The EOL-1 cell line as an *in vitro* model for the study of *FIP1L1-PDGFRA* positive chronic eosinophilic leukemia

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Abstract

We recently identified the chimeric kinase FIP1L1-PDGFRα as a cause of the hypereosinophilic syndrome and chronic eosinophilic leukemia. To investigate the role of FIP1L1-PDGFRα in the pathogenesis of acute leukemia, we screened 87 leukemia cell lines for the presence of FIP1L1-PDGFRα. One cell line, EOL-1, expressed the FIP1L1-PDGFRα fusion. The growth of EOL-1 cells was inhibited by 3 structurally divergent kinase inhibitors: imatinib (STI-571), PKC412, and SU5614. These results indicate that fusion of FIP1L1 to PDGFRα occurs rarely in leukemia cell lines, but identify EOL-1 as an in vitro model for the study of FIP1L1-PDGFRα-positive chronic eosinophilic leukemia and for the analysis of small molecule inhibitors of FIP1L1-PDGFRα.

Introduction

The hypereosinophilic syndrome (HES) is a hematological disease characterized by prolonged eosinophilia, exclusion of reactive eosinophilia and organ damage\(^1,2\). HES is reclassified as chronic eosinophilic leukemia (CEL) when clonality is demonstrated\(^3\). We recently identified the kinase FIP1L1-PDGFRα as the cause and the therapeutic target of imatinib in 56 % of HES cases\(^4\). These results demonstrate that most of the HES cases are clonal in origin, and could be reclassified as FIP1L1-PDGFRα-positive CEL. The FIP1L1-PDGFRα fusion gene is created by an interstitial chromosomal deletion on chromosome 4q12 that is not apparent using standard karyotypic analysis\(^4\). Expression of the FIP1L1-PDGFRα fusion is under control of the ubiquitous FIP1L1 promoter, suggesting the possibility that FIP1L1-PDGFRα may be involved in the pathogenesis of other hematological malignancies. To get insight into this, we screened 87 leukemia cell lines for the presence of the FIP1L1-PDGFRα fusion gene. Leukemia cell lines have been proven to be a valuable resource for the study of hematological malignancies\(^5\), and our results now identify the EOL-1 cell line as an in vitro model for the study of FIP1L1-PDGFRα-positive CEL.
Methods

Polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR)

The FIP1L1-PDGFRα fusion was amplified from DNA with primers FIP1L1-F9 (5'-tgtggcaattgatgttatcg) and PDGFRA-R112 (5'-gtgcaaggaaaaaggagtct). RNA was isolated from cell lines from the DSMZ collection (http://www.dsmz.de) as described⁶. RT-PCR was performed with primers FIP1L1-F7 (5'-acctggtgctgatctttctgat) and PDGFRA-R14 (5'-tgagagcttgtttttcactgga) for detection of FIP1L1-PDGFRα, and primers PDGFRA-F11 (5'-ggtgctgttggtgattgtga) and FIP1L1-R10 (5'-cagctctggagggaaaaac) for detection of PDGFRA-FIP1L1. Primers PDGFRA-F11 and PDGFRA-R14 were used to detect PDGFRA expression, and primers FIP1L1-F7 and FIP1L1-R10 were used to detect FIP1L1 expression.

Cell culture and dose response curves

The EOL-1 cell line (DSMZ ACC386) was grown in RPMI-1640 medium with 10 % fetal bovine serum. Imatinib and PKC412 were kindly provided by Novartis; SU5614 was purchased from Calbiochem. Kinase inhibitors were stored in water (imatinib) or DMSO (PKC412, SU5614) and diluted in RPMI-1640 medium. For dose response curves, EOL-1 cultures were initiated at 3 x 10⁵ cells/ml, and viable cell number was determined at the beginning and after 48 hours using the Celltiter AQeousOne solution (Promega). Dose response curves were fitted using Origin (OriginLab).

Detection of apoptosis

Apoptotic cells were detected by flow cytometric analysis using a FACSCalibur Cytometer (Becton Dickinson) after staining with annexin-V-fluorescein and propidium iodide (Roche).

Western blotting and immunoprecipitation

EOL-1 cells were treated with kinase inhibitors for 3 hours and then lysed in lysis buffer (Cell signaling) for immunoprecipitation or in SDS sample buffer (Cell Signaling) for whole cell lysates. Immunoprecipitation was performed using anti-PDGFRα(C-20) antibody (Santa Cruz) and Protein A agarose (Roche). Antibodies used were: anti-phospho-STAT5 (Cell Signaling); anti-PDGFRα(951), anti-STAT5a (Santa-Cruz); anti-phospho-tyrosine(4G10) (Upstate); anti-mouse-PO, anti-rabbit-PO (APBiotech).
Results and Discussion

We performed RT-PCR on RNA obtained from 67 acute myeloid leukemia (AML) and 20 acute lymphoid leukemia (ALL) cell lines, with a primer combination that would detect all known FIP1L1-PDGFRA fusion variants. In one AML cell line, EOL-1 (and derivative EOL-3), a fusion between FIP1L1 and PDGFRA was detected (Fig. 1). No other cell lines harbored this fusion gene (data not shown).

In consonance with our previous observations in patients with FIP1L1-PDGFRA-positive CEL, the EOL-1 cell line expressed in-frame FIP1L1-PDGFRA fusion transcripts, with fusion of exon 9 of FIP1L1 to a truncated exon 12 of PDGFRA. Due to splice variation within the FIP1L1 part, different fusion transcripts are observed in the EOL-1 cells (exon 8a can be present or absent between exon 8 and exon 9, but in both variants an open reading frame is present) (Fig. 1). Cloning of the fusion gene at the DNA level confirmed that the breakpoint in FIP1L1 was located in intron 9, and the breakpoint in PDGFRA was located in exon 12. No reciprocal PDGFRA-FIP1L1 fusion gene could be detected (Fig. 1). Taken together, these data indicate that the FIP1L1-PDGFRA fusion in the EOL-1 cell line is the consequence of the del(4)(q12q12) interstitial chromosomal deletion, as observed in FIP1L1-PDGFRA-positive CEL patients. The EOL-1 cell line did not express wild type PDGFRA, but did express wild type FIP1L1 (Fig. 1).

EOL-1 was originally derived from the blasts of a 33-year-old male with CEL, at a stage where he had progressed to AML (54% blasts, karyotype: 48, XY, +6, +8, 9q-)8. We recently discovered that this cell line harbors a partial tandem duplication within the MLL gene9. Translocations involving MLL and partial tandem duplication within the MLL gene were described as leukemogenic mutations involved in the pathogenesis of AML10,11. Mouse models have demonstrated that MLL fusion proteins are necessary, but not sufficient for leukemogenesis10. Based on these and other observations, it has been proposed that AML cells harbor at least 2 mutations, one that confers a proliferative and/or survival advantage and one that results in impaired differentiation of hematopoietic progenitors12. Taken together, these findings suggest that FIP1L1-PDGFRα and mutated MLL may cooperate to cause progression of CEL to AML.
We next tested for dose-dependent inhibition of the growth of EOL-1 cells by the kinase inhibitors imatinib\textsuperscript{13} and PKC412\textsuperscript{14}, both known to inhibit FIP1L1-PDGFR\textsubscript{\alpha}\textsuperscript{4,15}, and SU5614\textsuperscript{16}, known to inhibit FLT3\textsuperscript{17,18}. The growth of EOL-1 cells was inhibited by these drugs with a cellular IC\textsubscript{50} of approximately 0.8 nM for imatinib, 20 nM for PKC412, and 50 nM for SU5614 (Fig. 2-A). Twenty-four hours after drug treatment, most EOL-1 cells were apoptotic (Fig. 2-B), indicating that growth inhibition by these 3 drugs was due to apoptosis. FIP1L1-PDGFR\textsubscript{\alpha} has recently been identified as the major phosphorylated protein in EOL-1 cells\textsuperscript{19}. Our results suggest that all 3 inhibitors directly affect FIP1L1-PDGFR\textsubscript{\alpha} activity, as indicted by dose-dependent decrease of phosphorylation of both FIP1L1-PDGFR\textsubscript{\alpha} and STAT5, a downstream effector of FIP1L1-PDGFR\textsubscript{\alpha} mediated signal transduction. A 50% reduction of phosphorylation of FIP1L1-PDGFR\textsubscript{\alpha} was reached at approximately 5 nM of imatinib, 100 nM of PKC412 and 50 nM of SU5614 (Fig.2-C). As these 3 inhibitors also inhibit KIT, and as PKC412 and SU5614 also inhibit FLT3, we investigated if inhibition of FLT3 or KIT could be involved in the growth inhibition of the EOL-1 cells. However, we did not find evidence for phosphorylation of FLT3 or KIT. Although we cannot exclude that inhibition of other native tyrosine kinases may contribute inhibition of cell growth, our results suggest that growth inhibition and induction of apoptosis is primarily the result of direct FIP1L1-PDGFR\textsubscript{\alpha} inhibition.

In conclusion, although progression of CEL to AML has been observed\textsuperscript{4,8,20}, our results suggest that FIP1L1-PDGFR\textsubscript{\alpha} is not frequently involved in the pathogenesis of AML. We identified EOL-1 as the first cell line expressing the \textit{FIP1L1-PDGFRA} fusion gene, and as a valuable \textit{in vitro} model for the screening for new FIP1L1-PDGFR\textsubscript{\alpha} inhibitors in the context of a human cell line expressing the fusion protein from its endogenous promoter. EOL-1 cells may provide a unique reagent for understanding lineage involvement in \textit{FIP1L1-PDGFRA}-positive CEL, FIP1L1-PDGFR\textsubscript{\alpha} mediated signaling, and the transcriptional targets of FIP1L1-PDGFR\textsubscript{\alpha} that contribute to the CEL phenotype.
References


Figure Legends

Figure 1. Fusion of \textit{FIP1L1} to \textit{PDGFRA} in the EOL-1 cell line.
Detection of the \textit{FIP1L1-PDGFRA} fusion transcript in the EOL-1 and EOL-3 cell lines (A), detection of \textit{FIP1L1} expression in the EOL-1 cell line (B), and amplification of the \textit{FIP1L1-PDGFRA} fusion gene on DNA from the EOL-1 cell line (C). Different transcripts are observed for \textit{FIP1L1} and \textit{FIP1L1-PDGFRA} (A and B), due to alternative splicing. Expression of \textit{PDGFRA-FIP1L1} and native \textit{PDGFRA} were not detected (B). The sequence of the fusion gene surrounding the deletion is shown at the DNA level, at the RNA level (after splicing), and at the protein level (D). Splice donor and acceptor sites are underlined. A cryptic splice site is used in exon 12 of \textit{PDGFRA}. A schematic representation of FIP1L1, PDGFR\(\alpha\) and FIP1L1-PDGFR\(\alpha\) proteins is given in panel E. The points where the proteins are interrupted by the deletion are indicated by an arrowhead.

Figure 2. Inhibition of cell growth, induction of apoptosis and inhibition of FIP1L1-PDGFR\(\alpha\) kinase activity in EOL-1 cells treated with various kinase inhibitors.
(A) Dose response curves of EOL-1 cells treated with imatinib, PKC412 or SU5614. The percentage of growth relative to untreated cells is plotted for increasing drug concentrations (48 h incubation).
(B) Detection of apoptotic EOL-1 cells after 24 h incubation with imatinib (10 nM), PKC412 (100 nM) or SU5614 (100 nM). Apoptotic cells (lower right quadrant), necrotic cells (upper right quadrant) and viable cells (lower left quadrant) were detected.
(C) Phosphorylation of FIP1L1-PDGFR\(\alpha\) was analyzed using anti-phospho-tyrosine antibody on immunoprecipitated FIP1L1-PDGFR\(\alpha\). Phosphorylation of STAT5 was analyzed using anti-phospho-STAT5 antibody, which recognizes both phosphorylated STAT5a (upper band) and STAT5b (lower band). The membranes were blotted with anti-PDGFR\(\alpha\) or anti-STAT5a antibodies as loading control.
**Fig. 2**

**A**

- **Imatinib (nM)**
  - % growth relative to control
  - IC<sub>50</sub> = 0.6 nM
  - IC<sub>50</sub> = 20 nM
  - IC<sub>50</sub> = 50 nM

**B**

- **Annexin-V-Fluorescein**
  - No inhibitor
  - Imatinib (10 nM)
  - PKC412 (100 nM)
  - SU5614 (100 nM)

**C**

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- **IP**: anti-PDGFRα
- **Whole cell lysate**: anti-phospho-STAT5
- **Whole cell lysate**: anti-STAT5α
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