Overexpression and Constitutive Activation of FLT3 Induces STAT5 Activation in Primary Acute Myeloid Leukemia Blast Cells

Karsten Spiekermann, Ksenia Bagrintseva, Ruth Schwab, Karin Schmieja, and Wolfgang Hiddemann

Department of Medicine III, University Hospital Grosshadern, Clinical Cooperative Group “Leukemia”, GSF National Research Center for Environment and Health, 81377 Munich, Germany

ABSTRACT

**Purpose:** Activating length mutations in the juxtamembrane domain (FLT3-LM) and mutations in the tyrosine kinase domain (FLT3-TKD) of FLT3 represent the most frequent genetic alterations in acute myeloid leukemia (AML). However, the functional role of active FLT3 mutants in primary AML blast cells is not well characterized.

**Experimental Design:** We analyzed the transforming potential and the signaling of FLT3-ITD mutants in Ba/F3 cells and in primary AML blasts.

**Results:** FLT3-ITD mutants induce an autophosphorylation of the receptor, interleukin 3-independent growth in Ba/F3 cells, and a strong STAT5 and mitogen-activated protein kinase (MAPK) activation. In contrast to the FLT3-ITD mutants, the ligand-stimulated FLT3-WT receptor was unable to transduce a fully proliferative response in Ba/F3 and monocytic OCI-AML5 cells. The ligand-stimulated FLT3-WT receptor activated AKT and MAPK, but not STAT5. In primary blast cells from 60 patients with AML, FLT3 was expressed in 91.9% of patients carrying a FLT3-LM/TKD mutation compared with 77.8% in FLT3-LM/TKD-negative patients. STAT3 and STAT5 were constitutively activated in 76 and 63% of patients, respectively. In accordance with the results in Ba/F3 cells, a high FLT3 expression and the presence of a FLT3-LM was strongly associated with the STAT5 but not with the STAT3 activation in primary AML blast cells. Moreover, the constitutive tyrosine phosphorylation of STAT5 was efficiently downregulated by a FLT3 protein tyrosine kinase inhibitor in AML cells expressing an active FLT3 mutant.

**Conclusions:** Active FLT3 receptor mutants have transforming potential in hematopoietic cells and induce a strong activation of STAT5 in primary AML cells. The FLT3-STAT5 pathway contributes to the malignant phenotype and represents a promising molecular therapeutic target structure in AML.

INTRODUCTION

FLT3 belongs to the class III of RTKs and has an important function in the expansion of hematopoietic progenitors and in the pathogenesis of AML. Primary leukemic blasts from patients with AML express functional FLT3 receptors and stimulation with FL induces proliferation and inhibits apoptosis by up-regulation of BCL-2 (1). Activating mutations in the JM (FLT3-LMs) and in the TKD (FLT3-TKD) of FLT3 are found in 30–35% of patients with AML and represent the most frequent genetic alterations in AML (2–10). Genetically, FLT3-LMs are heterogeneous and consist of ITDs of 6–30 aa in most patients. These mutations result in an elongated FLT3 protein with constitutive PTK activity (11). Recent structural analyses of the Ephb2 family of RTK show that the JM domain forms an inhibitory loop that interacts with the catalytic domain of the kinase (12). Mutations in the JM region probably interfere with the inhibitory activity of this domain resulting in a constitutive catalytic activity of the kinase.

Analyses from our and other groups (4, 7) have shown that the frequency of the FLT3-LM differs significantly in cytogenetic subgroups of AML patients and is highest in patients with a normal karyotype and in patients carrying a t(15;17) translocation. In contrast, the FLT3-LMs are rarely found in patients with a complex karyotype and CBF-leukemias (CBFβ-MYH11 and AML1-ETO). In all cytogenetic subgroups, the presence of a FLT3-LM represents a negative prognostic risk factor for the overall and the event-free survival (4). Furthermore, the loss/deletion of the residual FLT3-WT allele represents an additional negative prognostic factor in FLT3-LM-positive AML (9). These findings are supported by data from our group showing that deletions of the FLT3-WT allele are more frequently found in patients at relapse as compared with patients at initial diagnosis, which suggests that these genetic alterations are associated with disease progression (7).

Constitutively active FLT3 mutants have transforming potential in IL-3-dependent cells and activate the STAT5 and MAPK pathways (13–15). The transforming capacity of active FLT3 mutants has been confirmed in animal models either by
overexpression of FLT3-LM in the bone marrow transplant model (16) or by expression of an active TEL-FLT3 fusion protein in transgenic animals (17). Both models show that the constitutively active FLT3 can induce a myeloproliferative syndrome in vivo and underline the potential transforming activity of these mutants in AML. However, these data also show that active FLT3 mutations alone are not sufficient to induce an AML phenotype in vivo and support the concept of a multistep pathogenesis in AML. Very recently, Kelly et al. (18) proposed a model that distinguishes two types of mutations in AML. According to this model, type I mutations represent genetic alterations that induce a proproliferative and antiapoptotic signal (e.g., gain of function mutations of PTK and ras), whereas type II mutations interfere with differentiation and often involve myeloid transcription factors (loss-of-function mutations).

Although studies in IL-3-dependent cell lines and also in mouse models support a model of a gain of function mutation of FLT3-LMs in AML, this hypothesis has to be directly verified in AML cells. In addition, it has been hypothesized that the constitutive activation of STAT5 in primary AML blasts might be related to the constitutive catalytic activity of leukemic fusion proteins with PTK activity, e.g., FLT3. Our results clearly indicate that STAT5 is an important target of constitutively active FLT3 mutants, but not of the ligand-stimulated FLT3-WT receptor. In addition, the constitutively active FLT3-ITD protein that is expressed in 92% of primary AML blasts substantially contributes to the constitutive STAT5, but not STAT3 activity in AML. These data have important clinical implications and support a role of the constitutively active FLT3-STAT5 pathway as a therapeutic target in AML.

PATIENTS, MATERIALS AND METHODS

Patient Samples. Bone marrow or blood samples from 60 adult patients with newly diagnosed and untreated AML were analyzed. All were diagnosed as having AML according to standard French-American-British (FAB) and WHO criteria and were referred to our clinic for central cytomorphological and cytogenetic diagnostics. The studies abide by the rules of the local internal review board and the tenets of the revised Helsinki protocol.

Cytogenetics. Cytogenetic G-banding analysis was performed with standard methods (19). The definition of the cytogenetic clone and descriptions of karyotypes followed the International System for Human Cytogenetic Nomenclature.

Reagents and Cell Lines. Recombinant murine IL-3 was purchased from Biosource International (Camarillo, CA). Restriction enzymes were obtained from New England Biolabs (Beverly, MA) and reagents for PCR were purchased from Applied Biosystems (Foster City, CA). Low-passage murine Ba/F3 cells were obtained from the DSMZ (Braunschweig, Germany) and were maintained in RPMI 1640 with 10% FBS. FLT3-ITD-Ba/F3 cells were obtained from the DSMZ (Braunschweig, Germany) and were maintained in RPMI 1640 with 10% FBS – (intermediate), 51–70% (strong), and 70% (very strong).

Cell Proliferation of Ba/F3 Cells. Cells were seeded at a density of 4 × 10^4/ml growth medium with or without murine IL-3. Viable cells were counted for 3 days in a standard Neubauer chamber by trypan blue exclusion.

Antibodies. The following antibodies were used: anti-FLT3/IT/2 (S18, sc-480; Santa Cruz, Heidelberg, Germany), anti-PY (PY99; Santa Cruz), anti-phospho-Thr202/Tyr204-p42/p44 MAPK-Ab, anti-phospho-STAT5-Tyr694, anti-phospho-STAT3-Tyr705, anti-phospho-STAT5-Tyr694 (all from New England Biolabs, Frankfurt, Germany), anti-STAT3 (C20; Santa Cruz), anti-STAT5 (sc-835; Santa Cruz).

DNA-Constructs and Vectors. The FLT3-ITD-NPOS construct contains a 28-aa-duplicated sequence (CSSDNEYFYVDFREYEDLKWEFPRENL) inserted between aa 610/611 and the FLT3-ITD-W51 constructs contains a 6-aa-duplicated sequence (REYEDL) inserted between aa 601/602 of human FLT3-WT (kindly provided by G. Gilliland, Howard Hughes Medical Institute and Brigham and Women’s Hospital Harvard Institutes of Medicine, Harvard Medical School, Boston, MA). Both the FLT3-ITD and the FLT3-WT construct were subcloned in the MSCV-ires-EYFP/EGFP retroviral expression vector (kindly provided by R. K. Humphries, The Terry Fox Laboratory, University of British Columbia, Vancouver, British Columbia, Canada).

Transient Transfection of BOSC23 Cells. One day before transfection, BOSC23 cells were seeded into 6-well plates at a density of 3.0 × 10^5/ml. Transient transfections were then carried out using the calcium-phosphate coprecipitation method with a total of 2 μg of plasmid DNA per well. Eighteen h after transfection, 2 ml of fresh medium were added, the cells were allowed to grow for another 30 h, and the retroviral supernatant was used for transduction of Ba/F3 cells.

Transduction of Ba/F3 Cells. Ba/F3 cells (2 × 10^5) were seeded in 1 ml of growth medium and were subsequently transduced once with 200 μl of retroviral supernatant in the presence of Polybrene (8 μg/ml). The FACS-Ventage system equipped with a Turbo-Sort device (Becton Dickinson, San Jose, CA) was used to highly purify EGFP/EYFP-positive well cells 48 h after infection.

Analysis of CD135 and CD117 Expression by Flow Cytometry. Expression of FLT3 and KIT was assessed by flow cytometry (FacsCalibur, Becton Dickinson) using anti-human CD135 and anti-human CD117 monoclonal antibodies (FLT3-PE/KIT-PE and a matched isotype IgG1-PE from Immunotech). The gate for the evaluation of the specific CD117/ CD135 antibody binding was set at a level in which less than 2% of isotype antibody-stained cells were defined as “positive.” Patient samples were grouped according to the percentage of CD117+CD135+ positive blast cells in five categories of expression intensity: 0–10% (negative), 11–25% (weak), 26–50% (intermediate), 51–70% (strong), and >70% (very strong).

Immunoprecipitation and Western Blot Analysis. Cell extracts were prepared and subjected to immunoprecipitation and immunoblotting as described previously (20). For densitometric analysis of Western blot results, films were scanned. Around each signal for FLT3 protein, a rectangle of equal area was defined on PY-blots as well as on FLT-3-reblots. On each
b remotiation of the particular background was subtracted from each measured value of protein containing lanes. Then the absorbance(p-Tyr):absorbance (re- blot) ratio was calculated and finally, the ratio for untreated controls was defined as 100%, and all of the other ratios were transformed into relative values.

RESULTS

FLT3-ITD Mutants, but not the FLT3-WT Receptor, Have a Direct Transforming Potential in Ba/F3 Cells. The pMSCV-IRES-GFP retroviral expression construct was used to evaluate the transforming capacity of the different FLT3 mutants after transduction of Ba/F3 cells. The presence of EGFP/EYFP as selectable markers enabled us to sort the cells by fluorescence-activated cell sorting after transduction and to avoid any selection by antibiotics. In addition to the human FLT3-WT construct, two FLT3-ITD mutants (N-POS and W51) carrying two different LMs (28 and 6 aa, respectively) which were introduced at two different positions (aa 610/611 and aa 601/602, respectively) were used.

Both FLT3-ITD mutants that were used in this study conferred long-term IL-3-independent growth to Ba/F3 cells (Fig. 1). In contrast, the FLT3-WT construct was unable to induce factor independency when stably expressed in Ba/F3 cells. Identical levels of the FLT3-ITD mutant and the FLT3-WT protein in the Ba/F3 cell lines were confirmed by flow cytometry (Fig. 2B) and by Western blot (Fig. 2A).

FLT3-ITD Mutants Are Hyperphosphorylated in Ba/F3 Cells. Given the profound biological differences between the FLT3-WT and FLT3-ITD constructs, we next analyzed the FLT3 receptor activation. In the presence of serum, the autophosphorylation of the cell surface fraction of FLT3 (Mr 165,000) did not significantly differ in FLT3-ITD and FLT3-WT expressing cells (Fig. 2A, upper panel). In contrast, the intracellular form of FLT3 (Mr 120,000) was hyperphosphorylated. After a 16-h starvation period with 0.3% FBS, the differences in the FLT3 autophosphorylation between the FLT3 mutants and the FLT3-WT receptor was even more pronounced. Although serum starvation resulted in a general reduction of the FLT3 tyrosine phosphorylation, the autophosphorylation of the Mr 120,000 and 165,000 forms of the FLT3-ITD constructs was still detectable. In contrast, the FLT3-WT receptor was completely dephosphorylated under serum starvation (Fig. 2A).

In Contrast to FLT3-ITD Mutants, the FLT3-WT Receptor Is Unable to Induce a Fully Proliferative Signal in Ba/F3 Cells and Activates MAPK and AKT, but not STAT3 or 5. To identify differences in receptor function and signal transduction that might be responsible for the transforming capacity of the FLT3 receptor mutants, we compared the function of the FLT3-ligand (FL)-stimulated FLT3-WT receptor...
with FLT3-ITD mutants. Stimulation of Ba/F3 cells expressing the FLT3-WT receptor with recombinant FL resulted in a dose-dependent increase of viable cells after 72 h compared with unstimulated cells (Fig. 3A). However, when the response of FL-stimulated cells was compared with IL-3 stimulated cells, even saturating concentrations of FL (10–20 ng/ml) induced only a minimal proliferative response (Fig. 3B).

Next, the biological effects of ligand-stimulated cells were compared with the signaling pathways that were activated by FL-stimulated FLT3-WT and FLT3-ITD mutants. Using phospho-specific antibodies, we could show that FL strongly activated AKT and MAPK (Fig. 3D). In contrast, FL activated neither STAT3 nor STAT5, which was minimally activated in mock-infected Ba/F3 cells grown in the presence of IL-3 (Fig. 3C). Analysis of the FLT3-ITD-expressing Ba/F3 cells revealed that both mutants activated MAPK and also, weakly, AKT which was dependent on the presence of serum (Fig. 3D). In contrast to the FL-stimulated FLT3-WT expressing cells, FLT3-ITDs induced a strong activation of STAT5 and a weak activation of STAT3 (Fig. 3C), independent of the concentration of FBS in the growth medium.

These results show that activation of FLT3 by FL can protect FLT3-WT-expressing Ba/F3 cells from apoptosis and activates AKT and MAPK. Unlike the activated IL-3 receptor or the FLT3-ITD receptor mutant, the FLT3-WT receptor is not able to induce a fully proliferative response in Ba/F3 cells and to activate STAT3 or STAT5.

**FL Activates FLT3 and MAPK, but not STAT5, in the AML Cell Line OCI-AML5.** To confirm our hypothesis that the FL-stimulated FLT3-WT receptor is unable to activate STAT5 in a myeloid cell model, we analyzed the AML-derived cell line OCI-AML5 in detail. In these cells, stimulation with FL has been described as inhibiting apoptosis in serum-free media (21). In contrast to the murine lymphoid Ba/F3 and myeloid 32D cells, the human OCI-AML5 cell line is of myeloid origin and expresses endogenous FLT3-WT receptor; stimulation with FL protects these cells from apoptosis and induces slight proliferation. Thereby these cells represent a valid model system and might adequately reflect the situation found in AML blast cells. We could clearly show that FL induces a strong tyrosine phosphorylation of the FLT3 receptor and MAPK (Fig. 4, A–C) that is specifically inhibited by the FLT3 PTK inhibitor SU5614 (Fig. 4, A and C). In contrast, no activation of STAT5 was detectable when aliquots from the same cellular lysates were analyzed using an antibody against STAT5-PY694 (Fig. 4D, Lanes 2–7), even at longer exposure of films (data not shown). In these experiments, Ba/F3 cells expressing a FLT3-ITD mutant were used as a positive control (PC) for the STAT5-Tyr694 specific antibody (Fig. 4D, Lane 1).

**FLT3-TKD Mutations Occur Independently of FLT3-LMs in Patients with AML.** To validate our hypothesis that active FLT3 mutants are responsible for the STAT5 activation in primary AML blasts, we analyzed the activation status of STAT3 and STAT5 in relation to the FLT3 protein expression and FLT3-LM/TKD mutations in 60 adult patients with AML.

In a first step, we screened genomic DNA from these AML patients for activating FLT3-LM and -TKD mutations (summarized in Table 1). The loss of the *Eco*RV restriction site at codon 835/836 by mutations (10) and the previously described PCR-based assay for detection of the FLT3-LMs (3) was used for a mutation screen. Abnormal restriction profiles at codon D835/836 were found in 6 (10%) of 60 of AML patients. The presence of a TKD mutation was confirmed in all cases by nucleotide sequencing. The mutations found at D835 were genetically heterogeneous and resulted in substitution of D835 by glutamine (n = 2), tyrosine, valine, alanine, and histidine (data not shown).

When related to the FLT3-LMs that were found in 27 (45%) of 60 patients for activating FLT3-LM and -TKD mutations (10) and the previously described PCR-based assay for detection of the FLT3-LMs (3) was used for a mutation screen. Abnormal restriction profiles at codon D835/836 were found in 6 (10%) of 60 of AML patients. The presence of a TKD mutation was confirmed in all cases by nucleotide sequencing. The mutations found at D835 were genetically heterogeneous and resulted in substitution of D835 by glutamine (n = 2), tyrosine, valine, alanine, and histidine (data not shown).

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60 patients, both genetic alterations occurred independently and none of the patients carried both mutations.

**FLT3 Is Strongly Expressed in Patients with FLT3-LM/TKD Positive AML.** To quantify the levels of FLT3 protein expression in primary AML blasts, we analyzed the CD135 expression by flow cytometry. Representative examples showing different levels of FLT3 expression are shown in Fig. 5A. Using a cutoff of 10% CD135 positive blast cells for the definition of “positive,” 85% of all patients expressed CD135 compared with 75% of patients for whom blast cells were positive for the CD117 antigen (Fig. 5B). Subclassification of the intensity of FLT3 expression levels showed that 18 (30%) of 60 patients expressed low levels (11–25% CD135+ cells), 16 (26.7%) of 60 expressed moderate levels (26–50% CD135+ cells), whereas in 17 (28%) of 60 of patients high levels of CD135 (>50% CD135+ cells) were detectable. When the CD135 expression was analyzed in relation to the FLT3 mutations, FLT3-LM/TKD-positive patients showed a slightly higher level of FLT3 expression [30 (91.9%) of 33] compared with FLT3-LM/TKD-negative patients [21 (72%) of 29; Fig. 5C].

**Overexpression and Constitutive Activation of FLT3 Is Related to the STAT5, but not the STAT3, Activation, in Primary AML Blasts.** To evaluate the activation status of STAT3 and STAT5 in primary AML, crude lysates from blast cells of the 60 patients were analyzed by Western blot analysis with commercially available phospho-specific antibodies raised against PY-694 of STAT5 and PY-705 of STAT3. Expression of STAT3 and STAT5 protein was found in a total of 49 (82%) of 60 and 43 (72%) of 60 patients, respectively (Fig. 6A). Of all patients who expressed STAT3 and STAT5 in their blast cells, a constitutive STAT activation was found in 37 (76%) of 49 and 27 (63%) of 43, respectively (Fig. 6B).

To validate our hypothesis that constitutively active FLT3-LMs induce a STAT5-activation in primary AML blast cells, we analyzed the activation of STAT3/5 in relation to the FLT3 expression and mutation status. These analyses were done in patients in whom STAT3 (n = 49) and STAT5 (n = 43), respectively, were expressed. Our results show that the percentage of patients with STAT5 activation was 2 (9.5%) of 21 in patients with low or absent FLT3 expression (<25% CD135+ cells), irrespective of the FLT3-status (Fig. 7A). In contrast, in patients with moderate to high FLT3 expression levels (>25% CD135+ cells) a clear correlation between STAT5 activation and the presence of a FLT3-LM was found (Fig. 7A). In this subgroup, a STAT5 activation was detectable in 3 (21.4%) of 14 patients without FLT3-LMs compared with a constitutive STAT5 activation in 4 (50%) of 8 of patients carrying a FLT3-LM.
In the Ba/F3 model, we could clearly show that FLT3-ITD mutants induce a strong activation of STAT5, but only a minimal activation of STAT3. To further validate these data in primary AML, we analyzed the STAT3 activation status in relation to the presence of FLT3-LMs and the protein expression levels of FLT3 (Fig. 7B). In contrast to the STAT5 activation, we found no correlation between the activation of STAT3 and the FLT3 protein expression or the presence of a FLT3-LM. The FLT3 PTK Inhibitor SU5614 Inhibits the Tyrosine Phosphorylation of FLT3 and STAT5 in the AML Cell Line MM6. Our findings in primary AML blasts suggest an important functional role of constitutively active FLT3 receptors in the regulation of STAT5 activity in AML. To confirm these findings in a well-defined model system, we analyzed the previously characterized MM6 AML cell line that carries an activating FLT3 mutation in the JM region (15). As shown in Fig. 8C, STAT5 is constitutively activated in this cell line. To provide direct evidence that FLT3 regulates STAT5 activity in AML cells, we used a recently characterized selective PTK inhibitor of FLT3, SU5614 (15).

SU5614 induces growth arrest, cell cycle arrest, and apoptosis in MM6 cells but not in AML cell lines expressing FLT3-WT or no FLT3 protein (15). The incubation of MM6 cells that express a strongly autophosphorylated FLT3 receptor for 4 h resulted in a complete down-regulation of the FLT3 tyrosine phosphorylation (Fig. 8, A and B) with an IC$_{50}$ of 0.2 µM. In parallel, STAT5 activation as analyzed by a STAT5-Tyr694 specific antibody was strongly down-regulated by FLT3 inhibition. As shown in Fig. 8A, targeting of FLT3 by SU5614 induced a complete down-regulation of STAT5 activity at identical concentrations required to inhibit FLT3 tyrosine phosphorylation (Fig. 8C).

These data clearly confirm the important role of active FLT3 mutants in the regulation of STAT5 activity in AML.

**DISCUSSION**

A constitutive activation of STAT3 and STAT5 has been described in the majority of primary AML blast cells, and...
because of their transforming potential, activation of STATs might directly contribute to the malignant phenotype of AML. The identification of the underlying mechanisms resulting in the STAT activation is, therefore, essential to develop therapeutic strategies that interfere with these essential mitogenic and antiapoptotic signaling pathways. We report here that activating mutations of FLT3 induce autophosphorylation of the receptor IL-3-independent growth in Ba/F3 cells and a strong constitutive activation of STAT5, but not of STAT3. In contrast, the ligand-stimulated FLT3-WT receptor is not sufficient to transduce a fully proliferative signal and is unable to activate STAT3 or STAT5. These in vitro findings were validated in primary blast from 60 patients with AML in which the levels of FLT3 expression and the presence of a FLT3-LM were strongly associated with the STAT5 but not the STAT3 activation.

Our results show that the FLT3-WT and the FLT3-ITD mutant receptor differ significantly in their biochemical and biological properties. The FLT3 receptor was constitutively autophosphorylated in Ba/F3 cells stably expressing FLT3-ITD

Fig. 5 Expression of CD135 and CD117 in primary AML blasts. A, primary AML blasts were stained with PE-CD135, PE-CD117, or PE-labeled matched isotype control antibody and were analyzed by flow cytometry. Representative examples showing different levels of FLT3 expression are shown. B, patient samples were grouped according to the percentage of CD135/CD117-positive blast cells in five categories: 0–10% (negative); 11–25% (weak); 26–50% (intermediate); 51–70% (strong); and >70% (very strong). C, FLT3 expression levels were evaluated as described in A, and results were analyzed according to the mutation status of FLT3 (FLT3-LM/TKD positive versus negative).

Fig. 6 Expression and activation of STAT3 and STAT5 in primary AML blasts. A, the expression of STAT3 and STAT5 was analyzed by Western blot using specific anti-STAT3 and anti-STAT5 antibodies. The intensity of antibody binding was grouped into five classes: −, absent; +, weak; ++, intermediate; +++, strong. B, the activation of STAT3 and STAT5 was analyzed by Western blot using phospho-specific anti-STAT3-PY705 and anti-STAT5-PY694 antibodies. The intensity of antibody binding was quantified as described in A. Only patients who expressed STAT3 (\(n = 49\)) and STAT5 (\(n = 43\)) were evaluated.
Fig. 7 Activation of STAT3 and STAT5 in primary AML blasts in relation to the FLT3 expression and the presence of a FLT3-LM. The activation of STAT5 (A) and STAT3 (B) was analyzed as described in Fig. 5A according to the levels of FLT3 expression by flow cytometry. Only patients expressing STAT3 (n = 49) and STAT5 (n = 43) were considered for this analysis.

Previous studies have shown that STAT3 and STAT5 are constitutively activated in primary blasts from 20–80% of patients with AML (25–31). However, the mechanisms underlying the constitutive STAT activation in AML are not well defined, but an autocrine production of IL-6 has been shown to contribute to the STAT3 activation in AML blasts (28). In addition, the STAT5 activation has very recently been linked to autophosphorylation of the FLT3 receptor in primary AML blasts (24).

On the basis of our findings in FLT3-ITD-transformed Ba/F3 cells we hypothesized that the FLT3-ITD mutant receptor might contribute to the STAT5 activation in AML. The results presented here clearly show that in primary AML blasts, the STAT5, but not the STAT3, activation is related to the presence of a FLT3-LM. Moreover, the constitutive activation of STAT5 was dependent on the levels of FLT3 receptor expression, because only low levels of constitutive STAT5 activation were detected in patients with low to absent FLT3 expression, irrespective of the presence of a FLT3-LM. These findings are in good accordance with the results in Ba/F3 cells showing that overexpression of a FLT3-WT receptor is not sufficient to induce a mitogenic signal. Although the overexpressed FLT3-WT receptor was also autophosphorylated at a low level, it was not able to transduce a fully proliferative signal and to activate STAT5, even in the presence of ligand.

Most experimental systems used thus far to characterize the function of active FLT3 mutants have important limitations. Overexpression of a constitutively active FLT3 mutant in Ba/F3 and 32D cells, which lack endogenous FLT3-WT, mimics a “hemizygous genotype” and the conclusions drawn from these model systems cannot easily be transferred to the heterozygous situation found in the majority of AML patients. Recently published data from a CALGB trial showed that the deletion of the FLT3-WT allele in patients with FLT3-LM further impairs the prognosis in AML patients (9). However, it is not known whether these additional FLT3-WT deletions have a pathophysiological meaning.

An important study from Birkenkamp et al. (24) could show that FL stimulation of AML blasts carrying a heterozygous FLT3 mutation, but not in AML cells harboring a homozgyous mutation, enhanced FLT3 tyrosine phosphorylation. Very recently, Grundler et al. have reported that Ba/F3 and 32D cells
coexpressing FLT3-ITD and FLT3-WT, compared with FLT3-ITD-expressing cells alone (mimicking the hemizygous situation in vivo), do not differ in their proliferative capacity (32). Interestingly, stimulation of FLT3-WT/ITD-expressing (“heterozygous”) cells with FL resulted in a slower growth rate, whereas FL stimulation did not affect the growth of FLT3-ITD (“hemizygous”) cells.

These results suggest an important functional role of the FLT3-WT receptor in AML blasts from patients with heterozygous FLT3-LM mutations that has to be clarified in the future. The frequency of FLT3 RNA and protein expression in primary AML blasts has been analyzed in several studies and ranges from 62.5 to 93% (33–36). The frequency of FLT3 expression in this report was 51/60 (85%) and is in line with these previous findings. Interestingly the FLT3 expression was higher in patients carrying a FLT3-LM/TKD mutation (91.9%) compared with patients expressing the FLT3-WT receptor (77.8%). These results show that virtually all of the patients who carry an activating FLT3 mutation express FLT3 protein on the surface of their blast cells and may indicate that higher FLT3 expression represents a growth advantage for FLT3-LM/TKD mutation-positive AML blast cells. In addition, the activated FLT3-STAT5 pathway might represent an important target structure for therapeutic interventions.

Several mechanisms, including autocrine production of growth factors and transforming mutations, have been shown to induce the activation of RTKs in solid tumors and hematological malignancies. In the case of FLT3, activating mutations have been described in 30–35% of AML patients. Our results clearly indicate that FLT3-LMs induce STAT5 activation in Ba/F3 cells and also in primary AML blasts. The frequency of the recently described FLT3-TKD mutations in AML was 6 of 60 (10%) in our patient population. These results were similar to those recently reported by Yamamoto et al. (10) and Abu-Duhier et al. (2), who found an incidence of FLT3-TKD mutations of 7.0 and 7.2%, respectively. However, the frequency of FLT3-LM in the present study was higher [27 (45%) of 60] than the previously described frequency in a large cohort of AML patients from our institution (23%; Ref. 7). This high incidence can be explained by a nonrepresentative distribution of cytogenetic subgroups in our study. Compared with the expected distribution, our patient population consisted of a low percentage of patients who rarely carry FLT3-LM mutations (AML1-ETO/H11001, CBFβ-MYH11, and complex karyotypes) thereby overrepresenting patients with a high expected frequency of FLT3-LMs.

Our findings have profound impact for the understanding of the pathogenetic role of the FLT3-LMs in primary AML cells. Although the transforming activity of the FLT3-LM has been shown in model systems, little is known about the function of the activated FLT3 receptor in primary AML blasts. Although our data from primary AML blasts are descriptive, we provide evidence that in AML cell lines, active FLT3 mutants play an important role of in the regulation of STAT5 activity. We could show that the constitutive activation of STAT5 in MM6 cells can be completely down-regulated by the selective FLT3 PTK inhibitor SU5614 (Fig. 8C). Moreover, down-regulation of
STAT5 activity parallels the FLT3 dephosphorylation, is already complete after 2–4 h of inhibitor treatment, and is followed by growth inhibition and apoptosis in AML cells expressing active FLT3 mutants. STAT5 itself activates several proproliferative and antiapoptotic pathways and directly binds to functionally relevant domains in the promoter of BCL-X<sub>L</sub> and Cyclin D1, thereby protecting cells from apoptosis and promoting cell cycle progression. The constitutively active STAT5, which has transforming activity in <i>in vitro</i> models (37, 38), might, therefore, be an important mediator of the malignant phenotype in FLT3-LM-positive AML. This hypothesis is further supported by two studies showing that FLT3-ITD mutants and constitutively active STAT5 mutants induce a very similar myeloproliferative syndrome in the mouse bone marrow transplant model (16, 39).

In conclusion, our data show that the FLT3-ITD mutant, but not the FLT3-WT receptor, has transforming potential <i>in vitro</i>. In contrast to the ligand-stimulated FLT3-WT receptor, the FLT3-ITD mutant receptor induces a strong activation of STAT5, but not STAT3 in Ba/F3 cells and in primary AML blasts. The active FLT3-STAT5 pathway provides an essential antiapoptotic and proproliferative signal in AML cells and represents a promising molecular target for therapeutic approaches in FLT3-LM/TKD-positive AML.

ACKNOWLEDGMENTS

We thank Ralf Dirschinger for performing the proliferation assays. We would also like to thank Dr. C. Schoch for performing the cytogenetic and Dr. T. Hafeler for the morphological analyses of patient samples.

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2150 STAT5 Activation by FLT3 in AML Blasts


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