Eradiation of Leukemia Stem Cells as a New Goal of Therapy in Leukemia

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Abstract

Leukemias have traditionally been classified and treated on the basis of phenotypic characteristics, such as morphology and cell-surface markers, and, more recently, cytogenetic aberrations. These classification systems are flawed because they do not take into account cellular function. The leukemia cell population is functionally heterogeneous: it consists of leukemia stem cells (LSC) and mature leukemia cells that differentiate abnormally to varying extents. Like normal hematopoietic stem cells, LSCs are quiescent and have self-renewal and clonogenic capacity. Because they are quiescent, LSCs do not respond to cell cycle-specific cytotoxic agents used to treat leukemia and so contribute to treatment failure. These cells may undergo mutations and epigenetic changes, further leading to drug resistance and relapse. Recent data suggest that mature leukemia cells may acquire LSC characteristics, thereby evading chemotherapeutic treatment and sustaining the disease. Ongoing research is likely to reveal the molecular mechanisms responsible for LSC characteristics and lead to novel strategies for eradicating leukemia.

The hematopoietic system is thought to originate from pluripotent hematopoietic stem cells (HSC) capable of producing a hierarchy of downstream multilineage and unilineage progenitor cells that differentiate into mature cells (1, 2). HSCs have self-renewal and clonogenic abilities and can differentiate into multiple lineages. HSCs with the capacity for both long-term and short-term repopulation of the mouse hematopoietic system have been characterized. These rare cells give rise to progenitor cells, which are destined to generate fully differentiated cells. HSC self-renewal is thought to be either symmetrical, producing two daughter HSCs, or asymmetrical, producing an identical HSC and a progenitor with diminished self-renewal capacity but with the ability to enact clonal expansion and maintain the circulating blood cell population (1–3).

It has recently been hypothesized that leukemogenesis, and, for that matter, carcinogenesis, arises from neoplastic stem cells. Leukemia stem cells (LSC) exhibit characteristics similar to those of normal HSCs and are thought to arise from normal stem cells through the accumulation of oncogenic insults (4, 5). However, LSCs may also arise from differentiated progenitor cells that have reacquired the capacity for self-renewal (6–8). Like normal HSCs, LSCs give rise to differentiated daughter cells that lose their self-renewal capacity. However, defects in their cellular machinery usually eliminate their ability to differentiate fully into morphologically and phenotypically mature cells.

As a result, the leukemic population consists of undifferentiated and variably differentiated leukemia cells. The degree of differentiation of leukemia cells has traditionally formed the basis of the morphologic classification of leukemias (9, 10).

The generally accepted paradigm of leukemogenesis rests on the theory that leukemia arises from a single cell and is maintained by a small population of LSCs (5, 11, 12). Studies of acute myeloid leukemia (AML) have shown that only 0.1% to 1% of AML cells have the capacity to initiate leukemia when injected into severe combined immunodeficient mice (4, 13). It has long been recognized that, like solid tumors, AML consists of a heterogeneous population of cells with a small percentage of noncycling, quiescent cells (14, 15). Two models of leukemogenesis have long been proposed. According to the stochastic model, leukemia consists of a homogeneous population of immature cells and a few cells that can either self-renew or proliferate in a stochastic manner (3, 16, 17). According to the hierarchy model, leukemia consists of a heterogeneous population, within which only a small percentage of LSCs sustain the disease. Recently, a third model was proposed. According to this model, mature leukemia cells can dedifferentiate and regain LSC capacity (6, 8, 18). Whereas the first two models hold that only LSCs sustain leukemia, the third model allows that mature cells can regain self-renewal capacity, thereby sustaining the disease.

Despite the development of multiple new agents that are effective at reducing the tumor burden in patients with leukemia, relapse continues to be the most common cause of death, particularly in patients with AML. Current efforts directed at detecting and quantifying minimal residual disease are based on the assumption that eradication of all cells capable of sustaining the disease will improve treatment outcomes. Modern techniques for detecting minimal residual disease, such
as PCR, are not sensitive enough to detect all residual leukemic cells. Therefore, a state of absolute disease eradication cannot be determined with the available tests. For example, disease recurrence has been reported after discontinuation of therapy with imatinib mesylate in patients with chronic myelogenous leukemia who have achieved a complete molecular remission (19). On the other hand, minimal residual disease will not lead to disease recurrence if the residual leukemic cells are not disease-sustaining or if the residual LSCs remain dormant (20, 21). Here, we examine the role of LSCs as applied to treatment strategies aimed at curing leukemia.

**Defining LSCs**

The phenotypic and functional properties of normal HSCs have been extensively studied (22, 23). Several combinations of cell-surface markers, such as Lin−/CD34+/CD38−/low, have been used to identify populations that are enriched with HSCs (24). Other techniques include the extrusion of Hoechst dye 33342 to identify side population cells, the use of immature cell-surface markers such as CD133 and CD150, and the use of Bodipy aminoacetaldheyde to assess aldehyde dehydrogenase activity (25–30). It was shown that, for most AML subtypes (with the exception of acute promyelocytic leukemia), the cells that initiate the disease in non-obese diabetic/severe combined immunodeficient mice have a CD34+/CD38− or CD34+/CD38−/low phenotype (4, 31). However, leukemic cells bearing these phenotypes are dissimilar functionally and include both short-term and long-term leukemia-initiating cells (Fig. 1). Several studies have suggested that HSCs and LSCs share some cell-surface markers but not others. For example, HSCs and LSCs both express CD34 but not CD71 and HLA-DR. However, Thy-1 (CD90) and c-Kit (CD117) are expressed on HSCs but not on LSCs, and CD123 and interleukin-3 receptor-α are expressed on LSCs but not on HSCs (31–33). Remarkably, cytogenetically abnormal leukemia-initiating cells have been found in the CD34+/CD90− population from several patients with AML and, in rare cases, CD34− cells as well as CD34+ cells have successfully engrafted and initiated human leukemia in mice (34–36). In acute promyelocytic leukemia, unlike in other myeloid leukemias, the characteristic translocation has been observed in CD34+CD38− but not in CD34−CD38− cells (4, 37). Therefore, a universal phenotype for LSCs may not exist and patient-to-patient variations in cell-surface protein expression may be the rule.

Of note, a similar heterogeneity exists in HSC gene expression: genes thought to form a stem cell gene signature have been identified; however, dissimilar data suggest that the existence of such a signature may be premature (38–40). Because stem cell–specific properties, such as self-renewal, quiescence, and proliferation, are not governed by genes that are specific to stem cells, it was proposed that there is a “stem cell state” rather than a “stem cell portrait” in the hematopoietic system (41). The stem cell state may represent a transient and potentially reversible state that cells can assume in response to the correct trigger. Using a similar concept, one can argue that there is an “LSC state” rather than an “LSC phenotype,” a concept that is supported by the well-described lineage “infidelity” in human leukemias (42). Although defining the sets of conditions that pertain to the LSC state may allow the development of strategies to eliminate LSCs or render them inconsequential, the identification of specific genes and surface markers expressed solely by LSCs may be difficult or impossible.

**Molecular Pathways Regulating HSCs and LSCs**

It is generally accepted that self-renewal is the hallmark property of stem cells in both normal and neoplastic tissues. Recent research has delineated molecular pathways that regulate the self-renewal capacity of HSCs. Overexpression and knockout experiments have identified several genes, transcription factors, and cell cycle regulators that modulate the self-renewal and differentiation of HSCs (43, 44). Genes such as SCL, GATA-2, LMO-2, and AML-1 (also known as CBFA2 or RUNX1) govern the transcriptional regulation of early hematopoiesis, and the deregulation of these genes through chromosomal aberrations leads to the genesis of several hematopoietic malignancies. For example, the gene encoding the transcription factor SCL is the most frequent target of chromosomal rearrangements in children with T-cell acute lymphoblastic leukemia (45). SCL is normally expressed in HSCs and immature progenitors and is down-regulated as differentiation proceeds. As a result of chromosomal translocations, SCL is inappropriately expressed and, through collaboration with other oncoproteins, initiates malignant transformation (46). Similarly, transcriptional activation of the AML-1 gene is required for definitive hematopoiesis. As a result of translocation t(8;21), the fusion protein AML-ETO, one of the most frequent chromosomal abnormalities in AML, is generated (47). Constitutive expression of AML-ETO has been shown to increase the frequency of self-renewal in stem cells (48). Of interest, such increased self-renewal is of no apparent pathogenic consequence, presumably because secondary mutations are necessary for the expression of the leukemic phenotype (49).

Other transcription factors such as the Homeobox (Hox) genes, including HoxB4, and the Wnt signaling pathway have
well-described roles in regulating the self-renewal and differentiation of HSCs whereas they retain their ability to differentiate into normal lymphoid and myeloid cells (51). It is abundantly expressed in HSCs but declines as terminal differentiation proceeds (51). Of note, deregulated expression of Hox family members such as HoxA9 is commonly observed in AML (52, 53). The Wnt signaling pathway has been shown to be critical to the development of several organs and recent studies have illustrated its important role in the regulation of hematopoietic stem and progenitor cell function (35, 54). Overexpression of β-catenin, a downstream activator of the Wnt signaling pathway, expands the transplantable HSC pool in long-term cultures (3). Furthermore, activation of Wnt signaling also increases the expression of other transcription factors and cell cycle regulators important in HSC renewal, such as HoxB4 and Notch-1 (35, 55).

The Notch/Jagged pathway is important in regulating the integration of extracellular regulatory signals controlling HSC fate (56). Ligand binding leads to proteolytic cleavage and transport of the intracellular domain of Notch into the nucleus, where it functions as a transcription activator. Members of the Notch family have critical roles in keeping HSCs in an undifferentiated state and may act as a gatekeeper for factors governing self-renewal and lineage commitment (57). Of interest, the gene encoding the Notch receptor was originally identified as the gene rearranged by recurrent chromosomal translocations in some patients with T-cell acute lymphoblastic leukemia (58). Other transcription factors and cell cycle regulators associated with oncogenesis, such as Bmi-1 and Sonic hedgehog (Shh), may play roles in the regulation of proliferation of both HSCs and LSCs (59, 60). Bmi-1 is a member of the Polycomb family of genes thought to be responsible for the preservation of gene silencing (61). It is highly expressed in purified HSCs and its expression declines with differentiation (62). It seems to regulate stem cell renewal by modulating other genes that are important in cellular functions such as proliferation, survival, and lineage commitment (62). Bmi-1 has an essential role in regulating the proliferative potential of leukemic stem cells (63). Although direct evidence for the role of Shh in the regulation of stem cell renewal is lacking, in vitro studies have shown increased self-renewal of HSCs in response to Shh albeit in combination with other growth factors (64).

Therefore, differential expression of several transcription factors controls the fate of HSCs and plays a critical role in the determination of self-renewal, differentiation, and lineage commitment. These pathways are under the control of various intracellular stimuli as well as cytokines and stromal factors from adjacent cells in the bone marrow microenvironment. Further studies of these transcription factors in HSCs and the mechanisms causing their deregulation are likely to provide us with better targets for development of disease-specific therapies.

The Role of LSCs in Drug Resistance and Relapse

Normal HSCs possess several characteristics that protect them from potential insults. LSCs have similar properties, including quiescence (65), resistance to drugs and toxins through the expression of ATP-associated transporters (66), and resistance to apoptotic stimuli (67). Data accumulated in recent years suggest that a small population of LSCs, through their HSC-like properties, survive chemotherapy and sustain the disease (3, 66). High levels of ATP-binding cassette transporters have been reported in both normal and cancer stem cells (68, 69). Indeed, HSCs, but not lineage-committed progenitors, express high levels of genes responsible for multidrug resistance-related transporters (70). As mentioned earlier, the efflux of fluorescent dyes such as Hoechst 33342 has been used to isolate HSCs (25, 71). Similarly, LSCs either inherently possess drug resistance mechanisms or acquire them through mutations (66).

Other properties of LSCs are also likely to contribute to drug resistance and relapse. Stem cell progenies such as long-term culture-initiating cells and AML colony-forming units are in an active cell cycle (72, 73) whereas several studies have clearly shown that LSCs remain quiescent (65, 74–76). For example, a study by Guzman et al. (75) showed that as much as 96% of the LSC population, as defined by the phenotype CD34+CD38+CD123+, were in the G0 phase of the cell cycle. This resting status of the putative LSCs protects them from the commonly used cell cycle–specific chemotherapeutic agents.

It is likely that secondary events, such as the development of mutations, further contribute to the intrinsic resistant properties of LSCs. In a multistep pathogenic process, quiescent LSCs may carry the initial mutagenic event leading to genomic instability and the induction of secondary mutations that are responsible for a more resistant phenotype. Alternatively, random secondary mutations or mutations occurring as a result of selective pressure caused by therapy may contribute to disease progression or resistance. This has been seen in patients with chronic myelogenous leukemia (CML) treated with imatinib mesylate, in whom mutations of the ATP-binding site of BCR-ABL are well documented (77).

Strategies to Overcome LSC Resistance to Therapy

As implied by the multistep theory of carcinogenesis, LSCs are likely to have the fewest number of molecular aberrations in the population of the malignant cells and, as such, biologically most similar to normal HSCs. The primary challenge in developing treatment strategies targeted toward LSCs is to identify proapoptotic stimuli that spare the normal HSCs while exerting the desired effect on LSCs.

A primary concern in the development of tumor stem cell–specific drugs is to overcome the inherent drug efflux pumps that are highly expressed in LSCs. Several agents effective in inhibiting the ATP-binding cassette transporters have been studied and found to have limited clinical efficacy (78, 79). The biggest obstacle to this approach is the similarly high expression of these transporters in normal HSCs, making them equally susceptible to the inhibitors (66). As such, strategies directed at pathways that specifically regulate LSC survival would probably be more fruitful (80). The search for identification of survival pathways that are preferentially overexpressed in LSCs is ongoing and several recent studies have described means of differential activation of apoptosis mechanisms in LSCs (75, 81, 82).

The transcription factor nuclear factor-κB was found to be constitutively activated in LSCs but not in normal HSCs (75). Therefore, nuclear factor-κB inhibitors were added to
antileukemic agents such as idarubicin in experimental models (75, 81). Recently, single-agent parthenolide, a potent inhibitor of nuclear factor κB, was found to induce apoptosis in AML and CML LSCs and progenitors while sparing normal HSCs (82). Notably, parthenolide was much more selective in eliminating LSCs and sparing normal hematopoietic cells than was the standard chemotherapy agent cytarabine (82). Constitutive activation of the phosphatidylinositol-3 kinase is also necessary for the survival of LSCs and its pharmacologic inhibition by LY294002 leads to a dose-dependent decrease in survival (83).

The fate of LSCs depends on the relative expression of transcription factors and their regulation, usually by aberrant signaling pathways (84, 85). Although it has been proposed that recruitment of LSCs from the G0 to the S phase of the cell cycle might contribute to their eradication by cell cycle–specific cytotoxic agents, it is possible that prolonging the “quiescent phase” could be beneficial. One could envision a scenario in which LSCs are maintained in a state of hibernation, thereby prolonging relapse-free survival. The best evidence for this possibility is the existence of patients with clinically distinct “indolent” and “proliferative” forms of AML as well as the wide variation in the duration of relapse-free interval in individual patients.

Future Directions
Over the past several decades, the mainstay of leukemia therapy (and treatment of other tumors) has been to induce a complete remission and to consolidate this with further courses of chemotherapy. Recently, flow cytometry and PCR have been used to monitor minimal residual disease. The clinical benefits of early identification of minimal residual disease using these techniques remain uncertain and are under investigation. Despite significant progress, relapse continues to be the most prominent factor responsible for the failure of therapy in leukemia, particularly in AML. Recent insights into the nature of normal and malignant stem cells have led to the identification of quiescent and drug-resistant LSCs as the likely minimal residual disease candidates responsible for relapse. In the appropriate setting, assays such as PCR may identify residual cells expressing leukemia-specific molecular abnormality; however, these techniques cannot currently distinguish LSCs from their more mature progeny. As such, further research should be focused on better defining the LSC state.

Characterization of the molecular and biological features of the cells that initiate and maintain leukemia is an essential step in the development of novel agents effective against the disease. Skeptics may ignore the above-mentioned evidence and argue that LSCs are simply leukemia cells previously referred to as resting in the G0 phase of the cell cycle. Others may concentrate their efforts on identifying a “stem cell phenotype,” thus ignoring the “stem cell state” and the potential fluidity of LSCs (a process in which the leukemia progenitors regain stem cell properties). Future studies of LSC biology, concentrating on the function of LSCs rather than strictly on their phenotype, are likely to identify new molecular targets and effective treatment modalities directed at LSCs.

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