Recent Advances in Characterization of Pre-Leukemic/Leukemic Stem Cells in Acute Myeloid Leukemia

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Abstract

Acute myeloid leukemia (AML) is a heterogeneous disease organized as a hierarchy similar to normal hematopoiesis. It is maintained and progresses by self-renewing leukemia stem cells (LSCs) that are usually quiescent and resistant to current chemotherapies and thus, contribute to leukemia relapse. Recently, more ancestral pre-leukemic hematopoietic stem cells (HSCs) were identified, suggesting that accumulation of mutations occurs in self-renewing HSCs. The pre-leukemic/leukemic stem cell model proposes that a non-genetic mechanism in leukemic process, harmonized together with the genetic model and recently identified epigenetic dysregulations, contributes to leukemia heterogeneity and treatment resistance. Here we review the recent advances in characterization of AML LSCs, identification of pre-leukemic HSCs, and emerging concept of cell origin that contributes to AML relapse. These profound insights into AML biology have clinical importance and great implications for future development of new anti-AML therapies.

Keywords: Acute myeloid leukemia; Leukemia stem cell; Pre-leukemic hematopoietic stem cell

Introduction

Acute myeloid leukemia (AML) is a biologically, molecularly, and clinically heterogeneous disease with variant clinical outcomes. Despite advances in treatment over recent decades, except acute promyelocytic leukemia (APL, M3 by FAB classification), sustained remission and long-term survival have not dramatically improved with more than 70% of AML patients dying of disease relapse in less than 5 years. Development of new therapies depends on our understanding of AML biology and genetics. For example, fundamental discoveries of chromosomal translocation and molecular mutations (e.g. Flt3, NPM1 and epigenetic modifiers DNMT3a, TET2, IDH1/2) in AML not only allow clinicians subgroup patients with favorable and adverse outcomes for therapeutic options and choices following induction treatment, but also provide opportunities for therapeutic targeting [1]. In parallel to the continuous effort to investigate the genetic abnormalities for understanding the pathophysiology of AML, the nature and existence of leukemia stem cells (LSCs) in leukemia were also identified and extensively characterized in last two decades [2, 3]. LSCs sit at the apex of leukemia to maintain leukemia clones and contribute to leukemia relapse. Recent identification of pre-leukemic stem cells (pre-leukemic HSCs) in AML further elucidated the cell of origin and biological consequences of initiating events in AML. Investigation of genetic, epigenetic, and proteomic profiles in AML is summarized in other articles. This review will mainly focus on the key findings of AML LSC biology, pre-leukemic HSCs, to understand how AML is approached and progresses as a cellular hierarchy driven by the ancestral cells.

Characteristics of LSCs

Although for a long time scientists consider tumors may arise from cancer stem cells (CSCs), as multiple types of cancers feature normal tissue organization with heterogeneity in the function of malignant cells, the existence of CSCs was not formally proved until the 1990s. Using xenograft combined with fluorescence activated cell sorting to analyze different subpopulations of cells from AML patients, John Dick laboratory first isolated AML LSCs. They demonstrate that AML is organized as a hierarchy originated from a primitive LSC with self-renewal ability to repopulate leukemia in immu-

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Kemia blasts. By this definition, LSCs are different from leukemia-initiating cells which are only defined by their abilities to initiate leukemia but with or without self-renewal ability. LSCs are rare primitive cells usually enriched in CD34+38- population and share multiple properties with normal HSCs: CD34+38- immunophenotype, the capacity of self-renewal, cell-cycle quiescence, and requirement of microenvironment (stem cell niche). However, the origin of LSCs is not only primitive HSCs as LSCs are also present in other populations based on CD34 and CD38 expression [8]. Studies in mouse MLL-AF9 leukemia suggest LSCs can originate either from HSCs or from myeloid progenitors, with different disease behaviors depending on cell context [9-11].

**Quiescence, self-renewal and clonal evolution of LSCs**

While LSCs share similar properties with normal HSC counterparts making them difficult to be eradicated from patients, substantial investigations have characterized the properties specific for LSCs (Fig. 1). LSC compartments, similar to other CSCs, are heterogeneous at self-renewal potential with enhanced self-renewal ability and evolutionary properties following treatment or in vivo passages [12, 13]. Minor dormant LSC clones evolve and fluctuate, and become dominant clones to contribute to leukemia progression and relapse. MicroRNA-126 is one of the important regulators for LSC functions as Lechman et al showed that through targeting the PI3K/AKT/MTOR signaling pathway, microRNA-126 preserved quiescence, increased self-renewal and promoted chemo-resistance of LSCs. Dorrance et al showed that LSCs can be targeted in vivo using antagoniR-126 nanoparticles, resulting in LSC reduction [14]. Id2/E-protein axis was shown as another key LSC regulator to orchestrate mouse LSC self-renewal and differentiation for initiation and maintenance of MLL-rearranged AML [15]. Patients with MLL-rearranged AML who had higher Id2 gene expression had better survival than those with lower Id2 expression. In contrast, overexpression of Id2 expanded normal human HSCs, and reduction of Id2 expression induced lymphoid lineage priming, suggesting that Id2 plays a converse role in normal HSCs and LSCs [16].

**LSC cell-surface markers**

During leukemogenesis LSCs aberrantly express certain cell-surface markers, phenotypically distinguished from HSCs. The first immunophenotype found for LSCs is the production of interleukin-3 receptor α (CD123) antigen, which is highly expressed in both leukemic blasts and CD34+38- LSC population. In contrast, CD123 is minimally expressed in the normal HSC population from adult bone marrow, making Cd123 a unique and interesting target for eradicating LSCs [17]. Multiple anti-CD123 antibody-based strategies have been developed, which include modified monoclonal antibodies, bispecific antibodies or conjugates of anti-CD123 antibody, toxins or electron-emitting radioisotope, and the creation of chimeric antigen receptor (CAR) modified T cells [18-22].
Other identified LSC cell-surface markers such as TIM3, C96, CLL-1, CD25, CD32 and CD47 [20-25], also provide interesting targets for eliminating LSCs. Increased CD47 expression on LSCs and other cancer CSCs enhances its binding to the receptor Sirpa on macrophages and induces innate immune tolerance. Disruption of CD47-Sirpa interaction induces macrophage-mediated phagocytosis of LSCs and other CSCs [25, 26]. The identification of LSC-specific antigens provides promising targets for developing antibody-based immunotherapies against LSCs, although the success of immunotherapies remains to be determined in clinical trials.

Microenvironment of LSCs

LSCs reside in endosteal microenvironment to maintain stemness, quiescence and resistance to chemotherapy. Using an antibody targeting adhesion molecule CD44 on LSCs, Jin et al demonstrated that LSCs require interactions with supportive microenvironment for self-renewal and proliferation [27]. This study, in parallel with a similar observation in CML LSCs, provided clear evidence that disruption of the supportive component of LSC niche is a new avenue to eliminate LSCs [28]. In a mouse study, gonadal adipose tissue was observed as an alternative niche for fatty acid transporter CD36+ LSCs to evade chemotherapy [29]. However, a recent study showed that AML cells compromised adipocyte bone marrow niche and leukemia growth can be repressed through therapeutically induced BM adipogenesis [30]. Collectively, these findings and other observations [31] have elucidated the importance of stem cell niche for LSC function, providing new means for developing novel therapies to target LSC stemness at the root through modulating the LSC-niche interaction.

Genetic and epigenetic dysregulations in LSCs

By genetic models, genetic lesions that drive carcinogenesis for continuous cancer growth, including substantial chromosomal translocations and gene mutations have been found in AML cases and in LSCs [32, 33]. While genetic changes and their roles in leukemic process are well documented in AML, LSCs also create LSC-specific transcription-factor-driven gene expression programs. Multiple intrinsic signaling pathways associated with stem cells such as HOX genes, Wnt/β-catenin, Hh pathways and inactivation of p53 tumor-suppressor pathways have been found to be critical for LSC survival and proliferation [34, 35].

In addition to genetic abnormalities extensively investigated in AML and LSCs, recently numerous studies have revealed the important role of epigenetic dysregulations in AML and LSCs. Most of driver mutations found in early stages of leukemogenesis are genes associated with epigenetic regulations including DNMT3A and TET2 [33, 36, 37]. An LSC DNA methylation signature derived from xenograft was shown largely mutation independent and associated with poor prognosis [38]. A recent study by Cimmino et al showed that genetic restoration of TET2 function or treatment with vitamin C blocked aberrant self-renewal of LSC in mouse TET2-deficient leukemia and suppressed leukemia progression [39]. Lysine methyltransferase DOT1L causes aberrant H3K79 methylation in MLL-rearranged leukemia. Inhibition of DOT1L selectively impairs LSCs with very limited side effects on normal hematopoiesis [40]. Lysing demethylase LSD1 is highly expressed in patients with AML and other cancers [41]. Inhibition of LSD1 demethylase reactivates the all-trans-retinoic acid differentiation pathways in AML, resulting in the regression of AML growth in xenograft with impairment of repopulating secondary mice [42]. Collectively, these studies demonstrate that both major areas of epigenetics, DNA methylation and histone modification are functionally involved in LSC function for leukemia development and progression.

Clinical Relevance of LSCs

LSCs are mostly defined in experimental xenograft assays based on their capacities of generating and maintaining malignant clones in mice. Although some studies showed LSCs may contribute to failure of treatment, metastasis and poor survival, for example, the frequency of putative AML LSCs (CD34+38-) has a strong prognostic impact on patients’ survival but the neoplastic population (CD34+38+ and CD34-) shows no prognostic impact [43, 44], the clinical relevance and importance of LSC concept has long been questioned. Using gene expression microarray analysis data generated from functionally validated LSC population of AML samples in xenograft assays, Eppert et al created an LSC signature that shares a core transcriptional program with normal HSCs and is a highly significant independent predictor of patient survival [8]. Patients with LSC gene expression signature have poorer prognosis than those without LSC profiles. This study provides the first clear evidence that the LSCs identified using the xenograft mouse model for human AML are clinically relevant. This was further confirmed by gene and epigenetic expression profiles generated from different groups [45, 46]. To develop a better predictive biomarker for identification of AML patients with high risk, Ng et al generated a list of genes related to stemness from functionally defined LSCs using the same strategy Eppert et al adopted but with a larger number of samples [47]. An optimal 17-gene LSC score (LSC17) was further generated from the list of the genes through sparse regression analysis against survival in a large training cohort. The LSC17 score was highly prognostic and predictive of initial therapy response/resistance to current treatments including allogeneic stem cell transplantation. These studies demonstrate that the LSC model in AML is not only clinical relevant but also important for clinicians to better determine the prognostic risk and evaluate novel strategies to improve the survival of patients with high risks.

Pre-Leukemic HSCs in AML

Like other malignancies AML is also considered a clonal disease growing from a single stem/progenitor cell that has sequential acquisition of fewer but certain genetic mutations.
than solid tumors [48, 49]. This stepwise evolutionary leuke-
mogenesis generates inter-tumor genetic diversity and cellular
heterogeneity consisting of LSCs with variable self-renewal
capacities, committed colony-forming AML progenitors, and
non-proliferative blasts. Recently, a more ancestral pre-leuke-
mic HSC was identified as the new source and compartment of
AML heterogeneity [36, 50, 51]. Pre-leukemic HSCs must be
defined as a condition that a HSC has some but not all leuke-
mia-specific mutations that cause clonal hematopoietic expan-
sion without disease, but are associated with progression to
AML when additional leukemia-specific mutations acquired.
The existence of pre-leukemic HSCs was indicated by clonal
analysis over three decades ago [52, 53] and was extensively
studied in pediatric twin pairs with concordant and discord-
ant AML, evidenced by the discovery of the same genetic al-
terations in the twin patients or in the normal differentiated
cells of the non-leukemia twin [54, 55]. Yasuda et al reported
a donor-derived leukemia in patients after allo-transplant of
HSCs from a HLA-matched sibling but the donor whose blood
cells carried
\[\text{IDH2}\]
and
\[\text{DNMT3A}\]
mutations remained free of
leukemia 10 years after his donation, suggesting the presence
of subclinical pre-leukemic hematopoiesis [56]. A recent study
by Slush et al directly identified pre-leukemic HSCs in AML at
diagnosis. When large-scale deep sequencing was performed
on highly purified HSC progenitors and mature cells from
AML patients, HSCs bearing \[\text{DNMT3A}\] mutation resulted in
competitive multilineage repopulation advantage over non-
mutated HSCs in xenograft. \[\text{DNMT3A}\] mutations were found
present in stem/progenitor cells at diagnosis and remission,
with increased allele frequency in late remission suggesting
that pre-leukemic HSCs are chemo-resistant. In contrast, both
\[\text{DNMT3A}\] and \[\text{NPM1}\] mutations were widely found in all kinds
of cells from relapse, demonstrating that \[\text{DNMT3A}\] mutation
occurs earlier in HSCs, leading to clonal expansion of pre-leu-
kemia before full leukemia developed by additional leukemia-
specific \[\text{NPM1}\] mutation [36]. Pre-leukemic HSCs were also
found in patients with other mutations such as \[\text{TET2}, \text{IDH2}, \text{IKZF1},\]
and \[\text{ASXL1}\] followed by secondary leukemia-specific
mutations like \[\text{NPM1}, \text{FLT3ITD}, \text{JAK2}, \text{NRAS}\] for the de-
velopment of full leukemia in patients and in relapse [50, 51].
Collectively, the identification of pre-leukemic HSCs has elu-
cidated both the cell of origin for mutations and the order of
mutation acquisition in leukemogenesis toward development
of leukemia (Fig. 2). Studies that examined the important role
of pre-leukemic HSCs in leukemia and relapse provide broad
clinical implications. In addition to LSCs, the more ancestral
pre-leukemic HSCs in patients should also be monitored and
targeted for the prevention of leukemia relapse.

**Cell Origin of AML Relapse**

Despite achieving remission, majority of AML patients die
from relapse. It used to be generally accepted that the mecha-
низm for leukemia relapse was that leukemic cells acquire
drug-resistant mutations or alterations, either as a consequence
of the mutagenic effect of chemo-drugs or due to the intrin-
sic instinct of leukemia cells to generate genetic alterations
for drug resistance [57, 58]. However, drug-resistant leu-
kemia cells are recently considered to pre-exist and survive
from chemotherapy as pre-leukemic HSCs, LSCs and their
associated progenitors in the heterogeneous AML respond

![Figure 2. Simplified model of multistep leukemogenesis and leukemia heterogeneity. Acquisition of primary mutations mainly
\[\text{DNMT3A}, \text{TET2}, \text{IDH1/2}\] and others modifies NDA methylation status in normal HSCs, resulting in enhanced self-renewal and
clonal hematopoiesis (pre-leukemic HSCs). Pre-leukemic HSCs with the primary mutations are responsible only for clonal hemat-
opoiiesis or early stage of leukemic process which is not irreversible to leukemia transformation. Acquisition of secondary muta-
tions like \[\text{FLT3ITD}\] and \[\text{JAK2}\] provides pre-leukemic HSCs the ability of proliferation. Mutated HSCs with both self-renewal and
proliferation abilities are considered LSCs that create leukemia clones, produce daughter progenitors and blasts cells. Together
distinct types of cells form the complex leukemia heterogeneity. Differentiation programs should also be affected by the genetic
alterations in LSCs for accumulation of immature blasts in frank leukemia.](image-url)
Pre-Leukemic/Leukemic Stem Cells in AML

While the study by Slush et al first elucidates cell origin of relapse and provides key insight into leukemia pathophysiology, progression of leukemia to relapse could be more complicated than what we have discovered. In their study, not all patients fell into these two patterns of relapse, indicating that either a more

HSCs/progenitors at diagnosis and repopulate in NSG mice. In another instance, relapse variants detected by variant allele frequency are not observed in any primitive HSCs/progenitors at diagnosis or in repopulated NSG mice. Those relapse variants present in CD33+ leukemia blasts at diagnosis demonstrate that this type of disease relapse originates from phenotypically more committed leukemia cells. Investigations of gene expression profile exposed both groups of disease require stem cell properties for relapse, either LSCs directly giving rise to relapse or stemness transcriptional programs being retained or regained in more matured leukemia cells. In summary, identification of pre-leukemic HSCs by Shlush et al sheds light on the mechanisms underlying AML progression and relapse (summarized in Fig. 3).

Figure 3. Speculated mechanism of leukemia relapse. Relapse can happen at any time in AML patients with complete remission (CR), from as early as a couple of months to a few or up to 5 years since CR is achieved. Proliferation of different types of residual cells in a patient’s BM may contribute to relapse. To summarize the studies by Shlush et al, relapse could arise from a rare pre-leukemic HSC (e.g. DNMT3A) that needs to acquire additional mutations (e.g. NPM1) to become LSCs and result in relapse (a), or rare dormant LSCs that are resistant to chemotherapies proliferate for leukemia relapse (b). Relapse from rare pre-leukemic HSCs or LSCs takes longer time than from more committed progenitor cells (c). Not all the patients tested in the studies by Shlush et al fit into the categories of cell of origin for relapse, indicating other mechanisms may be also involved in relapse (d).
sensitive sequencing technology needs to be adopted for detection of rarer relapse variants, or other types of cells or genetic events are responsible for leukemia relapse (Fig. 3). Also, the phenomenon of relapse from committed mature leukemic cells suggests that either founder dominant AML clones are incompletely eradicated by inductive therapies, or they inherit chemoresistance from their ancestral stem cells at diagnosis.

Conclusions

Like other solid cancers, AML also results from multistep pathogenesis, related to cytogenetic abnormalities, point mutations, aberrant gene expression, and epigenetic dysregulations. Numerous studies on AML LSC biology have indicated that non-genetic mechanisms together with genetic and epigenetic alterations are involved in leukemogenesis and leukemia progression. This review mainly summarized recent advances of studies on LSC properties to elucidate the non-genetic machinery and their clinical implications. Identification of more ancestral pre-leukemic HSCs provides key insight into the cell origin of leukemic initiation and the order of mutation acquisition during leukemia development. Genes that are first mutated are usually the ones (e.g. DNMT3A, TET2, IDH1/2, and ASXL1) involved in epigenetic regulation or cellular metabolism in HSCs causing defect in normal hematopoiesis and clonal evolution. Additional late mutations (e.g. FLT3-ITD, NPM1, JAK2, and NRAS) are more leukemia specific and usually contribute to leukemic cell growth through constitutive activation of signaling pathways for development of frank leukemia. The recognition of persistence of LSCs and pre-leukemic HSCs in leukemia development and remission, and their contribution to relapse will prompt the development of new therapies for the eradication of these ancestral malignant cells. Multiple inhibitors have been produced to target those mutations and are in the process of clinical trial but impressive efficacies like the phenomenon of relapse from committed mature leukemic cells at diagnosis.

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