Proteomic Profiling of Signaling Networks Modulated by G-CSF/Plerixafor/Busulfan-Fludarabine Conditioning in Acute Myeloid Leukemia Patients in Remission or with Active Disease prior to Allogeneic Stem Cell Transplantation

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Keywords

Acute myeloid leukemia · CXCR4 · Proteomic profiling of signaling · Plerixafor · Allogeneic stem cell transplantation

Abstract

To characterize intracellular signaling in peripheral blood (PB) cells of acute myeloid leukemia (AML) patients undergoing pretransplant conditioning with CXCR4 inhibitor plerixafor, granulocyte colony-stimulating factor (G-CSF), and busulfan plus fludarabine (Bu+Flu) chemotherapy, we profiled 153 proteins in 33 functional groups using reverse phase protein array. CXCR4 inhibition mobilized AML progenitors and clonal AML cells, and this was associated with molecular markers of cell cycle progression. G-CSF/plerixafor and G-CSF/plerixafor/Bu+Flu modulated distinct signaling networks in AML blasts of patients undergoing conditioning with active disease compared to nonleukemic PB

cells of patients in remission. We identified AML-specific proteins that remained aberrantly expressed after chemotherapy, representing putative chemoresistance markers in AML.

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Introduction

Inhibition of CXCR4 signaling using the small molecule CXCR4 inhibitor plerixafor alone or in combination with granulocyte colony-stimulating factor (G-CSF) is thought to disrupt stroma-leukemia interactions, mobilizing leukemia cells from their niche microenvironment. This combination (G+P), in conjunction with chemotherapy, has been investigated in preclinical and clinical studies for the treatment of hematological malignancies [1–5]. We have reported re-

Fig. 1. G-CSF/plerixafor/busulfan-fludarabine conditioning in AML prior to allo-SCT. Clinical trial schema and time points of PB sample collection. G, G-CSF (10 µg/kg); P, plerixafor (160 or 240

µg/kg); Bu, busulfan (130 mg/m²); Flu, fludarabine (40 mg/m²); ATG, antithymocyte globulin (0.5–2 mg/kg, given to patients with unrelated donors); PB, peripheral blood.

sults of the phase 1/2 study of G+P in combination with busulfan and fludarabine (Bu+Flu) conditioning regimen in patients with acute myeloid leukemia (AML) undergoing allogeneic stem cell transplantation (allo-SCT) [6]. AML patients who had not achieved remission at the time of allo-SCT had inferior outcomes compared to patients who were in remission at the time of transplantation. Similar results with other types of conditioning regimens have been reported in other clinical studies [7, 8]. Risk factors such as the presence of circulating blasts and unfavorable cytogenetics are associated with poor outcomes [9, 10], but the molecular wiring and its modulation associated with disease status have not been studied. In this study, we tested the hypothesis that the G+P – and G+P plus Bu+Flu – preparative regimen modulates distinct molecular signaling networks, which vary depending on disease status and may affect post allo-SCT outcomes.

Materials and Methods

Patients and Trial Study Group

All patients who enrolled in the clinical trial of G-CSF/plerixafor/busulfan-fludarabine conditioning provided written informed consent for biomarker sample collection in accordance with the Declaration of Helsinki and our institutional guidelines. Study approval was obtained from the Institutional Review Board at The University of Texas MD Anderson Cancer Center. This trial was registered at ClinicalTrials.gov with identifier NCT00822770. Additional details on patient eligibility, drug administration, disease evaluation, and toxicity assessment can be found in the published report [6].

Primary Samples, Flow Cytometry, and Fluorescence in situ Hybridization

Peripheral blood (PB) samples were collected from patients enrolled in the aforementioned clinical trial. Samples were collected at the indicated time points over the course of the trial regimen (Fig. 1).

Mononuclear cells were separated using Ficoll-Hypaque (Sigma Chemical, St. Louis, MO, USA) density-gradient centrifugation. Cells were stained with anti-human CD34 antibody and isotype control (BD Bioscience, San Jose, CA, USA) and subjected to flow cytometry to identify CD34+ cells. Cells with a cytogenetic abnormality detectable by fluorescence in situ hybridization (FISH) were plated on glass slides, stained with the appropriate probe (Vysis probes; Abbott Laboratories, Chicago, IL, USA) and subjected to microscopy to identify FISH+ cells. Details of the flow cytometry and FISH techniques used in this study were previously published [6].

Reverse Phase Protein Array

Mononuclear cells were lysed and subjected to reverse phase protein array (RPPA) analysis using previously described and validated methods [11, 12]. Raw signal intensities obtained from RPPA were processed with the SuperCurve to determine relative protein concentrations, and the results were further normalized to adjust for loading bias by median-centering each marker and each sample [13, 14].

Statistical Analysis

Two-tailed Student *t* tests were used to identify significant protein and biomarker alterations in samples from different treatment groups (with a significance value of $p \leq 0.05$) and in complete remission and in active disease samples with the same treatment (with a significance value of $p \leq 0.01$). Pearson correlation coefficient (*r*) was calculated to determine the correlation between AML blast percentage and overall survival duration after allo-SCT ($p \leq 0.05$ was considered significant). A Kaplan-Meier curve comparing overall survival in active disease and complete remission AML patients was generated using Prism software version 7 (GraphPad Software, La Jolla, CA, USA).

Results

To investigate the conditioning regimen-regulated signaling pathway, we profiled PB samples collected from 10 AML patients with sufficient material who participated in the aforementioned phase 1/2 trial. Five were in complete remission (CR) and 5 in active disease (non-CR) before conditioning. Four non-CR and 4 CR patients carried adverse cytogenetics; and 2 non-CR and 3 CR patients harbored unfavorable molecular markers (Fig. 1; online suppl. Table S1, for all online suppl. material, see

www.karger.com/doi/10.1159/000495456). Five CR patients had less than 1% blasts in bone marrow (BM) and no blasts in PB (Fig. 2a, left). The 5 non-CR patients had high blasts in BM and PB prior to conditioning (Day -9) and persistent blasts in PB following conditioning (Day -3) (Fig. 2a, left). A total of 4 of 5 non-CR and all 5 CR patients achieved a complete response to allo-SCT. The time to engraftment of donor cells did not differ significantly between CR and non-CR patients (online suppl. Fig. S1a). Disease progression following allo-SCT was observed in 4 of 5 non-CR patients, but in no CR patient.

Fig. 2. Clinical characteristics of 5 AML patients in CR and 5 non-CR patients from whom study samples were obtained. **a** Left: percentage of blasts in BM and PB in CR and non-CR samples at baseline (Day -9) and posttreatment with G+P plus Bu+Flu (Day -3). $^{\#} p = 0.002$; $^{\# \#} p = 0.017$; $^{\# \# \#} p = 0.028$. Right: correlation of blast percentage in BM and PB at baseline (Day -9) and in PB treated with G+P plus Bu+Flu (Day -3) with overall survival in CR and non-CR patients. $^{\#} p = 0.004$, $r = -0.813$; $^{\# \#} p = 0.047$, $r = -0.639$; $^{\# \# \#} p = 0.003$, $r = -0.855$. **b** Effects of treatment on defined cell

populations in samples collected at baseline (Day -9), after G+P treatment (Day -6), and after G+P plus Bu+Flu treatment (Day -3) in the 5 CR and 5 non-CR patients. Left: treatment effect on WBC count. $^{\#} p = 0.007$; $^{\# \#} p = 0.013$; $^{\# \# \#} p = 0.004$. Middle: treatment effect on FISH+ clonal AML cells (4 non-CR and 2 CR, $n = 6$). $^{\#} p = 0.027$; $^{\# \#} p = 0.019$. Right: G+P treatment effect on mobilization of CD34+ cells in non-CR AML patients. CR, complete remission; BM, bone marrow; PB, peripheral blood; WBC, white blood cell.

Overall survival of 5 non-CR patients was significantly shorter than that of 5 CR patients (online suppl. Fig. S1b), which is consistent with the overall study outcome [6]. The survival duration negatively correlated with blast percentage in BM and PB both before and after the conditioning (Fig. 2a, right). Together, our clinical data suggest an association between persistent circulating blasts and poor outcomes in non-CR patients undergoing allo-SCT, a similar finding was previously reported [9].

Treatment with G+P showed a trend in mobilizing white blood cells in both CR and non-CR AML patients (Fig. 2b, left). In 4 non-CR and 2 CR patients carrying cytogenetic markers detectable using FISH, G+P significantly mobilized clonal FISH+ AML cells (online suppl. Fig. S1c, left). These mobilized cells were reduced but not fully eliminated by Bu+Flu on Day -3 (Fig. 2b, middle). Flow cytometry analysis revealed that G+P mobilized

CD34+ cells in 4 of 5 non-CR AML patients (Fig. 2b, right; online suppl. Fig. S1c, right), but no CD34+ cells were detected in PB in any of the CR samples. Together, these results confirm that in these 10 selected patients, G+P mobilizes FISH+ clonal AML cells and AML progenitor cells, consistent with prior report [6].

Using the RPPA technology, we profiled 153 proteins in 33 pathways and functional groups in PB samples from the 5 CR and 5 non-CR patients at different time points (online suppl. Table S2). RPPA analysis of baseline samples taken prior to conditioning regimen (Day -9) revealed that 9 proteins in 9 pathways and functional groups were expressed differently in CR and non-CR samples. Among them, KIT, 53BP1 (a P53-binding DNA repair protein), CIAP (a SMAC and IAP family member), and BAK had significantly higher expression, while MIG6, MEK1, TUBERIN, GSK3AB, and ANNEXIN I had lower

Fig. 3. RPPA analysis of signaling pathways and functional groups. Proteins and associated pathways and functional groups that were significantly differentially expressed in baseline samples from 5 CR

and 5 non-CR AML patients are shown. Proteins in white had higher expression levels, and proteins in dark gray had lower expression levels in non-CR samples.

Fig. 4. Protein alterations triggered by treatment with G-CSF and plerixafor (G+P). **a** The Venn diagrams display the total number of proteins altered in CR (white) and non-CR (gray) samples. The number of proteins that were altered in both CR and non-CR samples is indicated in the region where the white and gray circles overlap. The stacked bar graphs show the expression difference between treatment and baseline of the identified proteins with up- and down-regulation in the same direction. Proteins altered in both sample groups are displayed in the table between the 2 bar graphs. Upregulated expression is indicated by black letters against white fill, and downregulated expression is indicated by white let-

ters against black fill. **b** Proteins significantly differentially expressed in the 5 CR and 5 non-CR samples after treatment of G-CSF and plerixafor (G+P). Proteins in white had higher expression and proteins in dark gray had lower expression in non-CR samples. Proteins that were persistently differentially expressed at baseline and in G+P treated samples are circled with a thick black line and highlighted in the light gray area. Proteins that were differentially expressed only in G+P-treated samples are circled with a thick gray line. The thin black dashed lines group proteins that belong to the same pathway or functional group.

(For figure see next page.)

G+P vs. baseline
(Day -6 vs. Day -9)

CR AML	PCNA	PKCA	p-RB S807/811
non-CR AML	PCNA	PKCA	p-RB S807/811

a

non-CR AML vs. CR AML
G+P (Day -6)

b

G+P plus Bu+Flu vs. baseline
(Day -3 vs. Day -9)

CR AML	PCNA	PKCA	MTOR	TAU
non-CR AML	PCNA	PKCA	MTOR	TAU

a

non-CR AML vs. CR AML
G+P plus Bu+Flu (Day -3)

b

(For legend see next page.)

expression in non-CR than in CR patient samples. The top 5 differentially expressed proteins between CR and non-CR samples were 53BP1, KIT, GSK3AB, CIAP, and MEK1 (Fig. 3; online suppl. Fig. S2). These data suggest that baseline differences in protein expression depend on disease status, resulting in divergent signaling network in the 5 CR and 5 non-CR AML, which could be the initial factor driving differential molecular responses to the G+P plus Bu+Flu conditioning.

Treatment with G+P significantly modulated 22 proteins in 13 pathways and functional groups in 5 CR AML samples; 17 of the 22 proteins were up- and down-regulated in the same direction (Fig. 4a; online suppl. Fig. S3a, c). In 5 non-CR samples, treatment with G+P significantly affected 7 proteins in 5 signaling and functional groups; all 7 were up- and down-regulated in the same direction

(Fig. 4a; online suppl. Fig. S3b, c). Three proteins – PCNA, PKCA, and p-RB S807/811 – were significantly affected by G+P in both CR and non-CR samples examined. Importantly, the upregulation of PCNA and p-RB S807/811 and downregulation of PKCA in G+P-treated patients indicated an increase in cell cycle activity and a decrease in PKCA-dependent CXCR4/SDF- α regulation, both of which are consistent with the mechanism of action of G+P-mediated disruption of stroma-leukemia interactions [15–17].

Comparison of proteins differentially expressed between the 5 non-CR and 5 CR samples after exposure to G+P (Day –6) revealed 22 differentially expressed proteins in 13 signaling and functional groups (Fig. 4b). Among the top 10 distinct proteins, 53BP1, KIT, PI3K-p110A, SMAD1, and p-JUN S73 had higher, and GSK-

Fig. 6. Persistently differentially expressed proteins in CR and non-CR AML. Bar graph displays the proteins that are persistently differentially expressed in non-CR and CR samples at baseline (Day –9), after treatment with G+P (Day –6), and after treatment with G+P plus Bu+Flu (Day –3). # $p = 0.015$.

Fig. 5. Protein alterations triggered by treatment with G+P plus busulfan and fludarabine (G+P plus Bu+Flu). **a** The Venn diagrams, the table, and stacked bars are described as above. **b** Proteins significantly differentially expressed in the 5 CR and 5 non-CR samples after treatment of G+P plus Bu+Flu. Proteins in white had higher expression and proteins in dark gray had lower expression in non-CR samples. Proteins that were persistently differentially expressed at baseline and in treated samples are circled with

a thick black line and highlighted in the light gray area. Proteins that were differentially expressed in both G+P- and G+P plus Bu+Flu-treated samples are circled with a thick gray line. Proteins that were differentially expressed only in G+P plus Bu+Flu-treated samples are circled with a thick black dashed line. The thin black dashed lines group proteins that belong to the same pathway or functional group.

3AB, P38MAPK, p-HER2 Y1248, P-CADHERIN, and SYK had lower expression in non-CR samples than in CR samples (online suppl. Fig. S3d).

Proteomics analysis of samples following G+P plus Bu+Flu conditioning showed modulation of 40 proteins in 14 signaling pathways in the 5 CR samples; 33 of 40 proteins were up- or down-regulated in the same direction in all CR samples (Fig. 5a; online suppl. Fig. S4a, c). In 5 non-CR samples, G+P plus Bu+Flu altered 10 proteins in 7 signaling and functional groups, all 10 were up- and down-regulated in the same direction (Fig. 5a; online suppl. Fig. S4b, c). Four proteins PCNA, PKCA, MTOR, and TAU were affected by G+P plus Bu+Flu in the same direction in both CR and non-CR samples examined. Interestingly, PKCA and p-PKCA S657 were downregulated in AML blasts from all 5 non-CR patients, both post G+P and G+P plus Bu+Flu. In nonleukemic cells from CR patients only PKCA but not its phospho-isoform was down-modulated. After G+P plus Bu+Flu, 16 proteins in 13 pathways and functional groups were found to be differentially expressed between non-CR and CR samples (Day -3) (Fig. 5b), a number of which were similar to those found to be distinct after G+P exposure. Among the top 10 distinct proteins, 53BP1, KIT, PI3Kp110A, XRCC1, and p-ERA S118 had higher expression, and GSK3AB, MIG6, SYK, TAU, and p-AKT T308 had lower expression in non-CR samples than in CR samples (online suppl. Fig. S4d).

Together, our data suggest that signaling networks modulated by G+P or G+P plus Bu+Flu vary among samples and are dependent on disease status.

Notably, 3 proteins, KIT, 53BP1, and GSK3AB, each involving a different pathway, were significantly differentially expressed in 5 CR and 5 non-CR samples at baseline, and were persistently differentially expressed in CR and non-CR AML posttreatment with G+P and G+P plus Bu+Flu. Expression of KIT and 53BP1 was higher, while GSK3AB was lower in non-CR than in CR samples (Fig. 6). High KIT expression in AML has been associated with the development of chemoresistance through drug efflux [18]. 53BP1-associated DNA damage repair is often compromised in AML because of dysfunctional upstream regulators of 53BP1, such as SIRT and the Ku complex [19]. GSK3AB suppression or deprivation is critical for both the initiation and progression of AML, and AML with low GSK3AB expression is associated with poor prognosis [20]. Importantly, these proteins were largely unaffected by the treatment with G+P plus Bu+Flu in non-CR AML, suggesting the need for an alternative therapy.

Conclusion

In summary, we observed that G+P mobilized AML progenitor cells and FISH+ clonal AML cells in both, PB blasts from AML patients with active disease and in non-leukemic PB cells from AML patients in remission. Our findings by RPPA indicate that the increase in circulating cells is not merely a result of mobilization but is also contributed by the increased cycling of both, AML and healthy cells, as shown by G+P-modulated PCNA, p-RB, and PKCA. Given simultaneous administration of plerixafor and G-CSF, we are unable to dissect whether this effect is attributable to P, G, or both; however, prior reports have suggested modulation of cell cycle by CXCR4 inhibition alone [21, 22]. As expected, our findings demonstrate that the signaling networks in active AML are distinct from those in AML in remission prior to the conditioning regimen; and additionally show that G+P and G+P plus Bu+Flu rewired signaling networks in a distinct fashion depending on the disease status. G+P plus Bu+Flu conditioning failed to effectively eliminate AML blasts which maintained aberrant expression of KIT, 53BP1, and GSK3 proteins, none of which were modulated by G+P plus Bu+Flu conditioning. These proteins could represent potential markers of chemoresistance and are putative therapeutic targets in AML. On the contrary, inhibition of PKCA signaling appears to be more selective in AML blasts, potentially representing a CXCR4-specific downstream target [23]. Of note, because cell type composition varies between CR and non-CR samples, population effects may mask rare but important signaling events in specific cell types, which is a limitation of this pilot study. Future cell-type specific analysis in a larger sample size is needed to validate our observations and advance the knowledge of cell type-dependent signaling modulation in AML.

Statement of Ethics

All patients have given their written informed consent. The study protocol has been approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conception and design: M.K., Z.Z., E.S., and R.C. Acquisition of data: Z.Z., H.L., R.-Y.W., J.C., and C.B.B. Analysis and interpretation of data: Z.Z., H.L., R.-Y.W., W.L., K.A.B., S.K., C.B.B., and M.K. Writing, revision, and editing of the manuscript: Z.Z. and M.K. Supervision and procurement of AML samples: E.S., R.C., and M.K.

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