Leukemia Stem Cells Shows the Way for Novel Target of Acute Myeloid Leukemia Therapy

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Abstract
Leukemic stem cells (LSCs) have been identified in acute myeloid leukemia (AML). Similar to haematopoietic stem cells, these LSCs are able to self-renew, differentiate, and proliferate extensively. Recent studies suggest that LSCs are critical for the initiation and maintenance of leukemia.

This review will describe the characteristic features of LSCs in AML and the possible targets expressed on the surface of AML, the intracellular targets and the novel molecular and flow cytometry methodologies being used to particularly ablate the LSC population.

Studies have shown the potential importance of ablating LSCs when treating leukemia. The unique characteristics of LSCs that differentiate them from their normal counterparts can be applied to specifically target the leukemic population.

Current therapeutic strategies may not effectively ablate the LSCs, leaving the potential for disease progression or recurrence. A better understanding of LSCs and molecular biology will allow the design of more effective therapies.

Keywords: Leukemia; Leukemic stem cells; Acute myeloid leukemia; Stem cell therapy; LSCs

Introduction
Acute myeloid leukemia (AML) is one of the most common leukemia’s in adults. AML is characterized by an accumulation of undifferentiated and functionally heterogeneous populations of cells [1,2]. Relapse of the disease is thought to occur because of the failure of chemotherapy to eradicate LSCs [3]. LSCs have been defined as CD34+/CD38- cells with the ability to reconstitute marrow of mice with severe combined immunodeficiency (SCID)—repopulating ability, which is a reflection of their capacity to self-renew [4,5].

In order for any AML therapy to be curative, it needs to be effective against the cells that propagate and sustain the disease, the so called LSCs. However, previous studies suggest that LSCs are biologically distinct from more mature leukemic blasts and may not be responsive to conventional chemotherapeutic regimens [6,7]. In 1997, Bonnet and Dick described the phenotype for LSCs as CD34+/CD38- [4]. Subsequent studies showed that LSCs are also CD34+/HLA-DR-/CD71+ and fail to express Thy-1 [5,8]. Numerous studies have attempted to distinguish leukemic from normal stem cells. One potential difference between normal and leukemic cells lies in their response to haematopoietic growth factors. Several studies have examined the cytokine response of primary leukemia cells and demonstrated mitogenic activity for interleukin-3 (IL-3), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), thrombopoietin (TPO) and other factors [9-11].

Over the last few years, the functional properties and phenotype of human LSCs have begun to be explored. Today, AML stem cells can be discriminated and separated from normal stem cells and from more mature AML cells [12-16]. Due to their repopulating and disease-maintaining effects, these cells appear to be a most important target cell population in the context of curative therapies. Therefore, a number of studies are presently focusing on AML stem cells and on effective drugs that can attack these cells in a target-dependent manner.

The currently available data are promising and point to the potential anti-leukemic effects of ‘stem cell-targeted’ drug therapy [14,17-22]. However, these data also show that many targets and pathways still need to be explored. The most promising approach for future concepts may be to target multiple extracellular and intracellular targets and unrelated downstream signalling pathways in AML stem cells by the co-administration of various targeted drugs (advanced targeted drug therapy). Whether these strategies will help in the eradication of AML clones and the management of minimal residual AML remains to be determined in future clinical trials.

Thus, there is compelling evidence that the precise predisposing or suppressing cellular and molecular factors of relapses in patients in complete remission (CR) remain to be determined. It is becoming evident that relapses occur in patients in whom the leukemic cells survive chemotherapy, which show evidence of functional properties of stem cells [23]. A number of observations proposed that AML clones in each patient represent a heterogeneous mixture of cells with varying phenotypic and functional properties [1,4,5,8,23-25]. In particular, despite differentiation and maturation arrest, AML clones are organized in a hierarchical manner similar to normal haematopoietic cells [1,4,24,26]. On the top of this hierarchy, LSCs act as AML-initiating and maintaining cells, whereas their more mature progeny-
cells are unable to maintain the long-term growth of leukemia [1,4,24]. This hypothesis has been confirmed for various subtypes of AML by utilizing repopulation assays [1,24].

LSCs obtained from patients with AML are defined by their AML repopulating capacity in vivo, i.e. their ability to give rise to leukemia’s in immunodeficient mice [1,4,24]. Over the last few years, these cells have been characterized in terms of their frequency in AML clones, their phenotype, and their functional properties. Similar to normal hematopoietic stem cells (HSCs), LSCs represent only a small fraction of cells within a given AML clone (0–2–100 cells in 10^6 cells) [4]. In common with their daughter cells, most LSCs express CD34 [1,27-29].

In addition, LSCs despite being present in low numbers may be responsible for the relapses in AML and therefore are considered to contribute essentially to the pathology and clinical outcome in these patients. So rather than monitoring the whole blasts cell population, it may be more relevant to search for the residual LSCs.

**Functional and Phenotypic Characterization of Leukemic Stem Cells**

**Functional characterization of LSCs**

AML-LSCs are found to be non-cycling and present in the endosteal region of the bone marrow [30]. Analogous to the repopulating potential of HSCs, LSCs have the potential to repopulate hematopoietic tissues in severe combined immunodeficient (SCID) mice [1,4,24,26]. Though, only a small sub-fraction of cells within leukemic clones represent LSCs, whereas the vast majority of AML cells in a given clone are unable to repopulate SCID mice with leukemia [1,4]. The capacity of LSCs to self-renew in vivo is further supported by the fact that the leukemic cells that can be generated from these cells in SCID mice can again give rise to leukemia when serially transplanted into ‘secondary’ cohorts of SCID mice [4]. The morphology and the immunophenotype of the LSC-derived AML cells in these SCID mice are identical to the original AML clone [4]. The capability of stem cells to be dormant and remain in their respective bone marrow niches protected from antagonistic influences allows for maintenance of their self-renewal activity [30]. These results demonstrate the potential of LSCs to initiate the re-growth of AML cells in vivo (Figure 1). Based on this finding, it is tempting to speculate that LSCs represent those residual AML cells (Minimal Residual Disease or MRD) [31-34] that can regrow to overt relapsing leukemia’s after chemotherapy or bone marrow transplantation.

An important aspect of LSCs in patients with AML is that a high percentage of them appear to be quiescent (Ki67-negative), thus contrasting with the high cell burden and potentially aggressive clinical course of these leukemia’s [35]. This observation may explain why these LSCs are often less responsive against cell cycle-active chemotherapeutic agents compared to their more mature progeny. Even though LSCs reportedly express receptors for IL-3 and other cytokines, it remains unknown whether such cytokines can induce the growth or differentiation of LSCs. Taussig et al. [36] reported that leukemia-initiating cells (LIC’s) were found to be present in the CD34 fraction in a significant amount of AML’s. They also showed that the phenotype of LIC’s is heterogeneous in AML. It is this feature of LIC’s which can make it difficult for targeting therapies against surface antigens. Furthermore, it was also shown that LIC’s were not found in the CD34/CD38 fraction in approximately half of the NPM (Nucleophosmin) gene-mutated AML’s [36]. NPM1 is a gene which has both tumor-suppressor and oncogenic functions. A disruption of the NPM nuclear-localization signal occurs with mutations of NPM, causing accumulation of NPM in the cytoplasm. The NPM1 gene rearranges with the retinoic acid receptor α (RARα) in AML [37,38]. Also, although Jordan et al. [35] have shown that IL-3α receptor (CD123) is detectable on LSCs and not HSCs, little is known about their exact functional role in LSCs.

**Phenotypic characterization of LSCs**

Previous studies demonstrate that LSCs are mainly found...
within the CD34+CD38−Lin− subset of the leukemic clone (Figure 2) [4,24,39]. Other clonal leukemic cells with a more mature phenotype are unable to initiate AML in vivo [1,24]. These data are similar to the repopulation characteristics of normal human HSCs in SCID mice [1,4,23,24,27,40,41]. However, despite phenotypic and functional similarities, it has to be highlighted that slight differences in the cell surface phenotype are found when comparing normal HSCs with LSCs. Similarly, LSCs, but not HSCs, express the IL-3 receptor α chain (CD123) [35,42,43]. Another marker that is detectable on LSCs in (a subset of) patients with AML, is Siglec-3 (CD33) [44,45]. In addition, LSCs may also show immunophenotypic heterogeneity concerning other surface molecules such as CD116 (GM-CSFRα), CD117 (SCFR), or CD71 [5,8,25,46].

C-type lectin-like molecule-1 (CLL-1) has also been shown to be expressed in LSCs and not HSCs [16]. CD34+CD38−CLL-1+ cells were found to engraft NOD/SCID mice and a high CLL-1+ fraction was associated with quick relapse. In this regard, CLL-1 may serve as a marker for quantification of minimal residual stem cell disease. Further, CD44, an adhesion molecule, has also recently been described as a target on CD34+CD38− cells [20]. It was shown that the activating antibody H90 results in differentiation of cells and in a major reduction of engraftment in NOD/SCID mice. However, CD44 is also weakly expressed on normal CD34+CD38− cells and on more differentiated haematopoietic cells. It is also a key regulator of AML-LSCs homing to microenvironmental niches [20].

Phenotypic differences between LSCs and HSCs may be of great value. These differences allow discrimination of these cells by flow cytometry assays and to separate LSCs from HSCs for genetic analyses. Markers specific for LSCs may prove suitable targets for the development of novel therapies; because otherwise normal HSCs would also be eradicated, and such an approach would then only be possible when combining with a transplantation strategy. As LSCs are responsible for relapse, they may also be used for MRD (Minimal Residual Disease) detection.

**Targets of AML Therapy Detectable in Leukemic Stem Cells**

The challenge is to identify proapoptotic stimuli that spare the normal HSCs while exerting the desired effect on LSCs. The key for targeting LSCs is by targeting drug efflux pumps, targeting cell cycle, targeting cell surface antigens, targeting NF-κB activity, targeting cell differentiation, targeting leukemia stem cells via active specific immunization and targeting other pathways involved in self-renewal. Molecular constructions in AML cells that may serve as targets of

![Figure 2: Schematic illustration of the normal and leukemic human haematopoietic hierarchies. Human haematopoietic cells are organized in a hierarchy that is sustained by a small population of self-renewing HSCs. HSCs give rise to progressively more lineage-restricted, differentiated progenitors with reduced self-renewal capacity (LTC-ICs, long-term culture-initiating cells; CFU, colony-forming units), which in turn produce functionally mature blood cells. Disruption of pathways regulating self-renewal and differentiation through the acquisition of transforming mutations generates LSCs capable of sustaining growth of the leukemic clone in vivo. LSCs possess an altered differentiation program, as demonstrated by aberrant expression of some cell-surface markers (indicated in purple) and give rise to an aberrant developmental hierarchy that retains aspects of its normal counterpart. In vivo, reconstitution assays using immune-deficient mouse recipients enable detection of HSCs and LSCs as SCID-repopulating cells (SRCs) and SCID leukemia-initiating cells (SL-ICs), respectively [39].](image-url)
specific therapy are located in various compartments of the leukemic cell. Generally, such targets are either detectable on the cell surface or within the cytoplasm (Table 1) [47].

**Targets expressed on the surface of AML leukemic stem cells**

Based on target molecules expressed on the surface of leukemia cells, a number of new treatment strategies have recently been established [22,45,48-51]. One of these concepts employs humanized antibodies (Ab) conjugated with a cytostatic drug. These conjugates bind to leukemic cells through an interaction of the Ab with the target structure on the surface of AML blasts. Consecutively, the Ab–drug conjugate is internalized by the leukemic cells. After internalization, the drug is released from the Ab and inhibits critical cell functions (depending on the nature of the cytostatic drug) and eventually leads to cell death [22,49,52,53]. An important example for such conjugates is Mylotarg (gemtuzumab/ozogamicin), which consists of a humanized anti-Siglec-3 antibody (CD33) and the highly potent (toxic) cytostatic drug calicheamicin [22,49,52,54]. However, it has been found that patients are prone to relapse despite being effective at inducing remission in some patients, which raises the question of the resistance of LSCs to the toxic drug [55].

Furthermore, anti-interleukin-3 (IL-3) receptor alpha chain (CD123)-neutralizing antibody (7G3) targeted AML-LSCs, impairing homing to bone marrow and activating innate immunity of NOD/SCID mice. 7G3 treatment profoundly reduced AML-LSCs engraftment and improved mouse survival. Mice with pre-established disease showed reduced AML burden in the bone marrow and periphery and impaired secondary transplantation upon treatment, establishing that AML-LSCs were directly targeted. 7G3 inhibited IL-3-mediated and impaired secondary transplantation upon treatment, establishing that AML-LSCs were directly targeted. 7G3 inhibited IL-3-mediated and impaired secondary transplantation upon treatment, establishing that AML-LSCs were directly targeted. 7G3 inhibited IL-3-mediated and impaired secondary transplantation upon treatment, establishing that AML-LSCs were directly targeted.
However, in a clinical setting, future studies are yet to determine whether or not the single chain fragment of the variable regions fusion proteins (scFv-based mini-antibodies) will be able to eradicate AML-LSCs [57].

Differential analysis of genes using microarrays as well as protein expression by flow cytometry revealed two cell surface markers, CD32 and CD25 (that are normally expressed on B and T cells) which were found to be highly expressed in human AML-LSCs and not expressed in normal HSCs. They were also found to be stably present in the all-important cell cycle-queuistic, AML initiating cells in the endosteal niche that might be the cause for AML relapse. Xenogeneic transplantation has shown that HSC function could be eliminated from the CD34+/CD38–CD25+ and CD34+/CD38–CD32+ fractions of human cord blood, thus confirming that targeting of CD32 or CD25 would not compromise normal HSC development [12]. This could facilitate the development of therapeutic strategies in AML because they were present in a significant number of AML patients, especially the poor-risk population [12, 13, 58].

CXC chemokine receptor (CXC4R), another marker for LSCs on the surface, is a cell membrane receptor is found on stem cells [59-61]. It has been found that SDF-1α (stromal cell-derived factor-1) / CXC4R interactions contribute to the resistance of LSCs to apoptosis in the microenvironment. Effective targeting of CXC4R and its interactions paves the way to eliminate and target cells that are usually protected by the bone marrow microenvironment [62]. Recently, it was suggested that CXCR4 expression is associated with poor prognosis in AML patients and a marker of more aggressive disease in a normal karyotype AML population [63]. This can even be incorporated into risk assessment of AML patients [18, 63].

Intracellular targets of AML therapy

Over the past few years, a large number of cytoplasmic and nuclear target structures in AML cells have been identified [17, 64-66]. Among these are DNA-methylating enzymes, histone deacetylases, leukemia-specific fusion gene-products (such as promyelocytic leukemia gene/ RARα (PML/RARα)), pro-oncogenic transcription factors (Signal Transducer and Activator of Transcription (STAT) family, Ets, c-Myb, HOX, NF-xB, others) and critical elements in pro-oncogenic signal transduction cascades (Receptor tyrosine kinases (RTKs), mutated oncogenic forms of Ras, others) [66].

Nuclear factor xB (NF-xB) is constitutively expressed in blast cells in a majority of patients with AML [64, 67, 68]. Additionally, NF-xB-activity is detectable in the quiescent LSC population in these patients, whereas normal HSCs do not express NF-xB activity [64, 68]. Thus, trying to eradicate LSCs by direct targeting using NF-xB pathway could be a potential therapeutic strategy [69, 70].

Recent reports have identified and applied a number of different tyrosine kinase inhibitors in clinical trials in leukemic patients [71-82]. Important stem cell RTKs expressed in AML cells are the SCF receptor KIT, macrophage colony-stimulating factor (M-CSF) receptor FMS, PDGFRβ, fms-related tyrosine kinase-1 (FLT1) and FLT3 [66, 81, 83, 84]. At least some of these tyrosine kinases are also expressed in LSCs [71].

Several previous and more recent observations suggest that these molecules do play an important role in leukemogenesis [66, 81, 83, 85, 86]. Similarly, the FLT3 gene is the most frequently mutated gene in patients with AML [66, 83, 84, 86, 87]. These mutations lead to ligand-independent dimerization of the receptor and its auto-phosphorylation with consecutive activation of multiple signal transduction pathways including the STAT5-, RAS/MAPK- and phosphoinositide 3-kinase/AKT-pathway (PI3/AKT) [88]. Since these mutations apparently act pro-oncogenically, it is appealing to speculate that they all take place and are detectable at the stem cell level in patients with AML.

A number of drugs targeting RTKs have recently been applied to AML cells in clinical and/or pre-clinical trials. Likewise, the inhibition of FLT3 by AG1296 or Herbimycin A in AML cells in mice was found to counteract the progression of leukemia [66, 83]. In addition, a number of targeting drugs directed against RTKs have been developed in recent years, including CEP701, CEP751, SU5614, SU5416, SU11248 and PKC412 [71, 76-82, 89]. These inhibitors may act on several RTKs including FLT3, thus inhibiting proliferation of leukemic cells. Additionally, some of these inhibitors have already been evaluated in vivo. Likewise, CEP701 has been reported to induce responses in AML patients’ refractory to conventional chemotherapy [79]. Interestingly, at least some of these RTK-type receptors are known to be expressed in AML-LSCs.

Recently, a novel and promising therapeutic strategy to preferentially target human AML-LSCs was uncovered by lysosome disruption [90]. An important finding of this study was that AML-LSCs were found to be enriched in bulk AML cells and their subsets which showed increased lysosomal size and biogenesis after lysosome disruption in human AML cells. This research study has shown that some common biological features and mechanisms remain open to selective targeting even though AML biology is so widely known as heterogeneous [90].

A breakthrough intracellular target for AML-LSCs is Aurora A kinases (AurA) which are a family of mitotic serine/threonine kinases that play a role in cytokinesis during mitosis and cell division [91, 92]. AurA showed a significant higher level of expression in AML-LSCs than in HSCs and can be used as a marker. Kim et al. [93] showed that specific AurA inhibitors could be used to reduce AML-LSCs. The study also further found that the reduction of AML-LSCs could be enhanced with stimulation with G-CSF and the use of AurA inhibitors [93]. Further studies confirmed that AurA inhibitors significantly inhibited proliferation, impaired self-renewal capacity and induced apoptosis of AML-LSCs as well as prolonged survival when AurA inhibitors were used during engraftment of CD34+/CD38+ AML cells in severely immunocompromised mice [94].

Another therapeutic target for AML-LSC therapy present intracellularly is myeloid leukemia-1 (Mcl-1), which has been found to be up-regulated during AML relapses in FLT3/ITD AML-LSCs. This could probably be due to the fact that Mcl-1 confers some sort of resistance to chemotherapy [95]. Mcl-1 has been suggested to promote survival of FLT3/ITD AML-LSCs via a STAT5-dependent pathway that is independent of normal FLT3 signaling [96]. Furthermore, deletion of Mcl-1 led to induced death of transformed AML and eradicated disease in the AML NOD/SCID mice [97]. So, the lowering of Mcl-1 through AKT-pathway (PI3/AKT) [88]. Since these mutations apparently act pro-oncogenically, it is appealing to speculate that they all take place and are detectable at the stem cell level in patients with AML.

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T cell immunoglobulin mucin-3 (TIM-3), normally found as...
a surface molecule expressed in CD4+ Th1 lymphocytes in mouse haematopoiesis, is another promising target to eradicate AML-LSCs as it has been found to be expressed on LSCs but not on HSCs. TIM-3 functions as a regulator of macrophage activation as well as having a role in complement-dependent and antibody dependent cell-mediated cellular cytotoxic activities. When TIM-3 was targeted using monoclonal antibody treatment, it inclined to eradicate AML-LSCs without affecting normal HSCs in mouse models [99,100].

**Summary and Future Directions**

AML populations are consisted of hierarchical structure and in recent years it has been possible to begin analyzing individual constituents of the leukemic clone. Although, varying AML subtypes differentiate to differing levels, it has become increasingly evident that important similarities exit at the top of the developmental hierarchy.

Given the quiescent status of LSCs and their relatively low frequency, ablation of this population is likely to be a significant challenge. Despite the fact that a variety of LSC characteristics are almost identical to normal HSCs, recent studies of AML molecular biology and immunophenotypic characteristics suggest that some differences between normal and leukemic cells are apparent in the stem cell/progenitor cell pool [14,43,101,102]. From a therapeutic perspective, this observation is extremely important because it suggests LSCs do have unique characteristics that may make them preferentially sensitive to apoptosis/ablation. This information also serves to emphasize the importance of better understanding LSCs and how they differ from normal HSCs.

By establishing general parameters for induction of LSC apoptosis, it should be potential to develop more effective clinical therapies. Given the heterogeneity of mutations that give rise to these malignancies, the ability to target the malignant population is not likely to be achieved by a single specific inhibitor. To this end, it is fundamental to completely understand the signaling pathways that regulate survival and death in LSC populations. Current studies have started to characterize molecular mechanisms that may be relevant to LSC survival. However, more comprehensive methodologies using multiparameter or combined approaches should be the priority for future studies [12-15,32,33,56,102,103].

Combining different MoAbs may also target a larger proportion of the heterogeneous AML population, overcoming possible clonal selection, as well as evasion by epitope down regulation, as has been shown in lymphoma after treatment with CD20 targeting rituximab [104]. An AML patient surface immunophenotype is relatively cost-effective to characterize, raising the possibility of tailored therapy based on a selection of available MoAbs. Indeed, we are entering a new and exciting era in the struggle to improve outcome in adult AML.

**Acknowledgements**

The author would like to thank Avinash Daniel Pinto for assistance and input in improving the quality of this manuscript. The author would also like to acknowledge The Research Council of Oman (TRC) for sponsoring this work (Grant Number 47).

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