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Targeting Bone Marrow Lymphoid Niches In Acute Lymphoblastic Leukemia

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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) potentially 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.

Keywords

Acute lymphoblastic leukemia; G-CSF; mobilization; lymphoid niche; CXCL12

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Declaration of Conflicts of Interests

The authors have no conflicts of interest to disclose.

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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.

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, 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.

METHODS

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 2x the institutional upper limit of normal. Treatment consisted of G-CSF (filgrastim) 10 mcg/kg/d SQ starting on day 1 and continuing for a minimum of 10 days or until ANC >1,000/mcL x 2 days, ifosfamide 10 gm/m² CIVI over days 4–6 with mesna, etoposide 150 mg/m² IV twice daily on days 4–6, dexamethasone 5 mg/m² 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.

Real-time quantitative RT-PCR

A bone marrow core was obtained and crushed using a mortar 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-CT 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).

CXCL12 ELISA

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

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 Cytfix/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).

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.

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 (Figure 1). Salvage chemotherapy consisted of ifosfamide 10 gm/m² CIVI on days 4–6 with mesna, etoposide 150 mg/m² IV twice daily on days 4–6, dexamethasone 5 mg/m² 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 (1 CR, 2CRi) for an overall response rate of 23%. Six patients were able to undergo a subsequent allogeneic stem cell transplant. The median OS is 139 days with three patients currently alive at 21, 27, and 47 months post treatment. The primary non-hematologic toxicities were fever and infection. Ifosfamide neurotoxicity was observed in three individuals. In each case, the toxicity resolved with temporary discontinuation of the medication and/or supportive care with methylene blue. In one patient, chemotherapy was started one day early due to increasing leukocytosis. One death occurred within 30 days of starting chemotherapy secondary to sepsis.

To assess the bone marrow microenvironment, we prepared RNA from bone marrow core biopsies and measured the expression of genes implicated in the proliferation and survival of ALL cells, including CXCL12, IL-7, and BAFF. We also measured expression of osteocalcin, as an indirect assessment of mature osteoblasts. We first analyzed bone marrow from healthy volunteers at baseline and from a separate group receiving G-CSF to mobilize stem cells for allogeneic stem cell transplantation. Compared to baseline specimens from untreated healthy subjects, G-CSF administration for 4 days was associated with a marked reduction in mRNA levels of CXCL12, osteocalcin, and IL-7, but not BAFF (Figure 2).

To assess whether the bone marrow microenvironment is altered in patients with ALL, we analyzed bone marrow specimens prior to G-CSF administration. Expression of each of these stromal genes was highly variable (Figure 2). For example, although in the majority of cases CXCL12 expression was markedly suppressed, it was in the normal range in 3 of 12 (25%) patients. A potential caveat of this study is the assessment of the bone marrow microenvironment based on a single bone marrow biopsy specimen, which may not reflect changes in the bone marrow at other sites. Nonetheless, our data suggest that the bone marrow microenvironment is markedly altered in most patients with relapsed or refractory ALL. Interestingly, the percentage of ALL blasts in the bone marrow did not correlate well

with CXCL12 mRNA expression, suggesting that simple replacement of bone marrow stromal cells by leukemia is not likely to account for the loss of CXCL12 (Figure 2E). To determine the effects of G-CSF treatment in patients with ALL, we also analyzed bone marrow specimens obtained after 4 days of G-CSF. No consistent effect on the expression of CXCL12, BAFF, IL-7, or osteocalcin mRNA was observed (Figure 3A). Protein expression of these genes in bone marrow extracellular fluid was not detected except for CXCL12 (Figure 3B and data not shown). Interestingly, in the three ALL cases where CXCL12 protein was measurable, it decreased following G-CSF treatment.

G-CSF treatment mobilizes hematopoietic progenitors and B cells into the blood in healthy subjects. To assess ALL blast mobilization, we quantified ALL blast number in the blood at baseline and after 4 days of G-CSF by flow cytometry. Mobilization of ALL blasts (at least two-fold over baseline) was observed in 5 of 8 cases where paired blood samples were available (Figure 3C). Suppression of CXCL12 expression in the bone marrow is a key step in the mobilization of normal hematopoietic progenitors.^{32,36,37} Surprisingly, we observed a poor correlation between the magnitude of the suppression of CXCL12 expression in the bone marrow after G-CSF treatment and ALL blast mobilization (Figure 3D).

In a limited number of cases, we were able to measure apoptosis in bone marrow resident ALL blasts before and after G-CSF treatment. At baseline, $2.9 \pm 0.47\%$ of ALL blasts were annexin V positive as compared to $13.2 \pm 2.32\%$ after G-CSF treatment ($P = 0.037$, Figure 3E & F). Likewise, the percentage of apoptotic ALL blasts, as assessed by activated caspase 3 expression, increased from $1.47 \pm 0.83\%$ at baseline to $9.43 \pm 2.42\%$ after G-CSF ($P = 0.038$, Figure 3G and Suppl. Figure 1).

DISCUSSION

In this study, we tested the hypothesis that upfront treatment with G-CSF, by altering the bone marrow microenvironment, would sensitize ALL cells to chemotherapy. While this study was not designed to detect differences in remission rates due to the limited sample size and patient heterogeneity, the observed rate of 23% is similar to previous studies in relapsed adult ALL of 20–40%.^{38,39} In one of the largest studies of G-CSF in adult ALL conducted by CALGB, 198 patients were randomized to G-CSF versus placebo beginning 4 days after starting induction and continuing until neutrophil recovery and again during 2 monthly courses of consolidation.⁴⁰ The study demonstrated significantly shorter durations of neutropenia and decreased hospitalization times. Interestingly, CR rates were higher in the G-CSF cohort, even though the total dose of chemotherapy was similar. Although the timing of the G-CSF in this trial was suboptimal for niche disruption, it is possible that alterations in the bone marrow microenvironment by G-CSF contributed to the higher response rate. Alternatively, it is possible that G-CSF may induce cycling of quiescent leukemic blasts, as has been suggested in AML.⁴¹

We show that treatment of healthy volunteers with G-CSF for 4 days is associated with decreased expression of key lymphoid trophic factors CXCL12 and IL-7, and it suppresses mature osteoblasts (as assessed by osteocalcin expression). These data suggest that, similar to mice, G-CSF treatment disrupts the lymphoid niche in the bone marrow of healthy

individuals. Our analysis of patients with relapsed/refractory ALL suggests that in most cases the lymphoid niche is disrupted and is consistent with a prior report showing decreased bone marrow CXCL12 expression in pediatric ALL.⁴² Whether this reflects stromal cell damage from prior chemotherapy or active suppression of stromal cells by ALL cells is uncertain. In support of the latter possibility, Zhang et al recently reported that chronic myelogenous leukemia cells alters the bone marrow microenvironment in mice, including suppression of stromal CXCL12 expression.⁴³

In contrast to healthy individuals, G-CSF induced changes in CXCL12, IL-7, and osteocalcin were only seen in a minority of patients with relapsed/refractory ALL. This is perhaps not surprising, given the marked suppression of these genes at baseline in most patients. Of note, we previously showed that G-CSF induced hematopoietic progenitor mobilization and disruption of the lymphoid niche are mediated by signaling in monocytic cells in the bone marrow.^{30,44} Whether ALL cells in the bone marrow alter the number or responsiveness of monocytic cells to G-CSF is unknown. Interestingly, we observed ALL cell mobilization into the blood in most of our patients. Although CXCL12 is a key retention signal for ALL cells in the bone marrow,²¹ other pathways have been shown to regulate ALL retention in the bone marrow, including the VLA4/VCAM1 axis.⁴⁵ Of note, G-CSF treatment decreases VCAM1 expression in bone marrow stromal cells in mice.⁴⁶ Whether G-CSF induced changes in VCAM1 or other signaling pathway induce ALL blast mobilization will require further study.

In summary, our data suggests that in healthy individuals, G-CSF treatment alters the bone marrow microenvironment, suppressing the production of several key lymphocytic trophic factors. However, in the majority of cases of relapsed/refractory ALL, the production of these lymphocytic trophic factors is markedly suppressed, likely limiting the effect of G-CSF on the bone marrow microenvironment. Thus, these data suggest that disruption of lymphoid niches by G-CSF to sensitize ALL cells to chemotherapy might be best accomplished in the consolidation setting, where the bone marrow microenvironment is more likely to be normal. Whether niche disruption by G-CSF (or other agents) augments the response to emerging immunotherapies for ALL is an open question.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

GLU and DCL designed the clinical trial and prepared the manuscript. GLU, WS, PW and JFD supervised the care of patients in this trial. TF managed patient data. YMSH, AS, and DCL analyzed the bone marrow specimens. This research was supported by the National Institutes of Health (P50CA171963 to DCL, CA152329 to JFD, and K23CA140707 to GLU) and by an Alliance for Clinical Trials in Oncology Foundation Clinical Scholar Award to GLU.

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HIGHLIGHTS

- G-CSF disrupts lymphoid niches in healthy human subjects
- The lymphoid niche is disrupted in most patients with relapsed/refractory ALL
- Treatment with G-CSF has modest and highly variable effects on the bone marrow microenvironment in patients with relapsed/refractory ALL
- G-CSF treatment mobilized ALL cells and induced apoptosis of bone marrow resident ALL cells

Figure 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 ifosfamide, 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).

Figure 2. The bone marrow microenvironment is altered in adult patients with relapsed/refractory ALL

RNA was prepared from bone marrow core biopsies obtained from healthy volunteers at baseline, healthy donors would had received 4–5 days of G-CSF (10 µg/kg/day), or patients with relapsed or refractory ALL. Shown is the expression of CXCL12 (A), osteocalcin (OSC, B), BAFF (C) or IL-7 (D) relative to GAPDH. The mean ± SEM is shown. The dashed red line represents the 95% confidence interval based on baseline healthy donor data. (E). Shown is the baseline CXCL12 mRNA levels versus the percentage of ALL blasts in the bone marrow. The Spearman rank correlation rho value is shown.

Figure 3. G-CSF mobilizes and induces apoptosis of bone marrow resident ALL blasts
 (A). RNA was prepared from bone marrow core biopsies of patients with relapsed/refractory ALL before (baseline) or after 4 days of G-CSF. Shown is expression of the indicated gene relative to GAPDH. (B). Bone marrow extracellular fluid was prepared from core biopsies and analyzed for CXCL12 protein expression by ELISA. (C). The number of CD45^{low} SCC^{dim} ALL blasts in the blood at baseline and after G-CSF is shown. (D). The fold change (baseline to G-CSF) in BM CXCL12 mRNA versus the fold change in blood ALL blast number is shown. The Spearman rank correlation rho value is shown. (E). Representative flow plots showing Annexin V and DAPI staining of ALL blasts at baseline or after G-CSF in a patient with B ALL; data are gated on CD45^{low} SCC^{dim} CD10⁺ cells. (F & G). The percentage of ALL blasts that were annexin V⁺ (F) or activated caspase 3⁺ (G) is shown.

Table 1

Patient Characteristics and Response Data

ID	Age	Sex	ALL Type	Treatment Indication	Prior alloHCT	Response
001-001	47	M	B-ALL, t(9;22)	1st relapse	N	CR
001-002	33	M	B-ALL	2nd relapse	Y	PD
002-005	24	M	B-ALL	3rd relapse	N	PD
001-007	56	F	B-ALL	1st relapse	Y	PD
002-008	46	M	T-ALL	1st relapse	Y	PD
001-010	21	M	T-ALL, hyperdiploid	1st relapse	N	CR
001-011	53	M	B-ALL, t(4;11)	1st relapse	N	PD
002-015	65	M	B-ALL	1st relapse	N	PD
001-019	69	M	B-ALL	1st relapse	N	TF /death
001-020	26	M	B-ALL	1st relapse	Y	CRi
001-021	21	M	B-ALL	Primary refractory	N	PD
001-026	23	M	B-ALL	1st relapse	N	PD
001-027	57	M	B-ALL	1st relapse	N	PD

alloHCT: Allogeneic Hematopoietic Cell Transplant, CR: Complete remission, PD: Persistent Disease, TF: Treatment Failure