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Impact of CXCR4 inhibition on FLT3-ITD−positive human AML blasts

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Objective. **Internal tandem duplication (ITD) mutations of the FLT3 receptor are associated with a high incidence of relapse in acute myeloid leukemia (AML). Expression of the CXCR4 receptor in FLT3-ITD−positive AML is correlated with poor outcome, and inhibition of CXCR4 was shown to sensitize AML blasts toward chemotherapy. The aim of this study was to evaluate the impact of FLT3-ITD on cell proliferation and CXCR4-dependent migration in human hematopoietic progenitor cells and to investigate their response to CXCR4 inhibition.**

Materials and Methods. **We used primary blasts from patients with FLT3-ITD or FLT3 wild-type AML. In addition, human $CD34^+$ hematopoietic progenitor cells were transduced to >70% with retroviral vectors containing human FLT3-ITD.**

Results. **We found that FLT3-ITD transgene overexpressing human hematopoietic progenitor cells show strongly reduced migration toward stromal-derived factor−1 in vitro and display significantly reduced bone marrow homing in nonobese diabetic severe combined immunodeficient mice. Cocultivation of FLT3-ITD−positive AML blasts or hematopoietic progenitor cells on bone marrow stromal cells resulted in a strong proliferation advantage and increased early cobblestone area−forming cells compared to FLT3−wild-type AML blasts. Addition of the CXCR4 inhibitor AMD3100 to the coculture significantly reduced both cobblestone area−forming cells and proliferation of FLT3-ITD−positive cells, but did not affect FLT3−wild-type cells—highlighting the critical interaction between CXCR4 and FLT3-ITD.**

Conclusion. **CXCR4 inhibition to decrease cell proliferation and to control the leukemic burden may provide a novel therapeutic strategy in patients with advanced FLT3-ITD−positive AML.** **© 2010 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder that originates from leukemic stem cells with the ability to generate an excessive amount of malignant myeloid blasts. A concert of genetic aberrations underlies the multistep pathogenesis of AML. It appears that one class of mutations, such as the internal tandem duplication of the receptor tyrosine kinase FLT3 (FLT3-ITD) results in uncontrolled proliferation of hematopoietic progenitors and that the other class of mutations, such as RUNX1-ETO or RUNX1-EVI1, induces a block in differentiation leading to consecutive blast cell accumulation [1,2]. The ITD mutation is located in the juxtamembrane domain of FLT3 and results in ligand-independent constitutive activation of the FLT3 receptor leading to activation of downstream signaling proteins, such as signal transducer and activator of transcription (STAT)5, STAT3, and extracellular signal-regulated kinase (ERK) [3]. ITD mutations of the FLT3 receptor occur in about 25% of patients with AML, and this mutation is associated with poor prognosis [4,5]. The high relapse rate is due, in part, to the inefficient elimination of leukemic stem cells in the bone marrow niche, where these cells seem to be

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protected from chemotherapeutic therapies through interactions with the microenvironment. While integrins such as very late antigen−4 and the adhesion molecule CD44 have been demonstrated to be crucial for the persistence of leukemic progenitors in the bone marrow, the chemokine receptor CXCR4 and its ligand stromal cell−derived factor-1 (SDF-1) have been identified as key players for homing of AML cells to their niche and their in vivo growth [6–8]. CXCR4 is a G-protein−coupled receptor that induces migration upon binding of SDF-1, activates adhesion molecules and integrins and induces cell proliferation and cell survival [9]. Inhibition of CXCR4 has been shown to sensitize leukemic blasts to chemotherapy [10–13]. Recent reports suggest a functional interaction between the FLT3 receptor and CXCR4. While CXCR4 expression in FLT3-ITD−positive $CD34^+$ AML cells was correlated with poor outcome [14], FLT3-ITD overexpression in murine hematopoietic cell lines was suggested to result in enhanced SDF-1−mediated cell migration [15].

We show that FLT3-ITD transgene overexpression in human hematopoietic progenitor cells (HPC) confers reduced SDF-1−mediated migration in vitro, as well as reduced homing of human HPC to the bone marrow of nonobese diabetic severe combined immunodeficient (NOD/SCID) mice. Furthermore, we demonstrate that the growth advantage of FLT3-ITD−positive HPC or AML blasts in coculture with bone marrow stromal cells largely depends on the activation of CXCR4. Inhibition of CXCR4 selectively reduced cell proliferation and formation of cobblestone area−forming cells (CAFCs) of FLT3-ITD transgene−positive HPC and FLT3-ITD−positive human AML blasts.

Materials and methods

Source of healthy human $CD34^+$ progenitor cells and primary AML blasts

$CD34^+$ HPC from mobilized peripheral blood or from cord blood of healthy donors were used. After informed consent following the Institutional Review Board–approved protocols, $CD34^+$ cells were selected by MACS MicroBeads (Miltenyi, Bergisch Gladbach, Germany). The purity of $CD34^+$ after selection was >97%. Primary AML cells were harvested from bone marrow aspirates after informed consent. Vials of $CD34^+$ cells and AML cells were stored in liquid nitrogen and thawed for each experiment.

Source of primary human bone marrow stromal cells (hBMSC)

Bone marrow aspirates were collected from healthy donors after informed consent and approval of the Institutional Review Board. After density centrifugation, human BMSC were isolated by plastic adherence and cultivation in Dulbecco's modified Eagle's medium/0.1% glucose and 10% fetal bovine serum (passaged maximally three times). After 1 week, cells were stained with peridinin-chlorophyll protein complex anti−human-CD45 immunoglobulin G (B&D, Heidelberg, Germany) to monitor possible contamination with hematopoietic cells. For further characterization of isolated hBMSC, osteogenic and adipogenic differentiation potential was confirmed by alkaline phosphatase activity and Oil Red staining, respectively.

Generation of γ-retroviral transfer vectors and virus vector production

FLT3-ITD complementary DNA was cloned from the human acute monocytic leukemia cell line MV4-11 [16]. FLT3 wild-type (FLT3-wt) complementary DNA was cloned from human HPC. To generate MFGS-FLT3-ITD-IRES-GFP and MFGS-FLT3-ITD-IRES-CFP, respectively, FLT3-ITD was cloned directionally into the XhoI-BamHI cloning sites of MFGS-IRES-GFP and MFGS-IRES-CFP vectors [17]. To generate MFGS-FLT3-wt-IRES-GFP, FLT3-wt was cloned directionally into the XhoI-BamHI cloning site of the MFGS-IRES-GFP vector. The MFGS-GFP, MFGS-CFP, and MFGS-IRES-GFP vectors were published elsewhere and served as controls [18]. To obtain various RD114-pseudotyped MFGS virus vectors from stable virus vector producer cell lines, FLYRD18 packaging cells were transduced with transiently produced virus vector, and for each vector a high-titer FLYRD18 producer clone was selected [17]. FLYRD18 cells with a FLT3-ITD−containing construct were cultured in medium supplemented with GTP-14564 at 2 μM. After ultracentrifugation, the virus vector pellet was suspended in fresh X-VIVO10/1% human serum albumin. The neat titers of the virus vector as determined on cord blood $CD34^+$ HPC were $2-5 \times 10^6$.

Transduction of HPCs

Cultures were initiated in Retronectin (Takara Shuzo Ltd., Otsu, Japan) precoated six-well plates with 3×10^5 $CD34^+$ HPC per well. HPC were cultured in X-VIVO10 (Bio Whittaker, Walkersville, MD, USA) supplemented with 1% human serum albumin, 50 ng/mL FLT3 ligand, 10 ng/mL stem cell factor, 10 ng/mL thrombopoietin, and 10 ng/mL interleukin-3. All cytokines were purchased from R&D Systems (Wiesbaden, Germany). HPC were transduced at 48 hours and 72 hours after culture initiation. Naïve and control-transduced HPC served as negative control. The transduction efficiency was controlled by flow cytometry and cell viability determined by trypan blue (Merck, Darmstadt, Germany) exclusion.

Analysis of STAT5a, STAT3, and ERK1/2 phosphorylation by flow cytometry

The 1×10^5 cells were fixed, permeabilized (BD PhosFlow Perm Buffer; B&D) and stained according to manufacturer's instructions with Alexa Fluor 647−conjugated antibodies directed against phospho-STAT5a, phospho-STAT3, and phospho-ERK1/2 (B&D), respectively, and subsequently analyzed by flow cytometry.

CXCR4 expression by flow cytometry

Cell surface and total (surface plus intracellular) CXCR4 expression were analyzed in transduced $CD34^+$ HPC by flow cytometry. Cells were stained with allophycocyanin-conjugated anti-human CD184 (anti-CXCR4; clone 12G5; B&D) or isotype control (Mouse IgG2a; B&D) after fixation and permeabilization (total CXCR4, Fix&Perm reagents; Caltag Laboratories, Hamburg, Germany) or without permeabilization (surface CXCR4).

In vitro migration assay

MFGS-FLT3-ITD-IRES-GFP or MFGS-FLT3-ITD-IRES-CFP transduced human $CD34^+$ HPC were pre-incubated for 3 hours in RPMI without FBS. The 5×10^4 of transduced HPC were