Extracellular KIT receptor mutants, commonly found in core binding factor AML, are constitutively active and respond to imatinib mesylate

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Supported by the Deutsche José Carreras Leukämie Stiftung (C.S., G.S.) and the Deutsche Krebshilfe (J.C., C.S.).

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Abstract

Multiple genetic alterations are required to induce acute myelogenous leukemia (AML). Mutations in the extracellular domain of the KIT receptor are almost exclusively found in AML patients carrying translocations or inversions affecting members of the core binding factor (CBF) gene family and correlate with a high risk of relapse. We demonstrate that these complex insertion and deletion mutations lead to constitutive activation of the KIT receptor, which induces factor-independent growth of IL3-dependent cells. Mutation of the evolutionary conserved amino acid D419 within the extracellular domain was sufficient to constitutively activate the KIT receptor, although high expression levels were required. Dose-dependent growth inhibition and apoptosis were observed using either the protein tyrosine kinase inhibitor imatinib mesylate or by blocking the PI3K-AKT pathway. Our data show that the addition of kinase inhibitors to conventional chemotherapy might be a new therapeutic option for CBF-AML expressing mutant KIT.

(143 words)
Introduction

Acute myelogenous leukemia (AML) is a complex disease that is classified by histochemical, cytochemical and genetic markers. Genetic alterations affecting members of the core binding factor (CBF), a heterodimeric transcription factor, are some of the most common events in AML and define a large group commonly designated CBF-AML, which includes patients with either t(8;21) or inv(16). Evidence from experimental studies support the hypothesis that these fusion proteins impair myeloid differentiation and/or expand a hematopoietic stem/progenitor cell pool, but are insufficient to cause leukemia. Secondary genetic alterations, such as mutations in receptor tyrosine kinases (RTK), are thus required for induction of an overt AML. Although the KIT receptor is expressed on the majority of AML blasts, mutations in the KIT gene are exclusively associated with CBF-AML. In addition to mutations affecting D816 within the KIT tyrosine kinase domain 2 (TK2), complex insertion/deletion mutations in exon8 affecting the extracellular (EC) domain are also observed in CBF-AML, with an especially high prevalence (26%) in inv(16) patients, and which correlates with a higher risk of relapse. In addition to EC mutations, a mutation in the transmembrane (TM) domain (V530I) has also been described in CBF-AML. Previous studies have shown that the KIT D816V mutation induces growth-factor independent growth of myeloid and mast cells and is insensitive to the RTK inhibitor imatinib mesylate. The purpose of this study was to test the functional activity of the EC and TM KIT mutations associated with CBF-AML, to determine their sensitivity to imatinib, and to delineate the activated signaling pathways.

Study design

Site-directed mutagenesis was used to create KIT mutants from either murine or human KIT cDNAs, as indicated in Table 1. KIT mutants were cloned into the SF91 retroviral vector co-expressing the yellow fluorescence protein (YFP), which were used to infect FDC-P1 cells in
the presence of interleukin-3 (IL3). Transduced cells were detected by FACS and directly sorted into 96-well plates with or without IL3 to calculate the incidence of factor-independent growth. KIT mutations in the factor-independent cells were verified by sequencing. To monitor proliferation rates, cells (10⁴/well) were pulsed for 8 hours with methyl-³H thymidine (0.5 μCi/well; S.A. 50 Ci/mmole) and incorporation levels were determined. Early and late apoptosis was evaluated using the apoptosis detection kit (R&D Systems, Minneapolis, MN, USA).

**Results and Discussion**

To analyze the oncogenic potential of KIT mutations that have been identified in CBF-AML, a series of KIT mutants were tested for their ability to stimulate proliferation and inhibit apoptosis of myeloid cells. Firstly, two KIT mutants were generated that have been cloned from patients with AML, one with a V530I substitution in the TM domain and a second with a complex deletion / insertion mutation affecting the EC domain (T417Δ,Y418Δ,D419Δ,InsV; designated "exon8"). As all EC in-frame deletion plus insertion mutations observed in AML lead to the loss of the evolutionary highly conserved amino acid D419, we also created a mutant (D419A), in which the acidic Asp is replaced with the neutral hydrophobic Ala. This mutant directly tests if loss of D419 is the critical mutation in these mutants. As a positive control for growth stimulation, we used the TK2 mutants hD816V and mD814V and the juxtamembrane (JX) mutant mV558D, identified in mastocytosis and gastro-intestinal-stroma tumors (GIST). These mutants also served as negative (mD814V/hD816V) and positive (mV558D) controls for the response to imatinib.

Expression of each of the KIT mutants in IL3-dependent FDC-P1 cells lead to factor-independent growth under stringent conditions, but with different efficiencies (Table 1). Whereas the TK2 and JX mutants gave rise to factor-independent cells at a frequency between 10⁻² and 10⁻³, the frequency was two to three orders of magnitude lower for EC and TM
Table 1. Summary of KIT mutants and frequency of conferring IL3-independent growth to FDC-P1 cells

<table>
<thead>
<tr>
<th>Vector / KIT Mutant</th>
<th>Frequency to IL3-independent growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vector (YFP alone)</td>
<td>&lt; 6.3 x 10^-8</td>
</tr>
<tr>
<td>wt hKIT</td>
<td></td>
</tr>
<tr>
<td>+SCF</td>
<td>1.8 x 10^-3</td>
</tr>
<tr>
<td>-SCF</td>
<td>&lt; 6.3 x 10^-8</td>
</tr>
<tr>
<td>Extracellular</td>
<td></td>
</tr>
<tr>
<td>mT417Δ,Y418Δ,D419Δ,InsV (exon8)</td>
<td>1.1 x 10^-4</td>
</tr>
<tr>
<td>hD419A</td>
<td>8.2 x 10^-5</td>
</tr>
<tr>
<td>Transmembrane</td>
<td></td>
</tr>
<tr>
<td>hV530I</td>
<td>&gt; 7 x 10^-6</td>
</tr>
<tr>
<td>Juxtamembrane</td>
<td></td>
</tr>
<tr>
<td>mV558D</td>
<td>1.6 x 10^-2</td>
</tr>
<tr>
<td>Tyrosine kinase domain II</td>
<td></td>
</tr>
<tr>
<td>mD814V</td>
<td>7.3 x 10^-3</td>
</tr>
<tr>
<td>hD816V</td>
<td>6.2 x 10^-3</td>
</tr>
</tbody>
</table>

The frequency to IL3 independent growth was calculated by end-point dilution of infected cells plated in the absence of IL3 and applying the Poisson distribution. The frequency was corrected for the plating efficiency in IL3 for each culture (between 42% and 96%). In two independent experiments, in which 10^7 cells were plated in the absence of IL3, no factor-independent growth was observed in FDC-P1 expressing the control vector or wt hKIT. The frequency of obtaining cell growth in hSCF (100 ng/ml) was determined in FDC-P1 cells infected with the wt hKIT vector. The exact incidence of factor-independent growth was not determined for mutant V530I, but is between 7 x 10^-6 and 5 x 10^-5.

Importantly, no spontaneous factor-independent growth was observed in control infected FDC-P1 cells expressing YFP alone or wild-type KIT, supporting the conclusion that expression of the EC and TM KIT mutants were responsible for the factor-independent growth. The low frequency of obtaining factor-independent growth with these mutants relative to the JX and TK2 mutations may reflect the necessity of high KIT expression levels. This hypothesis is supported by the fact that the transcription levels of the vectors (as
measured by YFP expression) was three- to six-fold higher in IL3-independent cultures expressing EC and TM mutants, as compared to the other mutants (Figure 1A). This was further documented by Western blot analysis in which KIT protein levels were examined, which correlated well with YFP expression levels (Figure 1B), although a direct comparison is complicated by potential differences in receptor turnover rates. Notably, all cultures proliferated at comparable rates and no significant difference in proliferation rates were observed if IL3 was added to the cultures, demonstrating optimal cell growth in the selected cultures (data not shown).

To determine if the EC and TM mutants are sensitive to RTK inhibitors, we tested the effect of imatinib on the factor-independent growth of FDC-P1 cells expressing the KIT mutants in proliferation assays. Under pharmacological doses of imatinib, FDC-P1 cultures expressing EC and TM mutants showed a drastic reduction in proliferation (Figure 1C), which could be rescued by addition of IL3 (data not shown). The IC$_{50}$ dose required for these mutants was lower (0.1µM) than that for wt KIT stimulated with the KIT ligand (0.5µM), but was similar to that required for V558D mutants, commonly observed in imatinib-responsive GIST patients. The inhibition of V530I is consistent with recent results with another TM mutant (F522C) identified in mastocytosis. Inhibition of EC and TM mutant KIT activity not only abrogated proliferation, but induced high frequencies of apoptosis, as detected by Annexin V staining (Figure 1D).

To confirm if growth inhibition and apoptosis was directly mediated by inhibiting KIT mutant activity, phosphorylation status of the different KIT mutants was evaluated in the absence and presence of different imatinib concentrations (Figure 1E). Consistent with the hypothesis that the KIT mutations lead to activation of the receptor, KIT phosphorylation was observed in all cultures expressing KIT mutants. At pharmacological doses of 1µM imatinib, inhibition of both TM and JX, but not TK2 mutant phosphorylation was observed, consistent
Figure 1. Analysis and comparison of FDC-P1 cells expressing different KIT receptor mutants. (A) High vector expression levels, as determined by FACS analysis of YFP expression, were observed in FDC-P1 cells expressing EC or TM mutant, as compared to other KIT mutants. Shown is the mean fluorescence of two independent experiments. (B) IL3-independent FDC-P1 cells expressing EC and TM KIT receptor mutants express high KIT protein levels, as compared to cells expressing other KIT mutants. Protein from total cell lysates was analyzed by Western blot analysis with anti-KIT antibody. Tubulin levels served as a loading control. (C) Proliferation response of FDC-P1 cells expressing wt or mutant KIT to imatinib. 3H-thymidine incorporation in controls (DMSO only) was set at 100% for each culture. Shown is one representative experiment of three, each performed in triplicate. (D) Apoptosis detection after imatinib treatment of parental
FDC-P1 cells with or without IL3 (FD +IL3 or FD –IL3) or factor-independent FDC-P1 cells expressing mutant KIT. Surface expression of annexin V was measured 12 h after drug treatment. IL3-withdrawal from control FDC-P1 cells showed significant slower kinetics of apoptosis. Shown is a representative experiments of three independent experiments. (E) Phosphorylation status of the KIT receptor and downstream signaling targets was determined by Western Blot analysis using a phospho-specific antibody after imatinib treatment (3 hrs). Due to the different levels of KIT protein in the various cell cultures, exposure times for the Kit blot were varied accordingly. For controls, protein extracts from FDC-P1 cells maintained in IL3 or FDC-P1 transduced with wt hKIT and maintained in SCF were analyzed. (F) Proliferation assays were performed in the presence of the indicated inhibitor for 48 hrs to determine importance of the different signaling pathways for proliferation.

with their response to imatinib in proliferation and apoptosis assays. Somewhat surprisingly, only slightly lower phosphorylation levels of the EC mutant were observed after imatinib treatment, despite its striking sensitivity to the drug in biological assays. Examination of KIT downstream signaling pathways clearly demonstrated that the imatinib-mediated inhibition of cell growth and induction of apoptosis correlated with phosphorylation of AKT, MAPK, and STAT5, but not STAT3 (Figure 1E). The critical importance of the phosphatidylinositol-3-kinase (PI3K)-AKT pathway was demonstrated with chemical inhibitors for the different pathways (Figure 1F). Strikingly, the PI3K-inhibitor LY294002 reduced proliferation rates of FDC-P1 cells expressing either the EC or JX KIT mutants to levels similar to that observed by directly inhibiting the KIT receptor using imatinib, in contrast to the MAPK-inhibitor PD98059, which only partially inhibited proliferation. Indeed, the PI3K inhibitor was alone sufficient to inhibit proliferation of the TK2 KIT mutants, resistant to imatinib. Rapamycin, which inhibits the AKT downstream effector mTOR, only partially inhibited proliferation, underlining the importance of other known downstream effectors of PI3K-AKT.

In summary, we show that both the EC (exon8) and TM mutants, which are found in CBF-AMLs at a high frequency, lead to constitutive activation of KIT. Furthermore, our data demonstrate that disruption of Asp419 is the critical event in EC mutations. Both the low transforming frequency in our assay, as well as their exclusive association with CBF-AMLs, suggest that high KIT expression levels or other secondary cooperating events may be a prerequisite for the transforming properties of EC or TM mutations. Interestingly, a recent study has shown that the fusion protein generated by t(8;21) induces high KIT expression levels in U937 cells. The striking responsiveness of both EC and TM KIT mutants to
imatinib demonstrate that RTK inhibitors in combination with conventional chemotherapy may be a novel therapeutic approach for the treatment of CBF-AML carrying EC or TM KIT mutations. Although recent studies have evaluated the response of AML to kinase inhibitors with only very limited response, the KIT status (wt or mutant) was only evaluated in one of these trials, where no EC or TM mutations were identified. Thus our data, together with studies investigating the use of kinase inhibitors in non-small cell lung cancer, argue for the importance of careful patient selection in clinical studies to evaluate the effectiveness of kinase inhibitors.

Acknowledgements

We would like to thank Marion Ziegler and Susanne Roscher for excellent technical assistance, Arne Düsedau for FACS support, and other members of the Stocking lab for helpful discussion. We also gratefully acknowledge P. De Sepulveda and P. Dubreuil, Marseille, France, for providing mutants D816V and V558D and Novartis (Basel, Switzerland) for providing imatinib mesylate.

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