Leukemia Research 34 (2010) 1358–1365

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Targeting integrin linked kinase and FMS-like tyrosine kinase-3 is cytotoxic to acute myeloid leukemia stem cells but spares normal progenitors

Andrew L. Muranyi[a], Shoukat Dedhar[b,c], Donna E. Hogge[a,d,*]

[a] Terry Fox Laboratory, British Columbia Cancer Research Centre, Vancouver, BC, Canada
[b] Department of Cancer Genetics, British Columbia Cancer Research Centre, Vancouver, BC, Canada
[c] Department of Biochemistry, University of British Columbia, Vancouver, BC, Canada
[d] Department of Medicine, University of British Columbia, Vancouver, BC, Canada

ARTICLE INFO

Article history:
Received 20 October 2009
Received in revised form
21 December 2009
Accepted 8 January 2010
Available online 2 March 2010

Keywords:
Acute myeloid leukemia
Signal transduction
Xenogeneic stem cell transplantation
Cellular proliferation

ABSTRACT

Acute myeloid leukemia (AML) is maintained by rare leukemia-initiating cells (L-ICs). FLT3 and/or PI3K pathways are often dysregulated in AML and may be important for L-IC survival. The presence of PI3K pathway intermediate integrin linked kinase (ILK), and FLT3 was confirmed in five L-IC-enriched AML patient samples. Treatment of AML cells with QLT0267, an inhibitor of ILK and FLT3, decreased survival of long-term suspension culture-initiating cells and NOD/SCID mouse L-IC. In contrast, little toxicity toward normal bone marrow progenitors was observed, demonstrating that candidate leukemic stem cells can be eliminated by inhibition of these targets while normal hematopoietic counterparts are spared.

1. Introduction

Among the malignant blast cells in patients with acute myeloid leukemia (AML) are progenitors that exhibit the capacity for self-renewal of their own numbers and a much greater proliferative capacity than the majority of leukemic cells [1]. These AML progenitors can be demonstrated by their capacity to engraft and proliferate in immunodeficient mice (NOD/SCID mouse leukemia-initiating cells or NOD/SL-IC) and to initiate long-term malignant hematopoiesis in tissue culture (long-term suspension culture-initiating cells or SC-IC) [2–4]. Cell surface phenotypes which enrich for progenitors that are detected in both assays are often similar to each other and to those exhibited by primitive normal progenitors [3–5]. It seems possible that such progenitors are important for maintenance of leukemia in patients.

Aberrant cell signaling is thought to play a role in maintenance of the leukemic clone in AML by providing a proliferative advantage to malignant cells and allowing them to escape mechanisms that lead to cell death or apoptosis. The FMS-like tyrosine kinase 3 (FLT3) and phosphatidylinositol-3-kinase (PI3K)-dependent pathways are two candidate signaling pathways thought to play such roles [6–8].

The FLT3 receptor is a member of the type III receptor tyrosine kinase (RTK) subfamily which also includes c-kit, c-FMS and PDGF [9]. Activation of FLT3 normally occurs when FLT3 ligand binds to the receptor, inducing formation of a homodimer which in turn activates the kinase domain of the receptor, resulting in signaling to downstream pathways such as Ras, and PI3K [10–12]. FLT3 is mutated in approximately one third of AML samples, the majority of these mutations being internal tandem duplications (ITD) of the juxtamembrane region of the receptor although other mutations exist which can, like the ITD, lead to aberrant and constitutive activation of the receptor tyrosine kinase and downstream signaling pathways [13,14].

The PI3K-dependent signaling pathway controls cell growth and proliferation via activation of Akt and downstream targets, and is activated in a large number of AML samples including those with poor prognostic features [8,15]. Inhibition of this pathway by compounds targeting various intermediates is cytotoxic to AML blasts. Integrin linked kinase (ILK) is an ankyrin-repeat containing serine/threonine kinase involved in phosphorylation of Akt (ser473) and GSK3. ILK is overexpressed or constitutively active in a large number of cancers, and is ubiquitously expressed in AML blast samples [16]. Inhibition of ILK by siRNA-mediated approaches or small molecule inhibitors in solid tumors results in apoptosis and/or impaired invasion of the cancer cells [17–19].

Here we demonstrate that specific inhibition of ILK using siRNA is toxic to primary human AML CFC progenitors, suggesting ILK as a relevant target for AML therapy. In addition, the expression of FLT3,

* Corresponding author at: Department of Medicine, University of British Columbia, Terry Fox Laboratory, British Columbia Cancer Research Centre, 675 West 10 Ave, V5Z 1L3, Vancouver, BC, Canada. Tel.: +1 604 675 8138; fax: +1 604 877 0712.
E-mail address: dhogge@bccancer.bc.ca (D.E. Hogge).

0145-2126/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2010.01.006

ILK and phosphorylated GSK3 (as a marker of activation of Akt) was measured in subpopulations of AML blasts enriched for NOD/SL-IC and SC-IC as well as quiescent and cycling leukemic cells. Furthermore, inhibition of these targets with QLT0267, a small molecule inhibitor of both ILK and FLT3 [16], is shown to inhibit the survival of both SC-IC and NOD/SL-IC but to have relatively little effect on normal bone marrow SC-IC, and NOD/SCID repopulating cells (RC). The combined effect on AML CFC of QLT0267 with conventional chemotherapy drugs often used in the treatment of AML (cytarabine or daunorubicin) was also investigated. In total, these data suggest that ILK and FLT3 expression are more important to the maintenance of leukemia-initiating cells in AML than to normal primitive progenitors and that their combined inhibition may be useful in eradicating the leukemic clone while sparing normal hematopoiesis.

2. Materials and methods

2.1. AML and normal cells

Peripheral blood (PB) cells and bone marrow samples were obtained from 11 newly-diagnosed AML patients and normal bone marrow donors after informed consent and with the approval of the Clinical Research Ethics Board of the University of British Columbia (Supplementary Table 1). Among the 11 AML patients ranging in age from 28 to 72 years, 10 had intermediate risk bone marrow cytogenetics (7 normal karyotype) and one (sample 3) good risk (inv(16)) cytogenetics at diagnosis according to Medical Research Council (UK) criteria [20]. AML samples 1, 2, 4, 5, 6, and 11 were FLT3-ITD+, while the remainder were FLT3 wildtype. Six of these AML patients achieved complete remission with conventional cytarabine and daunorubicin-based chemotherapy while 4 had chemotherapy refractory leukemia and one died during induction. One patient remains alive 7 years post-diagnosis while the median survival of the remainder was 8 months (Supplementary Table 1). Blood cells were Ficoll separated to obtain the mononuclear cell population and cryopreserved as described [21]. Prior to cryopreservation normal bone marrow from multiple donors was pooled and was enriched for $CD34^+$ cells using an immunomagnetic column (Easysep, StemCell Technologies Inc., Vancouver, Canada).

2.2. Cell culture

Thawed AML and normal $CD34^+$ bone marrow cells were resuspended in IMDM with 10% FCS at 1×10^6 cells/mL (1×10^4 cells/mL for $CD34^+$ normal bone marrow) and then cultured for 24 h (unless otherwise stated) with varying concentrations of the inhibitor QLT0267 (QLT Inc. Vancouver, BC, Canada), Ara-C, daunorubicin, or DMSO control. Cultured cells were washed with IMDM and plated in methylcellulose-based colony forming cell (CFC) assays as previously described [16], placed into long-term suspension culture-initiating cell assays, or injected into sublethally-irradiated immunodeficient mice.

For long-term suspension culture-initiating cell assays weekly half media changes were carried out during the 6-week culture period after which the entire culture was harvested, washed once in IMDM, counted and placed into CFC assays to measure progenitor cell output [22].

FACS isolated quiescent and cycling AML cells were cultured in serum free media (SFM) with or without QLT0267 for 24 h [23].

2.3. QLT0267

QLT0267 is a potent small molecule second generation kinase inhibitor which was originally characterized for its ability to inhibit ILK but not a variety of other kinases including Akt, PDK-1, DNA-PK and GSK3 [19]. More recently this compound has also been shown to inhibit both wildtype and mutant FLT3 with equivalent potency to that seen against ILK [16]. Treatment of AML blasts and CFCs with QLT0267 leads to inhibition of the PI3K pathway, and cell death [16]. In our previous work [16] concentrations of QLT0267 tested on AML cells were chosen based on previous studies in which 10 μM was the typical IC_{50} obtained for various cancer cell lines [19,24]. In sensitive AML samples 90% AML CFC kill was achieved after 48 h exposure to this concentration [16]. In the current study we wished to reduce the duration of ex vivo culture of AML cells prior to injection into NOD/SCID mice to 24 h to maximize the efficiency of engraftment of the treated cells [25,26]. In a series of preliminary experiments we determined that twice the concentration of QLT0267 (i.e. 20 μM) was needed in a 24 h culture to achieve a similar AML CFC kill to that which was achieved after 48 h exposure to 10 μM of this inhibitor. Thus, 20 μM was chosen as the lowest concentration of QLT0267 to test in long-term suspension cultures and mouse experiments.

2.4. siRNA inhibition

RNA inhibition experiments using Accell siRNA delivery system were performed on primary AML samples as instructed by the manufacturer (Dharmacon, Lafayette CO, USA). Briefly, cells were cultured for 1–2 h in IMDM with 10% FCS before being placed into Accell siRNA delivery media (B-005000-100. Dharmacon) supplemented with 100 ng/mL stem cell factor, 100 ng/mL FLT3 ligand, and 20 ng/mL IL-3 (StemCell Technologies) to maintain cell survival in the absence of serum, in the presence of either siRNA targeting ILK or non-targeting siRNA as control. After 72 h some cells were placed into CFC assays, while RNA from the remainder were analyzed by qRT-PCR for mRNA knockdown.

2.5. Fluorescence activated cell sorting (FACS)

Freshly-thawed AML cells were resuspended in Hanks' balanced salt solution modified (StemCell Technologies) with 2% FCS, and 0.04% sodium azide (HFN) with 5% human serum and then incubated with CD34-APC (clone 8G12, StemCell Technologies) and CD38-PE (clone HB-7, StemCell Technologies) antibodies on ice for 30 min, washed with HFN, followed by HFN with 2 μg/mL propidium iodide (PI), and resuspended in HFN. Cells were sorted into 3 populations ($CD34^-$, $CD34^+CD38^+$, and $CD34^+CD38^-$) and collected, using the FACSVantage SE or FACSVantage DiVa cell sorters (Becton Dickinson, San Jose, CA), after setting gates based on single antibody control stained cells.

For cell sorting based on cell cycle status AML samples were cultured overnight in SFM and then washed twice in HFN prewarmed to 37 °C. Cells were then incubated for 45 min at 37 °C in the dark in 3 μg/mL Hoechst 33342 (Hst). Pyronin Y (Py) was then added at 1 μg/mL for 45 min at 37 °C. Cells were then pelleted at 4 °C, washed with HFN with Hst, Py, and PI (1 μg/mL), and resuspended in HFN with Hst and Py. Two populations of cells were isolated; quiescent (Hst and Py dull) and cycling (Py bright) [26].

2.6. Intracellular staining for FACS analysis

Cells were resuspended in HFN and prepared for intracellular staining as per manufacture's instructions (Phosflow Perm Buffer III, Phosflow Fix Buffer I, BD Biosciences). Cells were resuspended in 100 μL wash/stain solution, to which pGSK3α/β Ser21/9, ILK1, or rabbit IgG isotype control (AlexaFluor-488 Conjugate) (37F11, 3862, and 4340, respectively, Cell Signaling) antibodies were added and incubated on ice for 30 min. Cells were washed and anti-pGSK3, and ILK treated cells were incubated with AlexaFluor-488 conjugated secondary Ab (A11070, Invitrogen) for a further 30 min on ice, followed by washing and resuspension in wash/stain solution and analysis on a FACScan flow cytometer using CellQuest™ software (BD Biosciences). Protein levels were quantified by calculating geographic mean fluorescent intensity (MFI) of the various cell stainings using FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.7. Quantitative RT-PCR

RNA was isolated from cells using the Arcturus PicoPure RNA isolation kit (KIT0202, MDS Analytical Technologies, Sunnyvale, CA, USA), and transcribed into cDNA using the superscript III first-strand synthesis system (Invitrogen, Burlington, ON, Canada) as per manufacturer's instructions. Control reactions were performed without RNA present.

Quantitative PCR was performed on the cDNA in MicroAmp Fast Optical 96-Well Reaction Plates (#4346906, Applied Biosystems) using an Applied Biosystems 7500 Real-Time PCR System under the following conditions. Reaction mixture: 3 μL cDNA, 1× SYBR Green PCR Master Mix (#4309155, Applied Biosystems, Foster City, CA), 375 nM forward and reverse primers, ddH_2O to 25 μL. cDNA was quantified using a two-step PCR reaction running 42 cycles with the cDNA of interest being measured relative to GAPDH. siRNA-mediated mRNA knockdown was measured relative to mRNA in untreated cells. Primers used: FLT3 forward (CCGCCAGGAACGTGCTTG), FLT3 reverse (ATGCCAGGGTAAGGATTCACACC); GAPDH forward (CCATCACCATCTTCCAGGAG), GAPDH reverse (TGAAGACGCCAGTGGACTC); ILK forward (GAATTCGTATGGACGACATTTTCACTCAGTGC), ILK reverse (CTCGAGCTACTTGTCCTGCATCTTCTCAAG).

Real-time PCR and data analysis were performed on a 7500 Fast Real-Time PCR system, using 7500 Fast Sequence Detection Software (Applied Biosystems). The relative quantification data of ILK and FLT3 in comparison to a reference gene (GAPDH) was generated on the basis of a mathematical model for relative quantification in real-time RT-PCR as described [27–29].

2.8. PCR to detect FLT3-ITD in AML CFC

Colonies were plucked from CFC assays using a glass pipette, and washed once in PBS. DNA was extracted from cell pellets using the Arcturus Pico Pure DNA extraction kit (KIT0103, MDS Analytical Technologies) as per manufacturer's instructions. PCR was performed on the DNA under the following conditions: 45 mM Tris–HCl (pH 8.8), 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM β-mercaptoethanol, 4.4 mM EDTA (pH 8.0), 1 mM each dATP, dCTP, dGTP, dTTP, 113 μg/mL BSA, 12.5 pM each forward and reverse primers, 0.375 μL Platinum Taq DNA Polymerase High Fidelity (Invit-