Erlotinib Effectively Inhibits JAK2V617F Activity and Polycythemia Vera Cell Growth

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JAK2V617F, a mutant of tyrosine kinase JAK2, is found in most patients with polycythemia vera (PV) and a substantial proportion of patients with idiopathic myelofibrosis or essential thrombocytemia. The JAK2 mutant displays a much increased kinase activity and generates a PV-like phenotype in mouse bone marrow transplant models. This study shows that the anti-cancer drug erlotinib (Tarceva™) is a potent inhibitor of JAK2V617F activity. In vitro colony formation assays revealed that erlotinib at micro-molar concentrations effectively suppresses the growth and expansion of PV hematopoietic progenitor cells while having little effect on normal cells. Furthermore, JAK2V617F-positive cells from PV patients show greater susceptibility to the inhibitor than their negative counterparts. Similar inhibitory effects were found with the JAK2V617F activity.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal anti-JAK2 and 4G10 monoclonal anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology and Upstate Biotechnology, Inc., respectively. Erlotinib, imatinib mesylate, and gefitinib were purchased from a local pharmacy. Tyrphostin AG490 was purchased from LC Laboratories, and 1,2,3,4,5,6-hexabromocyclohexane (C6H12Br6) was requested from the NCI Developmental Therapeutics Program.

Collection of Peripheral Blood and Purification of Human CD34+ Cells—Phlebotomized units of blood were obtained from patients who met the World Health Organization diagnostic criteria for PV and were treated with phlebotomy only. Normal peripheral blood samples were obtained from healthy donors after blood mobilization with granulocyte colony-stimulating factor. Institutional Review Board approvals have been obtained for the procedures, and each donor was consented. A CD34+ cell population was isolated from low density mononuclear cells of the blood by using the magnetic activated cell sorting CD34- isolation kit (Miltenyi Biotec, Auburn, CA).

Colonization Assays—CD34+ cells (1000 cells) were cultured in 1 ml of semisolid medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) containing α-minimal essential medium, 0.9% methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 0.05 mM 2-mercaptoethanol, and 0–50 μM erlotinib supplemented with 2 units/ml EPO alone or a mixture of six growth factors/cytokines (2 units/ml EPO, 100 ng/ml stem cell factor, 10 ng/ml interleukin 3, 100 ng/ml interleukin 6, 10 ng/ml granulocyte colony-stimulating factor, and 100 ng/ml thrombopoietin). All cultures were per-
formed in triplicate and various colony types enumerated using an inverted microscope at day 12–14 of culture according to the standard criteria.

**DNA Extraction and PCR Amplification**—Individual hematopoietic cell colonies were taken out from the semisolid phase culture media and diluted into 1 ml of α-minimal essential medium supplemented with 10% fetal bovine serum. After spin down, genomic DNAs were isolated from the pelleted cells by using the Extract-N-Amp™ blood PCR kit from Sigma. The JAK2<sup>V617F</sup> mutation was detected by using nested allele-specific PCR as described previously (18).

**Generation of a Protein Substrate for JAK2 Kinase Activity Assays**—A peptide fragment with a sequence of PQDKEYKYKE derived from the autophosphorylation sites of human JAK2 was expressed as a GST fusion protein by using the pGex-2T vector. The fusion protein designated GST-JAKS was expressed in *Escherichia coli* and then purified by using a glutathione-Sepharose column.

**JAK2 Kinase Activity Assays**—COS7 cells were transfected with pCDNA3 constructs carrying JAK2 or JAK2<sup>V617F</sup> as described previously (10). Cells were lysed in a buffer containing 25 mM β-glycerophosphate (pH 7.3), 5 mM EDTA, 2 mM EGTA, 5 mM β-mercaptoethanol, 1% Triton X-100, 0.1 mM NaCl, and a protease inhibitor mixture (Roche Applied Science). Cell extracts and anti-JAK2 immunoprecipitates were used for kinases assays in a buffer system containing 25 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 0.2 mM ATP, 2 mM dithiothreitol, and 0.2 mg/ml GST-JAKS. The reactions were allowed to proceed at room temperature for 20 min and then stopped by addition of the SDS gel sample buffer. Tyrosine phosphorylation of GST-JAKS was determined by Western blotting analysis with an anti-phosphotyrosine antibody. Capture of Western blot images and quantification of band signals were carried out by using FluorChem SP imaging system from Alpha Innotech.

**RESULTS**

**Development of an Assay for Kinase Activity of JAK2<sup>V617F</sup>**—Wild type JAK2 and the mutant JAK2<sup>V617F</sup> were expressed in COS7 cells. The overexpressed recombinant proteins were then immunopurified by using a specific anti-JAK2 antibody immobilized onto protein-A beads. Activity assays were carried out with substrate GST-JAKS, a GST fusion protein containing a C-terminal segment of JAK2 where the autophosphorylation sites reside. While JAK2<sup>V617F</sup> underwent strong autophosphorylation and caused marked phosphorylation of the added substrate, wild type JAK2 showed essentially no activity (Fig. 1, left panel). That GST-JAKS serves as a highly specific substrate for JAK2<sup>V617F</sup> was further demonstrated by the fact that when the tyrosine kinase activity assays were performed directly with crude cell extracts, only JAK2<sup>V617F</sup> transfected cells gave rise to a phosphorylation of the substrate, while cells transfected with the control vector or wild type JAK2 showed no activity (Fig. 1, right panel). Note that tyrosine phosphorylation by JAK2<sup>V617F</sup> caused a slight mobility shift of GST-JAKS. As expected, the phosphorylation reaction occurred in the JAK2 portion of the fusion protein because GST alone was not phosphorylated (data not shown). The data further demonstrate that JAK2<sup>V617F</sup> is a hyperactive tyrosine kinase and provide a good assay system to screen for inhibitors of the mutated enzyme.

**Identification of Erlotinib as a Potent Inhibitor of JAK2<sup>V617F</sup>**—Cell extracts obtained from JAK2<sup>V617F</sup>-transfected cells were employed to analyze the inhibitory effects of potential inhibitors. Included in our screening were the aforementioned anti-cancer drugs imatinib mesylate, gefitinib, and erlotinib and two other known inhibitors of JAK2, namely, AG490 and 1,2,3,4,5,6-hexabromocyclohexane. AG490, a putative JAK2 and EGFR inhibitor, had been shown to inhibit the growth of PV cells *in vitro* (8), and 1,2,3,4,5,6-hexabromocyclohexane was recently reported to be a specific JAK2 inhibitor as well (19). The data illustrated in Fig. 2 demonstrate that, among these chemicals, erlotinib was by far the most potent inhibitor. It displayed an IC<sub>50</sub> value of 4 μM, where IC<sub>50</sub> represents the concentration of compounds required to achieve a 50% reduction in the phosphorylation of the exogenous substrate. Imatinib mesylate, gefitinib, and AG490 also showed some inhibitory effects but only at much higher concentrations with IC<sub>50</sub> values in submillimolar to millimolar ranges. In contrast, 1,2,3,4,5,6-hexabromocyclohexane...
exhibited virtually no inhibitory effect on JAK2V617F. We also analyzed the inhibitory effects of erlotinib on wild type JAK2. However, as shown in Fig. 1, JAK2 displayed essentially no kinase activity in comparison with the JAK2V617F mutant when cell extracts or immunoprecipitates were employed for assays. For this reason, we enriched the enzyme from extracts of JAK2-transfected COS7 cells by using a Mono Q anion-exchange column. The JAK2 protein was eluted at around 0.3 M NaCl, and we were able to detect the activity of this partially purified JAK2 by using GST-JAKS as a substrate. As shown in supplemental Fig. S1, JAK2 was also inhibited by erlotinib but with an IC_{50} value beyond 20 \mu M. This suggests that the enzyme is less sensitive to the inhibitor. However, the data should not be overinterpreted since the data may only reflect the fact that wild type JAK2 stays in an inactive state.

**Inhibition of PV Hematopoietic Progenitor Cell Growth by Erlotinib**—The effectiveness of erlotinib in inhibiting the growth of JAK2V617F-positive PV hematopoietic progenitor cells was further analyzed with a cell-based system. For this purpose, CD34^{+} hematopoietic cells from normal and PV blood samples were cultured in a semisolid colony assay medium supplemented with erythropoietin to support the growth of erythroid cells or with a mixture of six growth factors/cytokines to stimulate growth of granulocytes/macrophages and megakaryocytes as well. Fig. 3 shows representative results obtained with one normal blood and two JAK2V617F-positive PV blood samples. Erlotinib effectively inhibited the growth of PV hematopoietic progenitor cells with an IC_{50} of \approx 5 \mu M, while it hardly affected the growth of normal cells at double that concentration. It should be pointed out that the reported IC_{50} values of erlotinib for inhibition of the EGF receptor and various mutants were at submicromolar to micromolar according to cell-based assays (20). In addition, imatinib mesylate, a highly effective drug for treatment of BCR-ABL-positive chronic myelogenous leukemia, inhibited the in vitro growth of BCR-ABL-positive cells with an IC_{50} value at the submicromolar to micromolar range (21, 22).

**Enhanced Sensitivity of JAK2V617F-positive Cells to Erlotinib**—To verify the selectivity of erlotinib toward JAK2V617F-positive cells, genomic DNAs were extracted from hematopoietic cell col-

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**FIGURE 2.** Erlotinib is a potent inhibitor of JAK2V617F. Tyrosine kinase activity of JAK2V617F was performed with GST-JAKS as a substrate in the presence of various concentrations of tyrosine kinase inhibitors. Tyrosine phosphorylation was detected by using anti-phosphotyrosine antibody and the protein level of GST-JAKS by anti-GST. The line graphs in the bottom panel are quantitative representations of GST-JAKS phosphorylation based on gel scanning of three independent experiments. Data represent relative band intensity with error bars denoting standard deviation.

**FIGURE 3.** Erlotinib inhibits in vitro growth of PV hematopoietic progenitor cells. CD34^{+} cells (1000) from normal control and PV blood samples were plated with indicated concentrations of erlotinib in the presence of EPO alone or a mixture of 6 growth factors/cytokines. Erythroid burst-forming unit (BFU-E, dashed bars), granulocyte-macrophage colony-forming unit (CFU-GM, open bars), and CFU-Mix (mixed colonies containing erythroid, granulocyte/macrophage cells, and megakaryocyte, solid bars) colonies were scored 12–14 days later. Representative data of independent experiments with four different normal and seven different PV samples are shown.
Erlotinib Inhibits JAK2V617F activity and JAK2V617F-positive PV cell growth, clinical trials of erlotinib for the treatment of PV and other related diseases appear to be well warranted. So far, there is no effective cure for the diseases.

Our study also included another EGFR inhibitor, namely, gefitinib, but this drug was found to be poorly effective. Likewise, AG490 and imatinib mesylate were found to be only moderate inhibitors of JAK2V617F despite the fact that previous studies had shown that both AG490 and imatinib mesylate inhibited autonornous erythropoiesis of PV cells in vitro (8, 24), an effect possibly attributable to their inhibition of the JAK2V617F activity. Imatinib mesylate at a concentration of 1 μM suppressed autonomous erythroid burst-forming unit growth in the absence of growth factors with a mean inhibition of 73% (24). Without a defined molecular target, this drug has been used in investigational treatment of PV and was shown to reduce phlebotomy.
requirements in polycythemia vera patients (25). Although imatinib mesylate-treated patients remained positive for JAK2V617F, there was a significant reduction in the percentage of mutant alleles that correlated with hematologic responses (26). We believe that erlotinib should be a more promising drug than AG490 and imatinib mesylate for the treatment of PV patients because: (a) it is a much more potent and selective inhibitor of JAK2V617F and (b) it inhibits growth of PV cells even in the presence of optimal concentrations of growth factors and cytokines. By analyzing the structures of the JAK2 kinase domain and existing tyrosine kinase inhibitors, we should be able to modify erlotinib to produce more potent and selective JAK2 inhibitors, which should serve as more effective drugs to treat diseases associated with the JAK2V617F mutation.

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