr>
<td>AML megakaryoblastic</td>
<td>M7</td>
</tr>
<tr>
<td>AML basophilic</td>
<td></td>
</tr>
<tr>
<td>AML panmyelosis with myelofibrosis</td>
<td></td>
</tr>
</tbody>
</table>

AML: acute myeloid leukemia
FAB: French-American-British
MDS: myelodysplastic syndrome
in first remission for intermediate and/or high-risk patients with an HLA-matched sibling or a matched-unrelated donor. Patients generally receive immunosuppressive chemotherapy in combination with total body irradiation (TBI). Although this strategy carries a high risk on short-term mortality and long-term morbidity as a result of graft versus host disease (GVHD), studies have been reported an improved overall survival for the patients with poor/high risk cytogenetics who underwent an allogeneic hematopoietic stem cell transplantation (HSCT), compared with those without a donor (22, 23). The reason for that probably lies in the immune graft versus leukemia (GVL) effect, as donor lymphocytes recognize the recipient leukemic cells as foreign with subsequent elimination of leukemic cells. This principle is also being applied in allogeneic transplanted patients with a relapse, in which the infusion of donor T-lymphocyte infusions (DLI) might lead to a second complete remission (24, 25).

Although about 80% of adult patients (<60 years old) achieve complete remission after above described intensive treatment strategies, only 30% to 40% of patients are alive after 5 years after diagnosis, mainly being the result of relapse of the disease. For elderly patients the prognosis is even worse (26). A relapse of the disease is now thought to be caused by the outgrowth of MRD. This concept has been supported by the finding that the detection of MRD after completion of therapy predicts relapse (27). Therefore, MRD detection after induction chemotherapy, might help to decide on which type of consolidation therapy. Currently, this decision is mainly based on molecular classification of AML as well as on the comorbidity score. However, it would be ideal to decide on the basis of the response of the disease to induction chemotherapy in order to improve efficacy where needed and to circumvent side effects in patients with a low risk for relapse.

2.3 Minimal residual disease
There are different hypotheses in order to explain the persistence of a minimal number of leukemic cells after the completion of chemotherapy treatment. The primary reason for treatment failure in AML is cellular drug resistance, which can be either caused by drug efflux, by inherent expression of apoptosis-resistance proteins or by micro-environment induced expression of apoptosis resistance proteins.

Resistance mediated by increased drug efflux in AML occurs via high expression of P-glycoprotein (Pgp/ABCB1), multidrug resistance–associated protein-1 (MRP1/ABCC1), lung resistance-related protein (LRP) and the breast cancer-resistant protein (BCRP/ABCG2). Especially, high expression of Pgp and BCRP has been
found to predict a poor prognosis (28-30). Moreover, patients with a multidrug resistant profile at diagnosis have higher MRD frequencies after chemotherapy than patients with a more sensitive phenotype (29). High expression of the anti-apoptotic protein Bcl-2 and/or low expression of the pro-apoptotic protein Bax at diagnosis, have been shown to predict poor survival in AML (31). This apoptotic profile is either an inherent characteristic of leukemic cells, but can also be induced by the bone marrow microenvironment. Again, an apoptosis-resistant profile is correlated with higher levels of MRD after chemotherapy (32).

2.3.1 The role of the bone marrow microenvironment in MRD
 Recently the bone marrow microenvironment has been proposed to be a key player in drug resistance in AML (33). Inhibition of chemotherapy-induced apoptosis is generally considered to underlie protection by BMSCs (34). However, both promotion of proliferation and inhibition of spontaneous apoptosis might add to the observed maintenance of leukemic cells by BMSCs (35-38). The latter is of particular interest in view of the reported prognostic significance of spontaneous apoptosis in AML (39). Moreover, in the situation of MRD, after the completion of chemotherapeutic treatments, the process of spontaneous apoptosis is likely to be of importance in the prevention of the outgrowth to relapse (40). The underlying mechanism in protection from apoptosis, is a reciprocal signaling dialogue between tumor cells and the surrounding microenvironment (37). For example, it has been shown that very late antigen-4 (VLA-4)-positive leukemic cells acquire resistance drug-induced apoptosis through the phosphatidylinositol-3-kinase (PI-3K)/AKT/Bcl-2 signaling pathway, which is activated by the interaction of VLA-4 and fibronectin (41). Accordingly, VLA-4 expression was found to predict prognosis of AML patients. Moreover, stromal cells have been found to prevent apoptosis of AML cells by up-regulating anti-apoptotic proteins in leukemic blasts (42).

In addition, discrete cellular spaces within the bone marrow microenvironment, termed “niches”, are thought to provide the mechanical support and extrinsic molecular factors that maintain stem-ness. Osteoblasts were identified as key cellular components, producing Wnt-proteins, thereby linking the concept of activated self-renewal pathways within stem cells and the concept of an inductive stem cell niche. Indeed, the re-plating efficiency of HSC, transduced with fusion proteins encoded by translocations commonly found in AML, was abrogated upon inhibition of γ-catenin, one of the mediating proteins in Wnt signaling (43). In view of the important role of bone marrow microenvironment-induced chemotherapy resistance and maintenance of malignant stem cells, it is important to elucidate the responsible mechanisms. Moreover, it is tempting to hypothesize that exposure of BMSCs to chemotherapy affects its leukemic supportive effect, however this has never been addressed. This is of importance taking into account the known hazardous effect of chemotherapeutics to BMSCs (44). Hopefully, this will lead to potential new therapeutic targets in AML.

2.3.2 MRD detection
 In patients with acute leukemia, detection and quantification of malignant cells after chemotherapy in remission BM provides powerful prognostic information for the identification of patient risk categories. The clinical importance of MRD detection and quantification in childhood ALL is already established and led to a risk-adapted approach in pediatric ALL trials (45, 46). A number of studies have reported on the clinical value of MRD detection in childhood AML (47, 48) and adult AML (27, 49, 50). The obtained knowledge is expected to facilitate the early detection of impending relapse, may result in risk-adapted therapies, and may offer a short-term endpoint to assess the effectiveness of new targeted therapies.

2.3.3 Different approaches in MRD detection
 If the leukemic cell at diagnosis carries an antigenic or molecular marker that distinguishes it from its normal counterpart, this marker can be used after chemotherapy to detect residual malignant cells in BM. This allows the application of two sensitive methods to identify MRD in both ALL and AML: polymerase chain reaction (PCR)–based and multiparameter flow cytometry (MFC) techniques (51). Each method has its advantages and disadvantages which will be discussed in more detail for AML.

2.3.3.1 PCR detection
 Real-time quantitative reverse transcriptase PCR (qRT-PCR) is the most sensitive molecular MRD detection method (cell frequency of $10^{-4}$ to $10^{-5}/-6$) used in recent years in AML (52, 53). It permits absolute quantification of target DNA or mRNA, in contrast to end-point quantification of the more classic PCR method. This technique is based on genetic aberrations such as mutations and fusion genes, occurring in subgroups of AML. The most common rearrangements producing fusion genes in AML are t(8;21), t(15;17), and inv(16)/t(16;16), which occur in a
minority (9% to 19%) of all patients with AML and have a favorable prognosis (54). The corresponding fusion transcripts AML1-ETO, PML-RARA, and CBFbeta-MYH11 can be detected by qRT-PCR at diagnosis and follow-up to identify MRD (54). Other potential targets for PCR-based MRD detection in AML are mutations in FLT3 (55, 56), MLL (57), and NPM1 (58, 59), and over-expression of WT1(60-62), EVI1 (63), and PRAME (64, 65). Recently published targets include c/EBPα (66), BAALC (67) and ETS-related gene (ERG) (67), which have not yet been studied for potential application in MRD detection.

QRT-PCR is the most sensitive molecular MRD detection method used in recent years in AML (52, 53). Although recent and future advances in identifying new AML-associated genetic aberrations eventually may provide such molecular targets for the majority of patients, currently, this technique is only applicable in about 40% of AML patients.

2.3.3.2 Prognostic impact of molecular abnormalities

An increasing number of studies have shown the clinical importance of PCR-based MRD detection in AML. Schnittger et al. showed that quantitative PCR can accurately identify prognostically unfavorable subgroups in overall relatively favorable PML-RARA+, AML1-ETO+, or CBFBM/YH11+ AML subgroups. Lower expression levels at diagnosis correlated with better overall and event-free survival in all 3 leukemia subtypes. Eight patients showed increasing levels of expression during follow-up and all had relapse (68). Other groups demonstrated the prognostic significances of qRT-PCR based MRD detection of the CBFbeta/ MYH11 and PML-RAR alpha fusion transcript in inv(16) AML and acute promyelocytic leukemia (APL), respectively (52, 69). Guerrasio et al. evaluated CBFbeta/MYH111 transcript copy numbers by quantitative real-time PCR assay. In continuous complete remission (CR) patients, the copy number dropped below the detection threshold after the treatment protocol was completed and remained undetectable in subsequent MRD analysis in accordance with results obtained by qualitative RT-PCR. On the contrary, in the all patients who relapsed, the copy number in CR never declined below the detection threshold (52).

2.3.3.3 Immunophenotypic detection

Immunophenotypical analysis of residual leukemic cells using MFC is an attractive approach for MRD detection: it is sensitive (cell frequency of 10^{-3} to 10^{-5}), fast, quantitative, relatively cheap, and, importantly applicable to more than 80% of patients with AML, compared to PCR-based MRD detection only being applicable in about 40% of these patients (27, 49-51, 68, 69). Applying quadruple marker combinations, MFC uses the presence of aberrant expression of markers, usually referred to as leukemia-associated immunophenotypes (LAIPs or LAPs) (27, 51). The pillar of immunophenotypic MRD detection is that LAPs are present on all leukemic cells or on a subset of them but absent or very infrequent on normal hematopoietic progenitors. These LAPs result from cross-lineage antigen expression, antigen over-expression, antigen under-expression, asynchronous antigen expression, and abnormal light scatter patterns (27). Unfortunately, in line with the immunophenotypical heterogeneity of AML cells at diagnosis, the LAPs are usually not expressed on the whole blast cell population (27). This heterogeneity affects the sensitivity of the approach, thereby introducing patient-dependent sensitivity. In addition, the absence of LAPs in part of patients, limits the applicability. Another pitfall is the occurrence of major or minor phenotypic shifts at relapse which potentially might result in false-negativity (27, 70-72). Lastly, background staining of the normal bone marrow is LAP-dependent thereby affecting the specificity (70).

2.3.3.4 Prognostic impact of immunophenotypic abnormalities

Several studies have shown that MRD detection based on MFC analysis provides strong prognostic information in AML after both induction and consolidation therapy (27, 47-51, 68). Venditti et al. showed that MRD detected level using MFC after consolidation therapy strongly predicts relapse in AML patients (50). An MRD level of 3.5 × 10^{-5} cells or higher after consolidation was significantly correlated with intermediate or unfavorable cytogenetic, a multidrug resistance 1 (MDR1) phenotype and short duration of overall and relapse-free survival. Seventy seven% of MRD-positive patients had relapse, compared with 17% of MRD-negative patients. Autologous stem-cell transplantation did not alter the prognostic effect of high MRD levels after consolidation: the relapse rate after transplantation was 70% (50). Feller et al. demonstrated that the MRD% in BM after the first cycle, second cycle and third cycle, as well as in peripheral blood stem cell (PBSC) products strongly correlated with relapse-free survival. At a cutoff level of 1% after the first cycle and median cutoff levels of 0.14% after the second, 0.11% after the third cycle and 0.13% for PBSC products, the relative risk of relapse was a factor 6.1, 3.4, 7.2 and 5.7, respectively, higher for patients in the high MRD group. Also, absolute MRD cell number/ml was highly predictive of the clinical outcome. After the treatment has ended, an increase of MRD% predicted forthcoming relapses, with MRD assessment intervals of < or =3 months (27).
2.3.3.5 Future perspectives in MRD detection
An overview of new approaches for the detection of MRD is shown in figure 1. New approaches include the use of six-color polychromatic flow cytometry (PFC) instead of four-color multiparameter flow cytometry and the use of PB instead of BM for MRD assessments. Importantly, the presence of MRD is expected to be incorporated in risk stratification, thereby guiding treatment strategies.

Figure 1 Overview of new approaches for the detection of minimal residual disease (MRD)

Overview of new approaches for the detection of MRD, showing established relationships (solid lines) and relationships under investigation (dashed lines). Molecular or phenotypic abnormalities at diagnosis are used to detect MRD in bone marrow after chemotherapy. The amount of MRD has an impact on survival of patients.

2.3.3.6 Multi-color flow cytometry
MFC techniques are being optimized by technologic advances in new fluorochromes, novel hardware capable of measuring more fluorescence parameters, and new software tools capable of analyzing, managing and reducing the large and complex data sets. Thus, PFC for the detection of different fluorochromes (using more than four colors) offers new possibilities for MRD detection (73). One of the key issues in MRD monitoring is specificity. Specificity can be increased by including additional markers e.g. exclusion markers (74). The other advantages of PFC would be to combine different four-color LAPs in one newly established composite LAP, thereby reducing the minimally required amount of BM, the number of tubes, costs, and time. Another development is offered by software that enables to merge results obtained from different test tubes allowing to produce a multi-color image even containing more than one antibody with the same fluorochromes (www.infinicyt.com). Specificity may also be increased by using PB, in which the ratio of malignant versus normal stem cells allows better detection of malignant cells. Whether this will be a valuable approach depends on the sensitivity: MRD may be lower in PB compared to BM (75).

2.3.3.7 Multi-center MRD analysis and prospective studies
The ultimate goal of MRD studies is to guide risk-adapted therapy in clinical practice. To this end, consensus in MRD analysis is necessary and guidelines should be published enabling uniformity of all procedures. In The Netherlands and Belgium, a working group, the Dutch/Belgium Task Force for MRD detection in AML, represented by several academic centers in cooperation with the European Society for Clinical Cell Analysis (ESCCA), has been initiated to prepare MRD guidelines. To definitely prove the value of MRD measurements, prospective MRD studies are needed. The Dutch hemato-oncology association, the HOVON, performed the HOVON-42A/SAKK protocol for the treatment of patients with AML who are aged under 60 years. In this multicenter, prospective, randomized study, a prospective study on the value of MRD has been included. The results are currently analyzed including a comparison between molecular and immunophenotypical MRD.

2.3.3.8 MRD as short-term endpoint for efficacy assessment in clinical studies
In recent years, many new targets have been identified that need testing in clinical practice. However, the study designs that are generally used require the accrual of many patients and a long follow-up. Short-term endpoints would be of great importance to evaluate new randomized phase II trials with new treatment modalities. Because MRD reflects the sum of all parameters contributing to quality of response to therapy, it might serve as an early endpoint to assess effectiveness...
of new therapeutic modalities. The correlation of multidrug resistance (29), apoptosis resistance (32), and presence of FLT3-ITD (76) and stem cell frequency (77) at diagnosis with MRD frequencies indicates that such an approach is very promising.

In conclusion, MRD detection by multi-color flow cytometry is technically possible in 80% of AML patients. There is considerable data on the predictive value of MRD in outcome of AML patients, which will allow risk-adapted therapy in AML in the near future. In order to imply multicolor flow detection of MRD on large scale outside clinical trials, standardisation of the technique is of utmost importance. This highlights the ongoing need for easier and even more specific quantification of MRD. Quantification of remaining malignant stem cells might be a promising tool, especially with regard to even more precise prediction of outcome, as there is increasing evidence on the importance of leukemic stem cells (LSCs) in the initiation, but also in relapse of AML.

3. LEUKEMIC STEM CELL

AML is generally regarded as a stem cell disease. Conclusive evidence for the existence of a LSC in AML has come from studies using the NOD/SCID mouse model showing that cells with leukemic characteristics, defined as capacity for self-renewal and engraftment potential, are present in AML (78, 79). These cells were referred to as SCID leukemia initiating cells or SL-IC.

3.1 The origin of the leukemic stem cell

It is likely that leukemia arises through the acquisition of defects in the HSCs. The current concept is that hematopoiesis in leukemia is hierarchically structured, similar to normal hematopoiesis. The concept of tumorigenic LSCs has emerged from findings that only a small subset of leukemic cells is capable of extensive proliferation in vitro and in vivo. The SL-IC were found exclusively in the primitive CD34+CD38- fraction of all patient samples regardless of the lineage markers expressed by the leukemic blasts, the percentage of CD34-positive blasts or FAB subtype (with exception of AML-M3). In addition, cytogenetically aberrant cells have been found in the CD34+CD38- compartment (80, 81). SL-IC were not found in more committed CD34+CD38+ progenitors or CD34-negative cells (82). These studies stress that leukemia initiating transformation and progression associated genetic events occur at the level of these primitive CD34+CD38- cells.

This parallels the hierarchy in normal BM in which a rare population of CD34+CD38- cells having stem cell characteristics (83), supporting the hypothesis that malignant transformation take place in normal HSC (figure 2). However, there is still uncertainty whether the transformation to a LSC occurs in the normal stem cell or the normal progenitor cell. Recent studies in mice models have shown that AML specific oncoproteins can transform both committed progenitors and HSC into LSC (84-87). Lavau et al. demonstrated that occurrence of a mutation of the HSC is not strictly necessary, i.e. mutation of more committed progenitors may also be sufficient (88). Mutations may confer self-renewal properties to progenitors that are normally quiescent and lead to second mutations and a subsequent transformed phenotype. Taking together, cumulative data suggests that LSCs may arise from mutations occurring in either the HSC or committed progenitor compartments, at least in murine models of disease (87, 88).

A similar role for the CD34+CD38- compartment in leukemogenesis has suggested for ALL, chronic myeloid leukemia (CML) and the myelodysplastic syndrome (MDS) (89-91). In the past years many other tumors have been shown to contain small populations with defined stem cell characteristics (the cancer stem cell hypothesis) (92). Also for these tumors is uncertain which cell undergoes malignant transformation.

3.2 LSC as a cause of therapy resistance

There is increasing body of evidence pointing towards the importance of the LSCs in the occurrence of MRD and relapse (figure 3). This might well be explained by proposed shared properties of LSCs with HSCs, such as being relatively quiet and resistant to apoptosis. Indeed, a high stem cell frequency in AML predicts MRD after chemotherapeutic treatment, resulting in poor prognosis. Moreover, the CD34+CD38- cell frequency at diagnosis was found to correlate with survival of patients (77).

Cell intrinsic mechanisms of resistance such as increased expression of the ATP-Binding-Cassette (ABC) transporters genes, being associated with MDR in AML, might play a role in therapy resistance of LSCs (93). There are different proteins which are encoded by ABC transporters genes and function as efflux pump for anti-leukemic drugs. Some of these proteins, such as P-glycoprotein (Pgp), multi drug resistance associated protein (MRP), lung resistance-related protein (LRP), and breast cancer resistance protein (BCRP) are involved in MDR (94-96). Costello et al. has reported that CD34+CD38- leukemia precursors have
Raaijmakers et al. reported that drug extrusion from LSCs in AML patients is mediated by BCRP and additional transporters (99). Other factors are also contributing to drug resistance and relapse. Firstly, because LSCs are quiescent, they do not respond to cell cycle-specific cytotoxic agents used to treat leukemia and so contribute to treatment failure. Secondly, LSCs may undergo mutations and epigenetic changes, further leading to drug resistance and relapse. Thirdly, anti-apoptotic proteins are being described to be highly expressed in non-proliferating leukemic cells, leading to apoptosis-resistance (100). Moreover, both stem cell specific molecular pathways and constitutively non-stem cell specific pathways are proposed to play a role not only in leukemogenesis and therapy-resistance (101). Non-stem cell specific molecular pathways, such as the phosphoinositide 3-kinase (PI3K)/Phosphoatase and TENsin homolog deleted on chromosome 10 (PTEN)/Akt/mammalian target of rapamycin (mTOR) and Raf/MEK/ERK, have been described to be constitutively activated in AML leading to proliferation of apoptosis resistance of malignant cells. This makes these pathways interesting candidates for targeted therapy. By example, the effectivity of inhibition of nuclear factor-kappa B (NF-κB) downstream of PI3K/PTEN/Akt/mTOR is now reduced in vitro sensitivity to daunorubicin, a major drug used in leukemia treatment, in comparison with the CD34+CD38- counterpart, and increased expression of MDR genes (MRP/LRP). These precursors show lower expression of Fas/Fas-Ligand and Fas-induced apoptosis than CD34-positive/CD38-positive blasts (97). It has been recently reported that the immature, self-renewable, and quiescent LSC population shows a high MDR transporters expression suggesting that MDR is one of the mechanisms responsible for treatment failure (98).
being evaluated. This is of particular interest, as it has been found to be particularly active in LSCs but not in HSCs (102-107). Secondly, stem cell specific molecular pathways, such as the Wnt and Notch signaling pathway are proposed to affect drug resistance; a high expression of Wnt proteins correlates with a worse prognosis (43, 108-111), whereas down regulation of Notch has been related to leukemogenesis (112, 113). Finally, like normal stem cells, malignant hematopoietic stem cells are thought to reside in discrete cellular spaces within the bone marrow microenvironment, termed “niches”, providing the mechanical support and extrinsic molecular mechanisms that maintain stem cell fate and inhibit differentiation. Recently, osteoblasts were found to be a key cellular component of such a niche, their number correlating with an increase in hematopoietic cells. Osteoblastic production of stromal derived factor-1α (SDF-1α) was found to partly underlie the maintenance and growth of hematopoietic stem cells (114). In view of the resemblance between benign and malignant stem cells the presence of a malignant stem cell niche, inducing drug resistance, can be proposed. This is supported by the fact that SDF-1α has been found to favor tumor-cell survival and growth. Indeed, CXCR4 expression, the only receptor for SDF-1α, is a prognostic marker in AML (115). In this respect it is of interest that both chemotherapeutic treatment and radiotherapy increase SDF-1α in the bone marrow microenvironment (116). It can therefore be postulated that even after chemotherapeutic treatment, the tumor microenvironment preferentially maintains LSCs. These LSC characteristics leading to therapy resistance, highlight the need for new therapies overcoming drug resistance in LSC thereby eradicating LSCs and preventing relapse out of MRD. In order to do so both the detection of LSCs and knowledge on the physiological regulators of malignant stem cell behaviour are prerequisites. Insight in the immunophenotypical characteristics of normal stem cells and in the regulators of normal stem cell maintenance may serve as a paradigm.

3.3 Identification of stem cells

Specific identification of LSCs is important for the quantitation of the stem cell compartment at diagnosis and after chemotherapy and for further functional characterization. Therefore, similar to described for MRD, markers are needed to distinguish LSCs from normal HSCs. This information would enable not only identification of patients at risk of relapse, but also would help to identify new targets for therapy. Notably, the LSC distinct phenotype may also serve as a tool to separate HSCs from LSCs (purging) for transplantation purposes.

3.3.1 Differentiation of LSCs and HSCs by immunophenotypical detection

HSCs and committed progenitor cells in human BM can be identified by their expression of the CD34 membrane phosphoglycoprotein. Reconstitution of BM by CD34-positive cells suggests that the HSC is within this specific population (117). HSCs in humans have been shown to have a CD34+CD38- phenotype as shown by long-term in vitro cultures (118, 119) and in vivo transplantation experiments in which the CD34+CD38- cells from adult BM were able to engraft and generate sustained (re)transplantable multilineage human hematopoiesis in non-obese diabetic/sever-combined immunodeficiency (NOD/SCID) mice (120, 121). CD34+CD38- have a very low frequency, only comprising 1-10 % of CD34+ cells (122) and have a lymphocyte-like morphology (119), weekly express Thy1(CD90) and the tyrosine kinase receptors c-kit (CD117) and Flt-3 (123-125), but lack antigens displayed on mature hematopoietic cells (126). CD38 is a 45 kDa transmembrane glycoprotein that appears to play a role as a differentiation molecule (127) and inductor of apoptosis (128, 129). It appears that CD38 is a better marker for HSC purification than lineage markers because CD34-positive cells undergoing early differentiation firstly express CD38 followed by lineage markers (119).

Although at least in part of the AML cases both HSC and LSC are found within the immature CD34+CD38- fraction of normal and CD34-positive AML cells respectively (78), there are distinct markers that can separate these two cell groups. The majority of studies have shown that unlike normal HSCs, AML LSCs usually lack the expressions of CD117, CD90, CD71 and HLA-DR (130, 131, 82). In contrast, AML LSCs do express CD33, IL-3R-α (CD123) and CD96 (132-134). However, CD33 and CD123 are also expressed on the cell surface of some normal HSCs (135). This also accounts for the expression of the adhesion molecule CD44 (receptor for hyaluronan). Although its expression on LSCs might play an important role in transport of LSCs (homing) to stem cell-supportive microenvironmental niches and therefore would be an interesting target for therapy, normal HSCs do weakly express CD44. Moreover, the different CD44-isoforms are expressed in many different tissues (136, 137).

Interestingly, the antigen C-type lectine-like molecule-1 (CLL-1) is exclusively expressed on malignant CD34+CD38- cells and not on the normal counterpart, although normal more mature progenitor cells do express CLL-1 (138). This might allow LSC specific therapy in the future. CD47 is a membrane protein that functions in neutrophil trafficking and T cell costimulation (139). CD47 also interacts with a specific macrophage receptor, SIRPα to downregulate the phagocytic potential of macrophages (140). It has
been reported that myeloid LSC population have increased expression of CD47 relative to normal HSCs (141). This suggests that LSCs are likely to enjoy enhanced survival due to decreased clearance by macrophages. Majeti et al. reported that CD47 expression on human LSCs results in decreased phagocytosis by macrophages, an effect that can be reversed by the presence of antibodies directed against CD47 or SIRPα (142).

Taken together, a number of markers have been described that can discriminate between normal and AML stem cells, however the lack of expression on all LSCs hampers its overall usefulness in the detection of LSCs in patients with AML. Moreover, regenerating and follow-up BM controls, important for specific detection of LSCs at follow-up as well as for future intervention using anti-LSC therapies, are lacking which enables to detect and study normal stem cells in bone marrow of AML patients recovering from chemotherapy.

3.3.2 Differentiation of LSCs and HSCs by functional assays

Recent evidence suggests that not all HSCs or progenitors express CD34 at all times (143). Firstly, it is known that 5% of patients with AML who have a CD34-positive phenotype lack a detectable CD34+CD38– compartment (< 0.01%) and about 20% of AML patient do not express CD34 on the leukemic cells and thereby, by definition, CD34+CD38– negative (77). Secondly, cell immuno-phenotype may alter during cell cycle progression or ex vivo in liquid culture assay (144-146). Therefore, identification on the basis of conserved stem cell function might help to detect LSCs at follow-up as well as for anti-LSC therapies.

3.3.2.1 Side population

An alternative stem cell compartment is offered by the so-called side population (SP). A decade ago, Goodell et al. reported the presence of an extremely small but distinct population of cells in normal BM of mice, capable of efficient Hoechst 33342 dye efflux (147). This population was highly enriched for HSCs and was identified too in human BM. SP cells shown to be largely CD34-negative with a high repopulation potential and successful engraftment in mice (148). SP cells are also present in BM obtained from patients with AML, and these were capable of initiating leukemia after transplantation into NOD/SCID mice, suggesting that these cells might be candidate LSCs (149). Moreover, AML-specific cytogenetic abnormalities were present in SP cells obtained both at diagnosis and after chemotherapy in remission BM. These findings suggested that SP cells in normal BM are candidates to transform in leukemia initiating cells.

3.3.2.2 ALDH

A promising complementary strategy for identifying and studying HSCs and progenitors is to rely on the expression of intracellular enzymes that may be important during development. One such cytosolic enzyme is aldehyde dehydrogenase (ALDH) that protects cells from the toxic effects of peroxidic aldehydes. ALDH may play an important role in retinoid metabolism, and it appears to be relatively highly expressed in both normal progenitor and CD34-positive/CD38-negative LSCs of a subgroup of AML (150-152). High ALDH activity results in resistance to alkylating agents such as the active derivatives of cyclophosphamide which may have important implications for resistance to chemotherapy. The incidence of LSCs with an extremely high ALDH activity is correlated significantly with poor clinical outcome (153). Identification and isolation of LSCs on the basis of ALDH activity provides a tool for their isolation and further analysis.

3.3.2.3 Rhodamine

It has been reported that quiescent normal CD34-positive progenitors showed rhodamine efflux. These cells appeared noncycling, in contrast to the proliferating rhodamine bright cells. Also in AML, the proliferation rate (percentage S/G2+M and Iododeoxyuridine labelings index) was significantly less in the rhodamine dull blasts. This makes the efflux of rhodamine an alternative marker for quiescent cells (154).

3.4 Leukemic stem cells as a target for therapy

Despite major efforts during the past 40 years, limited improvement in disease survival have been made and less than one third of adults with AML are cured by current treatments, emphasizing the need for new approaches to therapy. The discovery over a decade ago that myeloid leukemias originate from rare stem-like cells that can propagate the disease in immune deficient mice (78, 79) suggested that these LSCs are responsible for relapse of leukemia following conventional or targeted cancer therapy and that eradication of LSCs might be necessary to cure the disease permanently. As mentioned before, eradication of LSCs requires targets specific for LSCs versus HSCs. Antigenic expression might enable antibody-directed therapy. In addition, it might be interesting to further explore the possibility of therapy targeting the differential expression of functional characteristics such as drug-efflux pumps and molecular pathways involved in “stemness”. Thirdly, targeting the interaction...
between LSCs and the stem cell niche has been recently explored, from which the disruption between CXCR4 on LSCs and SDF-1α is an example (155, 156).

3.4.1 Antigenic targets
Cytotoxic antibodies can be used as a form of AML therapy to target the cell surface molecules that are differentially expressed between HSCs and LSCs. CD33 is expressed on the CD34+CD38- stem cells of AML samples at diagnosis (132). The anti-CD33 MoAb conjugated with the cytotoxic antibiotic calicheamicin induces remission in some cases of AML (157). However, in the long term, patient on this drug have developed considerable hematological toxicity and prolonged cytopenia, which may be due to its adverse effects on normal HSCs, which are CD33-positive (158).

Another potential target on AML stem cells is CD123, a receptor for IL-3 (133), as diphtheria toxin-IL-3 fusion protein selectively target AML LSCs in comparison with normal HSC in vitro (159). This immunocojugate was further shown to decrease the effectiveness of AML engraftment in NOD-SCID mice, indicative of an effect on LSCs.

The adhesion molecule CD44 has emerged recently as a promising target that is over-expressed on both AML and CML stem cells relative to normal HSCs and can be used to target the LSCs in AML. In vivo administration of this antibody to NOD-SCID mice transplanted with human AML markedly reduced leukemic repopulation. Absence of leukemia in serially transplanted mice demonstrated that AML LSCs are directly targeted (136). However, the different CD44 isoforms are expressed on many different tissues which might limit therapeutic efficacy (137). Furthermore, it has been shown that the combination of anti-VLA-4 antibody and cytarabine can eradicate residual AML in xenografted mice (41). Thus, addition of anti-VLA-4 antibodies to standard AML consolidation therapy might help to eliminate LSCs and lead to lower relapse rates in VLA-4-positive AML.

Finally, CD96, which is a member of Ig gene superfamily has been described to be expressed differentially expressed between HSCs and LSCs. Therefore, this LSC-specific antigen might serve as a therapeutic target (134).

Little is known about the expression of the antigens on normal stem cells in the BM of AML patients at diagnosis and during/after therapy. CD123, but not CLL-1 seems to be upregulated in treated normal BM stem cells (160). Nevertheless this is a very important issue from a standpoint of BM toxicity of anti-stem cell therapies. Because of its very high specificity for AML stem cells, CLL-1 is a promising target too (138). Studies are underway in which the CLL-1 antibody has been coupled to toxins.

3.4.2 Functional targets
Another approach to eliminating LSCs is to targeting LSCs on the basis of stem cell function.

3.4.2.1 Therapy targeting drug efflux mechanism in LSCs
The drug efflux in LSCs provides a mechanism for long-term survival of LSC population via an enhanced ability to pump cytotoxic compounds out of the cell. LSCs display MDR that is conferred by ABC transporters (98). Targeted inactivation of ABC transporters could reinstate the drug sensitivity in LSCs resulting in LSC killing. For example, ABCB5 has been reported as a marker for a subset of CD133+ melanoma stem cells. ABCB5 provides resistance to doxorubicin by functioning as an efflux pump. When it was blocked by anti-ABCB5 monoclonal antibody, doxorubicin sensitivity was restored in these malignant stem cells (161). Moreover, the expression of ABCG2 has been implicated in MDR of AML (162). A variety of inhibitors for ABCG2 may prove useful for sensitizing LSCs to chemotherapy. Tyrosine kinase inhibitors, such as imatinib and gefitinib, are both inactivators of ABCG2 and, therefore, serve as candidates to reverse LSC chemoresistance and potentially target them (163).

ALDHs have been known to provide resistance to hematopoietic stem cells against alkylating agents of the oxazaphosphorines family, such as cyclophosphamide and its derivatives. AML stem cells do not express ALDH-1, whereas normal HSCs do (150, 151). Since ALDH-3 is overexpressed in various malignancies (164), it may be that the majority of AML samples that do not express detectable ALDH-1, actually express ALDH-3. This difference could allow a therapeutic reagent to be developed that is specific to ALDH-3 and hence AML stem cells (165).

3.4.2.2 Targeting LSCs with drugs interfering with signal transduction pathways
Other approach to eliminating LSCs is to determine the target pathways that are aberrantly expressed and to identify drugs, which selectively target LSCs and not the normal HSCs. The NF-κB transcription factor is of particular interest because it is found to be particularly active in LSCs but not in HSCs (102, 105), allowing selective intervention (105, 166). The proteasome-inhibitor MG-132, which inhibits NF-κB activation, has been proposed for stem cell targeted therapy (105, 167). Another approach to blocking NF-κB is through inhibition of IκB kinase (IKK),
which phosphorylates and inactivates IkB. Parthenolide, a sesquiterpene lactone naturally occurring small molecule with IKK-inhibitory activity, has been proposed to selectively target LSCs while sparing normal HSCs (168).

Another attractive therapeutic target in AML stem cells is the mTOR pathway. The kinases Akt and mTOR are activated in human AML blasts and treatment with inhibitors of PI3K or mTOR, in combination with cytarabine or etoposide, induces apoptosis in AML cells and decreased the abundance of LSCs (102). Recent studies in mouse models also implicate mTOR as an attractive drug target for the selective elimination of LSCs (103, 104).

3.4.3 Therapy targeting the microenvironment

In normal hematopoiesis, the bone marrow microenvironment is known to be involved in the maintenance of stem cells. Normal HSC reside in the BM and interact with a highly organized microenvironment compromised of a diverse population of stromal cells and an extracellular matrix rich in fibronectin, collagens, and various proteoglycans. The interaction between HSCs and the bone marrow microenvironment is critical in regulating HSC processes such as trafficking, self-renewal (stemness), proliferation, and differentiation. Without direct contact with the cellular niche, HSC tend to differentiate and lose their stemness (169-173).

In AML, the bone marrow microenvironment provides the primary site of MRD after chemotherapy (156, 174-176). There is increasing evidence that LSCs occupy and receive important signals from the microenvironment that support self-renewal and may use the normal homeostatic mechanisms that preserve long term HSCs (155, 177-179). Recent studies have demonstrated the involvement of the SDF-1/CXCR4 axis, the VLA-4, and the adhesion molecule CD44 in the homing process of the AML LSCs to the microenvironment, chemotherapy resistance (cell- adhesion mediated drug resistance) and cell cycle regulation (136, 155, 156, 173-179). These observations offer the possibility that perhaps AML resistance can be overcome by altering the microenvironment.

4. SCOPE OF THIS THESIS

AML is generally regarded as a disease likely to originate from the hematopoietic stem cells and it has been shown that high LSCs frequency in AML at diagnosis predicts poor survival (77).

The increasing evidence that LSCs play an important role in the occurrence of MRD and relapse urges for an unmet need for LSC-targeted therapy. In order to develop such new treatment strategies in the future, the ability to specifically detect LSCs, in particular to discriminate between LSCs and HSCs is a prerequisite. This is the main scope of this thesis.

In chapter two we aim to investigate whether leukemia specific lineage markers as used for immunophenotypic MRD detection can also be used to discriminate between LSC and HSC in the CD34+CD38- defined stem cell compartment.

It has been reported that not in all patients with AML, the CD34+CD38- compartment contains the malignant stem cell. Therefore, we investigated the use of the side-population (SP) as an alternative stem cell compartment. In chapter three it is investigated whether leukemia associated antigens, including CLL-1 and lineage markers, also specifically mark the LSCs in the SP compartment. This would be instrumental to discriminate LSCs and HSCs in the SP compartment as well.

Whether LSCs harboured in the CD34+CD38- and SP defined compartments are biologically similar is still unknown. Insights into the putative relationship between both defined stem cell compartments may provide clues for further characterization of these cells with the aim of developing new therapies specifically directed towards LSCs. In chapter four we asked ourselves whether a relation between the CD34+CD38- and SP defined LSC and HSC compartments exists.

A main problem in the design of new therapies that target the LSC specifically, while leaving the HSC unharmed, is the large overlap in properties of HSC and LSC. Different cell surface markers have been shown to mark LICs and may have potential for anti-LIC therapy in AML. However, in most studies attempts were made to validate the specificity of expression i.e. to show that these markers are not only absent or lowly expressed on normal BM HSC, but also on HSC present in AML BM under conditions of disease and treatment relevant for target finding and specificity of antibody/ligand therapy as well as for monitoring purposes. In

In chapter one we discuss the general introduction to the thesis.
chapter five we compared the specificity of stem cell markers aimed for therapeutic use, defined by us (CLL-1) and by others (CD33, CD44, CD47, CD96, and CD123). This was performed for both CD34+CD38- and SP defined HSC present in diagnosis AML BM, and normal BM as well as regenerating BM of the patients being recently treated with chemotherapy.

Apart from inherent stem cell characteristics, therapy failure may also result from bone marrow microenvironment induced apoptosis resistance. The effect of chemotherapy exposure of BMSCs, however, is unknown. Therefore, we aim to investigate the supportive role of chemotherapeutically-treated BMSCs in maintenance of leukemic cells. In chapter six the role of the bone marrow microenvironment in both chemotherapy-induced and spontaneous apoptosis resistance of leukemic cells has been determined.

Finally, in chapter seven the findings described in this thesis are summarized, discussed and future perspectives are outlined.

REFERENCE LIST


81. Mehrtra B, George TF, Kavanau K, Moore D, Willman CL, Sutherland HJ. Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)CD38(-). Cancer Res 2000;60:6067-6075.

82. Blair A, Hogge DE, Sutherland HJ. Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)CD38(-). Cancer Res 2000;60:6067-6075.


