Extrinsic targeting strategies against acute myeloid leukemic stem cells

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Abstract

Despite advances in the treatment of acute myeloid leukemia (AML), patients still show high relapse and resistance against conventional chemotherapy. This resistance is related to a small clone referred to as Leukemia Stem Cells (LSCs). New targeted strategies are directed against the LSCs' extrinsic regulators including their microenvironment such as a CXCR4 antagonist that is used to interfere with LSCs' homing. Targeting LSCs' surface molecules such as CD33 for selective elimination of LSCs has variable degrees of success that may require further assessments. Trials with CARs cells were effective in eradication of acute lymphoblastic leukemia, and they may have an effective role also in AML. Other strategies are directed against the intrinsic regulators such as self-renewal mechanisms and epigenetic reprogramming of LSCs. This review highlights targeting of the extrinsic regulators of the LSCs and identifies biological differences between them and normal hematopoietic stem cells.

Introduction

Acute myeloid leukemia (AML) is a hematological disorder characterized by a malignant clone thought to be derived from a small number of cells known as leukemic stem cells (LSCs). LSCs have a great ability for limitless self-renewal and also generation of leukemic progenitors that actively divide and produce a large number of blasts [1].

Most AML patients treated with conventional chemotherapies can achieve remission, but about 60% of remission patients still relapse. AML relapse may originate from LSCs (the chemo-resistant clone), and therefore targeting LSCs should be a main aim in the search for a cure for human AML. LSCs and normal hematopoietic stem cells (HSCs) share different properties and surface markers, so it is necessary to identify a signaling pathway that is specific to the AML LSC stage to eliminate them without damaging normal HSCs [2]. For targeting LSCs, it is important to overcome the properties that make LSCs resistant to therapy such as quiescence in the bone marrow niche and ability for limitless self-renewal [3].

Different trials have been performed to isolate LSCs, and it was reported that LSCs were present within the CD34+/CD38- clone; this clone is known to establish human AML in a xenograft model. In contrast, it has been observed that CD34- clones from the majority of nucleophosmin-1 (NPM-1) mutated AML are able to initiate human AML in a xenograft model. Thus, the isolation of LSCs on the basis of their phenotypic expression alone might not be enough [4].

In this review we present the newer therapeutic approaches that target the extrinsic LSC regulators, including manipulation of LSCs' microenvironment. Cellular therapy depending on genetic engineering of T cells with chimeric antigen receptors (CARs) showed good results in pediatric acute lymphoblastic leukemia (ALL) and may have a role in the management of AML [5]. Other trials that utilized surface markers achieved different levels of elimination of AML [6].

LSCs theory and properties

LSCs are able to divide to progeny clonogenic blast cells, leading to the concept that AML is arranged in a hierarchy, with the LSCs present at the apex and the more "differentiated" blasts representing the main tumor bulk [7]. Some researchers argue that normal HSCs are the "cells of origin" for LSCs as a result of the similarity between normal and leukemic stem cells [8].

In general, LSCs widely differ in their self-renewal potential (long-term and short-term LSCs), demonstrating that the LSC still retains some aspects of normal HSC properties. This information supports the concept that HSCs are the cellular targets for transformations, not progenitors or the blast population. LSCs and HSCs share several properties that render them as stem cells, especially limitless self-renewal activities. With the discovery of the role of Bmi-1 as a key regulator for self-renewal mechanisms in LSCs, the stem cell model of leukemogenesis was given a strong boost [7].

Other researchers had argued that non-stem cells could be converted into LSCs if the correct self-renewal machinery were activated in these cells. Using retrovirus vectors with fusion oncogene MLL-AF9 in murine models provided evidence that the cell of origin of LSC may be a progenitor, related to the more phenotypically mature cells in comparison to the primitive HSC [9]. With regard to retroviral transformation in a murine model, LSC also did not express the stem

cell antigen Sca1 and had an immune phenotype more related to normal granulocyte-macrophage progenitors (GMP) [7].

So from those two models, the transforming events (*i.e.,* oncogenic mutations) may occur in either HSC (long-term HSC) or in progenitors (such as GMP) [8]. In relation to the long-term HSC, mutations may give rise to a pre-leukemic condition associated with expansion of HSCs and increase the genetic instability. Second hit (genetic event) will be needed to develop the full leukemic phenotype (Figure 1) [10]. However, mutations that occur within the progenitors may confer the property of limitless self-renewal to those cells (e.g., expression of the MLL-AF9 fusion oncogene), giving rise to the full leukemic phenotype (Figure 1) [8].

Relevance of bone marrow microenvironment

Bone marrow microenvironment is important for HSCs and LSCs. Two anatomical HSC niches that may have overlapping roles can be defined: the endosteal niche [11] and the perivascular niche [12]. Osteoblast, osteoclasts, and osteopontin are essential for regulation of HSC count and engraftment within the niche [13]. Osteoblast in endosteal niche provides essential support to HSCs because the manipulation of osteoblasts could increase HSC number [14]. Other observations support that abnormal osteoblast has no effect on HSCs. For example, the increase of the number of osteoblasts under treatment with strontium was not associated with effects on HSC numbers [15]. In contrast, the perivascular niche depends on cell/cell interactions. The interactions between chemokine receptor 4 (CXCR4) on HSCs' surface and chemokine ligand 12 (CXCL12, also known as stromal-derived factor 1 alpha, SDF1-α) that expressed on mesenchymal stromal cells, and also the interactions between integrins (such as VLA-4) on HSCs and VCAM-1 on stromal cells [16] appear to be essential for HSCs' homing [17].

The bone marrow microenvironment appears to be responsible for protecting LSCs by keeping them at quiescent state since many chemotherapeutic drugs target actively cycling cells [18]. LSCs close to the endosteal cells appear more quiescent and more resistant to the conventional chemotherapy drug cytarabine [18]. Manipulation of this microenvironment may be effective in the elimination of LSCs [19].
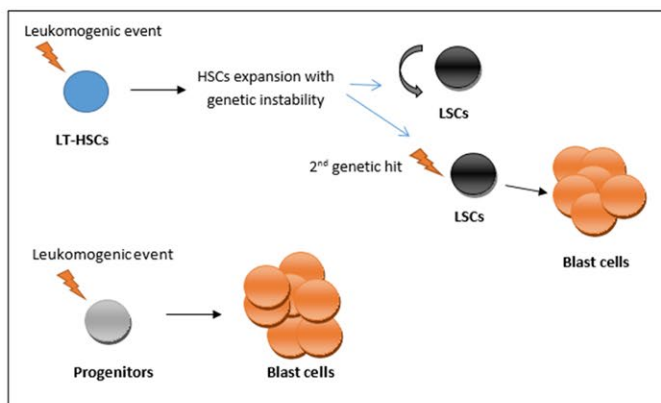

Figure 1. Leukemia stem cells (LSC) hierarchy
The transforming events (*i.e.,* oncogenic mutations) may occur in either long-term hematopoietic stem cells (LT-HSC) or in progenitors (such as granulocyte-macrophage progenitors). In relation to the LT-HSC, mutations may give rise to a preleukemic condition associated with expansion of HSCs and increase the genetic instability. Second hit (genetic event) will be needed to develop the full leukemic phenotype. On the other hand, mutations that occur within the progenitors may confer the property of limitless self-renewal to those cells (e.g., expression of the MLL-AF9 fusion oncogene), giving rise to the full leukemic phenotype.

Strategies for AML treatment

Targeting LSCs is the new hope to eradicate AML without harm to normal HSCs. It is important to overcome the properties that make LSCs resistant to therapy such as their ability for limitless self-renewal and quiescence in bone marrow niche [20].

Disrupting LSCs' microenvironment

LSCs are able to receive and send several signals within bone marrow niche that play an important role in maintaining their quiescence. Several factors and signals such as Wnt/β-catenin and CXCL12/SDF1-α are involved in the relation between bone marrow microenvironment and LSCs. Also, the bone marrow niche is composed of both well vascularized zones and hypoxic zones that may affect LSCs' activity [21].

LSCs have demonstrated over-expression of CXCR4, which is related to a poor prognosis. Inhibition of the CXCR4–SDF-1 interaction was noticed to decrease the engraftment of the Nalm-6 (ALL cell line), after the transplantation in mouse bone marrow [22].

AMD3465 is a CXCR4 antagonist that is used to interfere with CXCR4/SDF1-α and demonstrates a good effect in addition to conventional chemotherapy [6]. AMD3465 inhibits stromal cell-induced pro-survival signals in AML cells. Also, it was shown that AMD3465 increases the sensitivity of Flt-3-mutated AML cells to sorafenib (Flt-3 inhibitor). Both mobilization of AML cells into the peripheral circulation and the increased sensitivity to sorafenib decreased the AML burden in mice following sorafenib treatment [23]. AMD3100, another CXCR4 antagonist, was shown to increase the mobilization of murine acute promyelocytic leukemia cells. The combination of AMD3100 with chemotherapy diminished AML tumor burden and increased mice survival compared with chemotherapy alone [24].

Interaction between integrin VLA-4 on the LSCs and fibronectin on the stromal cells is associated with activation of the PI3K pathway (pro-survival) in AML cells. The effect of interfering with the interaction between VLA-4 and fibronectin and its role in sensitizing AML cells to drugs (e.g. cytosine arabinoside) has been demonstrated by administration of VLA-4 neutralizing antibodies that eliminate the relative resistance of VLA-4 (+) AML cells to drug-induced apoptosis [25]. The effector of the previous interactions, the Rac family (especially Rac1 and Rac2), also may be a target to inhibit LSCs' homing in bone marrow niche. The Rac1 inhibitor NSC23766 has been found to succeed in delaying leukemia development in a mouse model [26].

Leukemic cells have been shown to express vascular endothelial growth factor (VEGF) and its receptor VEGFR-2. Recent studies illustrated the autocrine and paracrine functions of VEGF that mediate leukemic cell proliferation and the role of anti-VEGFR-2 in inhibition of leukemic propagation in a NOD/SCID xenograft model. Thus, VEGFR-2 signal targeting may be a promising strategy against AML in the near future [24].

Inhibition of LSC homing by blocking the interleukin-3 (IL-3) receptor has been tested. *Ex vivo* culture of LSCs with anti-IL-3 α chain neutralizing antibody, 7G3, significantly reduced the engraftment of transplanted AML cells in NOD/SCID mice, resulting in prolonged survival. However, transplantation of cord blood hematopoietic cells after co-culture with 7G3 did not affect the engraftment in comparison with AML-treated cells, which related to the significantly lower expression of IL-3 on CD34+CD38-HSCs compared with LSCs

[27]. CD44, a hyaluronic acid receptor, is also one of the important hematopoietic stem and progenitor cell homing receptors. Treatment with an anti-CD44 antibody resulted in LSC mobilization in both a mouse model of chronic myelogenous leukemia and a xenograft model of human AML [28].

Another strategy depends on interfering with the ability of LSCs to thrive within the bone marrow microenvironment. Hypoxia inducible factor-1 alpha (HIF-1α) is a transcription factor that is induced in hypoxic conditions such as those typically found in the bone marrow microenvironment [6]. A HIF-1α inhibitor, echinomycin, was shown to eliminate leukemia stem cells in a xenograft transplant model without marked damage to the normal HSCs. Other HIF-1α inhibitors such as EZN-2968 and PX-478 are currently in clinical trials [29]. Other researchers have studied forced cell cycle induction by interferon-alfa (IFN-α) that will decrease LSCs' resistance to treatment with chemotherapeutic agents such as 5-fluorouracil by overcoming their quiescence state [30].

Raaijmakers *et al.* demonstrated that deletion of certain bone marrow mesenchymal cells' genes such as the Schwachmann–Bodian–Diamond syndrome gene led to development of myelodysplasia and AML in mice. These results strongly suggest that the development of certain hematologic malignancies cells may be derived by the transformation of bone marrow niche cells [31].

Targets expressed on the surface of AML LSCs (Monoclonal antibody)

CD33: Many trials have been designed to target CD33. The Gemtuzumab Ozogamicin (GO, Mylotarg®) trial tested a humanized anti-CD33 monoclonal antibody (mAb) conjugated with calicheamicin (DNA-damaging toxin) [32]. However, recently this drug was withdrawn because of its toxic effects on normal cells. The presence of CD33- LSCs and CD33+ normal HSCs has been reported; this observation lowers the therapeutic role of the anti-CD33 antibodies [33]. The remission rate can be increased with a combination of GO and valproic acid (epigenetic modifying drug) thereby facilitating the intercalation of calicheamicin with DNA, followed by induction of synergistic apoptosis (Table 1) [21].

CD123: Several trials to target CD123 have had successful results related to the mAb anti-CD123 (7G3) (Table 1) [27]. Use of fusion protein 26292(Fv)-PE38-KDEL, consisting of a CD123-specific single chain fragment of the variable regions (scFv) antibody that is fused to a pseudomonas exotoxin A fragment (PE38), had a potent apoptotic effect on different CD123+ leukemia cell lines [34]. Other 7G3-derived recombinant mAbs, CSL360 and CSL362, had a more potent effect [5]. The recombinant immunotoxin DT388IL3, consisting of a truncated diphtheria toxin fused to human IL-3 ligand, was studied but the clinical results were not promising [35]. The expression of CD123 on both normal HSCs and regenerating bone marrow was reported, so that the combination with other target markers may be safer [33].

CD44: CD44 plays an important role in engraftment of LSCs and HSCs [36]. Because the expression of CD44 is high in LSCs, targeting of CD44 (using the H90 mAb) might impair the LSCs' engraftment in the bone marrow (Table 1) [28]. Jin *et al.* showed that treatment with H90 significantly increased survival of NOD/SCID mice transplanted with AML LSCs (CD34+CD38-) and reduced the LSC burden in the bone marrow of those mice. In contrast, the leukemic cells obtained from primary mice (H90 treated mice) failed to engraft into the secondary recipient mice [28]. Zhou and Chng explained the efficacy of the anti-CD44 mAb to eliminate LSC by three mechanisms: induction of leukemic cell differentiation, inhibition of cell proliferation, and disruption of LSCs' homing [37].

Table 1. Leukemia Stem Cell (LSC) markers.

Markers	Function	Distribution	Expression		Targeting	Mechanism
			HSCs	LSCs		
CD123	High affinity IL-3 receptor (IL-3α)	Mainly, hematopoietic cells (myeloid lineage) Also expressed by regenerating bone marrow	Absent	Present	7G3 (mouse IgG2a)	Inhibits homing and engraftment to the bone marrow niche
CD44	Signal transduction Mediates cell adhesion, key regulator of AML-LSCs homing to bone marrow microenvironment Cancer stem cell marker on a number of solid tumors	Widely expressed at low levels	Present in low levels	Highly expressed	H90 (mouse IgG1)	Inhibits homing and engraftment to the bone marrow niche Blocks signal transduction
CD47	Inhibits phagocytosis via CD47/SIRPa interaction (Don't eat me strategy)	Widely expressed at low levels	Absent	Present	Monoclonal Ab (mAb) against CD47	Allows phagocytosis process
CLL-1	Unknown (may be involved in intracellular activating and inhibiting pathways)	Mainly, hematopoietic cells (myeloid lineage)	Present in low levels	Highly expressed	Monoclonal Ab (mAb) against CD47	Inhibits homing and engraftment to the bone marrow niche - Blocks signal transduction
CD96	May have a function in NK cell adhesion and/or activation	Resting and activated T cells and NK cells, possibly intestinal epithelium	Present in low levels	Highly expressed	Monoclonal Ab (mAb) against CD96	Activation of antibody dependent cell-mediated toxicity
CD32	Fc-g receptor 2 (FCGR2)	Mainly, to hematopoietic cells Not expressed on functional HSC	Absent	Present		
CD25	High-affinity IL-2 receptor (IL2RA)	Mainly, to hematopoietic cells	Absent	Present		
TIM-3	Appears to have immune regulatory function, as controlling macrophage activity and controlling antibody dependent and complement-dependent cell-mediated cellular cytotoxicity	T cells, Th-1, Tc-1 Macrophage, dendritic cells	Absent	Highly expressed		

CD47: CD47 is a transmembrane protein that interacts with signal regulatory protein (SIRPα) on phagocytes [38]. CD47 presents also in high expression in LSCs and has an important role in protection of LSCs from phagocytosis (Table 1). The interaction between CD47 of LSCs with SIRPα on phagocytes is a defense strategy ("Don't eat me strategy"), enabling LSCs to escape phagocytosis, so targeting of CD47 has strongly enabled phagocytosis of LSCs [39]. Anti-CD47 mAbs have been used in xenograft mice models to block the CD47/SIRPα interaction, resulting in induction of phagocytosis of leukemic cells and eradication of LSCs [37]. The use of soluble SIRPα-Fc fusion proteins is another promising strategy, which leads to initiation of macrophages-mediated phagocytosis, resulting in a strong anti-leukemic effect and eradication of LSCs [40].

C-type lectin-like molecule-1: C-type lectin-like molecule-1 (CLL-1) has been found to be expressed in more than 87% of AML patients, while it is weakly expressed by normal hematopoietic cells [38]. For AML, both blast cells and LSCs express CLL-1 (86.5% vs. 54.5, respectively) (Table 1) [41]. However, the anti–CLL-1 antibody didn't show the expected anti-leukemic effect, since anti-CLL-1 mAbs have been tested *in vitro* against the CLL-1+HL60 cells and had no effect on cell proliferation. Combination of a toxic moiety to the anti–CLL-1 antibody might be a promising targeting strategy to eradicate LSCs [42].

T cell immunoglobulin mucin-3: T cell immunoglobulin mucin-3 (TIM-3) is one of the most recent markers that has been found to be highly expressed in LSCs with no expression in normal HSCs (Table 1) [43]. Kikushige *et al.* demonstrated that TIM-3+AML population engrafted and initiated human AML in NRG mice, while TIM-3-AML population did not, suggesting that LSCs mostly present within the TIM-3+ population [44].

Anti-TIM-3 antibodies (ATIK2a) tested in mice models succeeded in blocking the human AML engraftment and development of leukemia in NOD/SCID mice, without any effect on normal HSC engraftment. Furthermore, ATIK2a treatment reduced or eliminated the re-engraftment of primary recipients' bone marrow cells into secondary recipients [44].

FMS-related tyrosine kinase 3: FMS-related tyrosine kinase 3 (FLT-3) is one of the most frequent mutations in AML [45]. A neutralizing mAb specific for FLT3, named IMC-EB10, is effective in inhibiting growth and expansion of leukemic cells [46]. FLT-3 inhibitors such as lestaurtinib have been used in clinical trials involving patients with refractory AML, showing a great ability to induce rapid clearance of blasts from peripheral and bone marrow without associated normal cell toxicity [47]. Combined therapy of FLT-3 inhibitors with conventional chemotherapy was associated with about 92% complete remission [48].

Cellular therapy (Genetic engineering of T cells with chimeric antigen receptors)

Genetic engineering of T cells with chimeric antigen receptors (CARs) represents a revolution in targeted cellular therapies. The extracellular antigen-binding domain of CARs consists of the variable regions of the light and heavy chains of immunoglobulins, and the intracellular domain usually consists of the CD3 zeta chain of the T cell receptor complex (T cell activating domain). This unique combination increases efficacy of the engineered T cells against malignant cells [5]. For AML, a few CARs have been developed against different target molecules such as CARs specific for CD44v6 and CD123 [5].

Cytokine-induced killer cells, a natural killer like cells population, emerged as a novel therapy against chemo-resistant AML through the intrinsic non-specific antitumor activity of these cells [49]. Combination therapy of cytokine-induced killer cells and CARs showed a potent killing activity against leukemic cells, with a promising strategy for safe and effective eradication of LSCs [5].

There are many publications demonstrating various clinical trials of CAR cells in hematologic malignancies including AML by targeting Lewis-Y antigen (tumor-associated carbohydrate antigen on AML cells). One of those trials included 4 AML patients and resulted in improvement for 2 patients (1 transient reduction in blasts, 1 transient cytogenetic remission) [50].

Conclusions

For decades, researchers have been seeking to identify LSCs, the leukemic resistance clone that is responsible for initiation and recurrence of AML. Recent studies have shown that a high level of LSCs in patients is associated with poor prognosis such that effective eradication of these cells is needed in order to cure AML.

Different strategies are established based on understanding LSCs' behaviors. Some researchers study and target the LSCs' microenvironment, others study survival, growth, and apoptotic pathways, and other groups are interested in isolation of LSCs through different cell surface antigens such as CD33, CD123, CLL-1, CD44, CD47, and TIM-3 that are preferentially expressed on LSCs as compared to HSCs. But still the variability in expression of the target molecules and also the differences in response from patient to patient represent a major challenge for scientists, and further studies for the detection of the ideal weapon against this resistance clone are needed.

As we discussed in this review, until now there have been many trials with different levels of success because of the difference in expression of each target from one patient to another. Therefore, development of personalized therapies may be a promising way to design an ideal strategy for each patient.

Authors' contributions

N. D. wrote the initial draft of the manuscript. Both N.D. and S. M. revised the manuscript, and both authors have approved the final version.

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