Leukemia Research 39 (2015) 1437–1442

Contents lists available at ScienceDirect

Leukemia Research

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

Targeting bone marrow lymphoid niches in acute lymphoblastic leukemia

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ARTICLE INFO

Article history:
Received 13 July 2015
Received in revised form
15 September 2015
Accepted 28 September 2015
Available online 1 October 2015

Keywords:
Acute lymphoblastic leukemia
G-CSF
Mobilization
Lymphoid niche
CXCL12

ABSTRACT

In acute lymphoblastic leukemia (ALL) the bone marrow microenvironment provides growth and survival signals that may confer resistance to chemotherapy. Granulocyte colony-stimulating factor (G-CSF) potently inhibits lymphopoiesis by targeting stromal cells that comprise the lymphoid niche in the bone marrow. To determine whether lymphoid niche disruption by G-CSF sensitizes ALL cells to chemotherapy, we conducted a pilot study of G-CSF in combination with chemotherapy in patients with relapsed or refractory ALL. Thirteen patients were treated on study; three patients achieved a complete remission (CR/CRi) for an overall response rate of 23%. In the healthy volunteers, G-CSF treatment disrupted the lymphoid niche, as evidenced by reduced expression of CXCL12, interleukin-7, and osteocalcin. However, in most patients with relapsed/refractory ALL expression of these genes was markedly suppressed at baseline. Thus, although G-CSF treatment was associated with ALL cell mobilization into the blood, and increased apoptosis of bone marrow resident ALL cells, alterations in the bone marrow microenvironment were modest and highly variable. These data suggest that disruption of lymphoid niches by G-CSF to sensitize ALL cells to chemotherapy may be best accomplished in the consolidation where the bone marrow microenvironment is more likely to be normal.

1. Introduction

Lymphopoiesis is dependent on the production of supportive signals by bone marrow stromal cells. CXCL12-abundant reticular (CAR) cells, osteoblasts, and other bone marrow stromal cells produce growth factors and chemokines that contribute to lymphopoiesis [1–4]. CXCL12 is constitutively expressed at high levels by CAR cells and osteoblasts and is required for early stages of B lymphopoiesis [5–7]. Interleukin-7 is produced by a subset of CAR cells and is required for the maintenance of pro-B cells [8–10]. Insulin-like growth factor-1 (IGF-1) is produced by CAR cells and osteoblasts and cooperates with IL-7 to promote B cell expansion and pre-B maturation [11,12]. Other factors produced by bone marrow stromal cells that support lymphopoiesis include IL-6 [13], RANK ligand (RANKL) [14,15], B cell activating factor (BAFF) [16], kit ligand [17], flt3 ligand [18], and delta-like ligand 4 [19]. The importance of CAR cells and osteoblasts to lymphopoiesis is supported by the finding of profound lymphopenia in mice after conditional deletion of these cell populations [2,4,20]. Acute lymphoblastic leukemia (ALL) cells, similar to normal B cell progenitors, receive key signals from bone marrow stromal cells that regulate their growth and survival [10]. Specifically, CXCL12, Interleukin-7, IGF-1, and BAFF have been shown to support ALL cell survival in vitro [21–24]. Moreover, direct contact of ALL cell lines with stromal cells confers resistance to chemotherapy [25]. Together, these data suggest that CAR cells and osteoblasts contribute to a lymphoid niche in the bone marrow that supports both normal and malignant B cell progenitors.

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http://dx.doi.org/10.1016/j.leukres.2015.09.020
0145-2126/

There is emerging evidence that granulocyte colony-stimulating factor (G-CSF) disrupts the lymphoid niche in the bone marrow. G-CSF is widely used in the clinical setting to stimulate granulopoiesis and mobilize hematopoietic progenitors into the blood for stem cell transplantation. Less well appreciated, treatment with G-CSF also results in a marked loss of B cells, T cells, and NK cells in the bone marrow [26–29]. G-CSF results in a significant decrease in pro-B and pre-B cells, suggesting that G-CSF disrupts B lymphopoiesis at an early stage of development [29,30]. Recent studies show that G-CSF suppresses B lymphopoiesis in a non-cell autonomous fashion by altering the bone marrow microenvironment [29,30]. Specifically, G-CSF decreases the number and activity of mature osteoblasts [31–33]. It also decreases bone marrow stromal cell production of a number of B trophic factors, including CXCL12, kit ligand, IL-6, IL-7, IGF-1, and BAFF [30].

Collectively, these data suggest the hypothesis that disruption of lymphoid niches by G-CSF may sensitize ALL cells to chemotherapy. To test this hypothesis, we conducted a pilot study of upfront G-CSF treatment in patients with relapsed or refractory ALL. To determine the effects on the bone marrow microenvironment in patients with ALL, bone marrow specimens were analyzed at baseline and after G-CSF treatment.

2. Methods

2.1. Clinical trial

We conducted a multicenter, open-label pilot study of adults (age ≥ 18 years) with relapsed or refractory ALL. Participants were required to have adequate performance status (ECOG ≤ 3), and organ function defined as a creatinine clearance ≥50 ml/min and AST, ALT, total bilirubin ≤2× the institutional upper limit of normal. Treatment consisted of G-CSF (filgrastim) 10 mcg/kg/day, SQ starting on day 1 and continuing for a minimum of 10 days or until ANC >1000/mcL × 2 days, ifosfamide 10 gm/m^2 CIVI over days 4–6 with mesna, etoposide 150 mg/m^2 IV twice daily on days 4–6, dexamethasone 5 mg/m^2 PO or IV twice daily on days 4–10. Bone marrow from healthy donors receiving G-CSF was obtained under protocol WU-201103258. All subjects provided written informed consent prior to enrollment. This study was conducted in accordance with the ethical standards in the Declaration of Helsinki, approved by the Human Research Protection Office of Washington University and registered at ClinicalTrials.gov, Identifier NCT01331590.

2.2. Real-time quantitative RT-PCR.

A bone marrow core was obtained and crushed using a mortal and pestle in 1 ml of TRIzol reagent (Invitrogen). RNA was isolated according to the manufacturer's instructions. Real-time RT-PCR was performed using the TaqMan RNA-to-C_T 1-Step kit (Applied Biosystems) on a StepOne Plus Real-time PCR System (Applied Biosystems). RNA content was normalized to human GAPDH. The following primer/probe mixes were purchased from Life Technologies: GAPDH (Hs02758991_g1), TNFSF13B (BAFF) (Hs00198106_m1), IL7 (Hs00174202_m1), and BGLAP (OC) (Hs01587814_g1).

2.3. CXCL12 ELISA

Bone marrow extracellular fluid was obtained by crushing bone marrow core biopsies wash crushed using a mortal and pestle in 1 ml of sterile saline. Bone fragments and cells were removed by centrifugation at 500 × *g* for 3 min at 4 °C. CXCL12 protein expression was measured using the Human CXCL12 Quantikine ELISA kit from R&D Systems, as per manufacturer's recommendation.

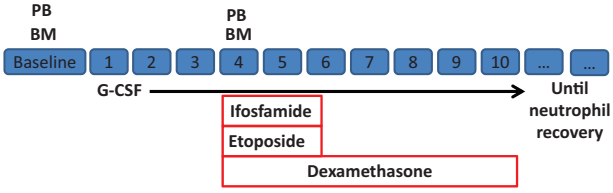

Fig. 1. Clinical schema.
Adults with relapsed or refractory ALL were treated with G-CSF (10 μg/kg) per day starting on day 1 and continuing through neutrophil recovery. Salvage chemotherapy with ifosphamide, etoposide, and dexamethasone was given starting on day 4. Peripheral blood (PB) and bone marrow (BM) were obtained at baseline and after 4 days of G-CSF (but before starting salvage chemotherapy).

2.4. Flow cytometry

Red blood cells were lysed in Tris-buffered ammonium chloride (pH 7.4) and the remaining white blood cells were incubated with cocktails containing antibodies against CD45 (clone HI30) and CD10 (clone eBioCB-CALLA) from eBioscience, and CD19 (clone HIB19) from BD Biosciences. An Annexin V stain and binding buffer were used to assess apoptosis according to manufacturer's protocol, (BD Biosciences #556422, 556454). For activated caspase 3 detection, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences #554714). Fixed cells were incubated with an anti-activated caspase 3 (clone# C92-6050). Cells were analyzed on a FACScan flow cytometer (Beckman Coulter).

2.5. Statistical analysis

T-tests were used to compare mRNA expression of CXCL12, BAFF, IL-7 and osteocalcin in healthy donors with G-CSF and additional healthy subjects who did not receive G-CSF. Expression values in healthy subjects are reasonably Gaussian but those in ALL patients are moderately strongly skewed. Nonparametric Wilcoxon rank-sum tests were used for comparisons of expression in healthy donors vs. ALL patients and for comparison of baseline CXCL12 expression in ALL patients with and without response to treatment. Wilcoxon signed rank tests for comparing paired values measured before and after G-CSF in ALL patients. Rank correlations were used to estimate the strength of association between CXCL12 mRNA and ALL blast percentage or number.

3. Results

Preclinical studies show that at least 4 days of G-CSF are required to maximally suppress bone marrow stromal production of CXCL12 [32,34]. Thus, in this study, G-CSF was administered for 4 daily doses prior to the start of chemotherapy and continuing until neutrophil recovery (Fig. 1). Salvage chemotherapy consisted of ifosfamide 10 gm/m^2 CIVI on days 4–6 with mesna, etoposide 150 mg/m^2 IV twice daily on days 4–6, dexamethasone 5 mg/m^2 PO or IV twice daily on days 4–10 [35]. The study population consisted of 13 patients, 11 males and 2 females, with a median age of 46 years (range 19–69). Eleven patients had a B-precursor immunophenotype, and one patient each had a *t*(9;22) and 11q23 rearrangement (Table 1). Ten of the 14 patients were being treated for their first relapse with an additional 1 patient each treated for primary refractory disease, 2nd relapse and 3rd relapse. Prior therapies include HyperCVAD ($n = 7$), a pediatric based ALL induction regimen ($n = 3$), the CALGB 9551 five drug induction regimen ($n = 1$), or a combination of fludarabine, cytarabine, G-CSF and idarubicin ($n = 1$). Four of the patients had relapsed following allogeneic stem cell transplantation. Three patients achieved a complete remission (2CR, 1CRi) for an overall response rate of 23%. Six patients were able to