Identification and characterization of acute myeloid leukemia stem cells
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Summary, concluding remarks and future perspectives
Summary

Acute myeloid leukemia (AML) is a clonal expansion of immature hematopoietic cells, characterized by an arrest in differentiation, which results in an accumulation of immature abnormal myeloid cells in bone marrow and peripheral blood. Although in general chemotherapeutic treatment leads to complete remission of the disease, reflecting a high proliferation rate of malignant cells conferring chemotherapy sensitivity, in the majority of patients a relapse occurs. This is thought to be the result of outgrowth of minimal residual disease (MRD). MRD might be caused by therapy resistance of whole leukemic blast population at diagnosis. Indeed, we previously showed that an anti-apoptosis protein profile at diagnosis was linked to the emergence of high frequencies of MRD followed by relapse. However, there is increasing body of evidence pointing towards the importance of leukemic stem cells (LSCs) in the occurrence of MRD and relapse. The concept of LSCs originating from a transformed CD34+CD38- hematopoietic stem cell (HSC) has been challenged by the existence of a more mature progenitor-like cell with acquired stem cell properties as the source of AML. LSCs share properties with normal stem cells, such as being relatively quiescent, resistant to apoptosis and having a high expression of ABC transporters, such as ABCB1, ABCC1, and ABCG2, leading to increased drug efflux. 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. Such characteristics lead to therapy resistance, which highlights 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.

Both immunophenotypic and functional definitions of LSCs have been proposed, but the relation between both types of LSCs defined as such has not been studied in depth. The detection of LSCs is hampered by the fact that both LSCs and HSCs share the same immunophenotype and functional characteristics being used to define stem cells. The studies in this thesis describe how to differentiate between normal and leukemic stem cells and whether different classes of stem cells exist, with different biological impact. Finally, the role of the protective bone marrow microenvironment is being studied.
In Chapter 2 we describe the possibilities to discriminate leukemic CD34+CD38- cells from normal HSC both at diagnosis and in complete remission. A set of (mostly lymphoid) lineage markers was found to be differentially expressed on LSCs. These markers add to a similar role of the already described LSC specific marker C-type lectin-like molecule-1 (CLL-1). Normal bone marrow (NBM) and regenerating BM (RBM) samples served as negative controls. Follow-up analyses showed their predictive value in AML samples with a detectable CD34+CD38- population. Our results showed that normal and leukemic CD34+CD38- cells can be discriminated in the majority of AML patients, not only at diagnosis but also after chemotherapy. This approach thus delivers the possibility to detect, parallel to whole blast MRD, the frequency of residual malignant CD34+CD38- cells, which are responsible for relapse.

In addition, to CD34+CD38- LSC, new definitions of LSCs should be aimed for because not all LSCs have a CD34+CD38- phenotype, as for example in CD34-negative AML. An alternative stem cell compartment is the so-called side population (SP). In chapter 3 we determined that the CLL-1 and lineage markers as described in chapter 2 can also be used to discriminate HSC from LSC within the SP population. Apart from mature compartments (high forward and sideward scatter and high CD38 expression) and lymphoid population consisting of B-, T- and NK-cells, two primitive compartments were identified, both characterized by low forward and sideward scatter and low CD38 expression and one of these with and the other without aberrant marker expression. Clonogenic malignant cells were enriched in the primitive cell fraction, as shown by outgrowth of these cells to cytogenetically abnormal colonies. This detailed characterization enabled us to identify a low frequency SP subfraction (median 0.0016%) likely to be enriched for leukemic stem cells.

In chapter 4 we report a detailed analysis of both the CD34+CD38- and SP compartments in AML patients. We found that the interrelationship is dependent on the type of AML in terms of CD34 expression. In patients with CD34-negative AML, almost always a very small CD34-positive population was present containing CD34+CD38- cells, which were presumably normal by virtue of their low forward and sideward scatter and the absence of aberrant markers. As CD34-CD38- cells had low FSC/SSC with the absence of aberrant markers and have been shown in the past to have hematopoietic reconstitution ability; this population may also represent the HSC. We provide evidence that in this type of AML, the LSC is present in the CD34-negative compartment. Since the frequency of primitive cells assessed with clonogenic assays was found to be much higher in the SP compartment, LSCs are likely present in the CD34- negative SP compartment. Together with literature data on cytogenetics and NOD/SCID engrafting ability, this strongly suggests that in CD34-negative AML the biological important LSCs are present in the CD34-38+ SP compartment.

In contrast, in CD34-positive AML the HSC may still be present in the CD34+CD38- compartment, but most important, the LSC can be present in CD34-positive and CD34-negative fraction irrespective of CD38 expression. Similar to CD34-negative AML, the SP fraction had a very high clonogenic potential, suggesting that LSCs are harbored within all four CD34/CD38 defined SP compartments.

In the previous chapters we have used stem cell markers like CLL-1 to enable discrimination between LSC and HSC under different conditions. In chapter 5 we compare 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 LSC and HSC present in diagnosis AML BM, NBM as well as RBM of the patients being recently treated with chemotherapy. Absolute expression on the HSC present under these condition as well as expression differences with LSC revealed that some antigens (CLL-1 and CD96) are highly specific (expression on LSC but not on HSC), but their expression on LSC is usually not high. Other antigens may have low but clear expression on HSC, but expression on LSC is also relatively high (CD123). A third class represents antigens with high expression on LSC and HSC (CD33, CD44, CD47). Despite this, there may still be differences between LSC and HSC in the latter class, allowing to discriminate LSC and HSC (CD44). Since CLL-1 was highly LSC specific in all sources of HSC (NBM, RBM and HSC in diagnosis AML BM), a therapeutic program for anti-CLL-1 treatment seems promising and has already started.

Normal hematopoiesis is maintained by dynamic interactions between hematopoietic cells and the BM microenvironment. In hematological malignancies, there are reciprocal interactions between leukemic cells and the bone marrow stromal cells (BMSCs) provide a sanctuary for leukemic cells to evade chemotherapy-induced inhibition of cell proliferation and cell death. In chapter 6, we investigated the putative mechanisms responsible for enhanced AML survival in both leukemic cell lines and primary AML cells, by either untreated or chemotherapeutically-treated
BMSCs. Both chemotherapy-treated and untreated AML cells were protected against spontaneous and chemotherapy induced apoptosis, with the Akt-Bcl-2 axis of signal transduction involved. Protection was partly mediated by soluble factors. Moreover, after exposure of BMSCs to chemotherapy, there was a partial loss of the protective effect. Therefore, in vivo outgrowth of MRD may be dependent on the extent of chemotherapy-induced damage to the BM microenvironment. Although this has to be confirmed for LSCs, the results stress the importance of including the microenvironment in new target finding.

Concluding remarks and future perspectives

We now know that a series of markers allows to identify both LSC and HSC, concomitantly present in the patients BM. These markers include lineage markers as well as a series of reported LSC markers. This is possible in both immunophenotypically as well as functionally defined stem cells. This opens perspectives for specific monitoring of LSC during treatment/disease, with possible prognostic impact, e.g. prediction of impending relapses. Also the prospective isolation of LSC and HSC from the same patients BM, and subsequent molecular and/or functional characterization, may well offer new therapeutic targets with the highest possible specificity. The marker studies may also be convenient in studies on the fate of LSC and HSC in the context of the BM microenvironment. Since in vivo outgrowth of MRD may be dependent on the extent of chemotherapy-induced damage to the BM microenvironment, future studies should include such models instead of normal functioning BM or stromal cell lines. This will help finding new drugs targeting interactions between LSCs and the BM microenvironment and might lead to prevention of leukemic outgrowth in AML patients. The work on stem cell markers has also made clear that several of these may be suitable to serve as therapeutic targets. Above that, this work has shown how heterogeneous marker expression may be on the presumed LSCs. Whether it is the absolute marker expression on LSC that is the main determinant of efficacy of therapy or the difference in expression of the marker on LSC and HSC or both, is currently unknown.

Our work has also revealed two separate entities of AML cases: CD34 negative and CD34 positive AML, characterized by different types of LSCs. These findings, together with reported literature on cytogenetics and NOD/SCID engrafting ability, suggest that that CD34-positive AML harbours a more therapy resistant and leukemogenic LSC compartment compared to CD34-negative AML. Indeed, in other studies we have found that the prognosis of CD34-positive AML is worse compared to CD34-negative AML patients. Also, based on the history of NOD/SCID mouse models, we propose that CD34-positive AML may preferably be studied in immunologically less restrained mouse models, while CD34-negative AML may better be studied in immunologically restrained mouse models.