Juxtamembrane Mutant V560GKit Is More Sensitive to Imatinib (STI571) Compared with Wild-Type c-Kit Whereas the Kinase Domain Mutant D816VKit Is Resistant[1]

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Abstract

Imatinib (Glivec; STI571) is an ATP-competitive kinase inhibitor of c-Abl, BCR/ABL, c-Kit, and platelet-derived growth factor receptor. Overexpression or constitutive activation of Kit by mutations have been associated with various malignancies. Mutations in the intracellular juxtamembrane region of Kit (*e.g.,* V560G) are common in gastrointestinal stromal tumors and have been linked to poor prognosis. Mutations in the kinase domain of Kit (*e.g.,* D816V) have been detected in mastocytosis, acute myeloid leukemia, and germ-cell tumors. To determine the sensitivity of Kit mutants to Imatinib in the same cellular background, wild-type Kit (WTKit), V560GKit and D816VKit were expressed in FDC-P1 cells. Growth of FDC(WTKit) was inhibited by Imatinib with GI_{50} (a concentration of drug at which 50% inhibition of growth occurs) of 0.1–0.2 μM but FDC(V560GKit) were more sensitive to Imatinib with a GI_{50} of 0.01–0.025 μM and FDC(D816VKit) were resistant to Imatinib with a GI_{50} greater than 5 μM. The naturally occurring isoforms of c-Kit did not differ in their sensitivity to Imatinib. Immunoprecipitation and Western blot analysis indicated that 1 μM Imatinib reduced phosphorylation of WTKit and completely blocked phosphorylation of V560GKit but did not affect D816VKit phosphorylation. In signaling studies, addition of stem cell factor (SCF) induced phosphorylation of ERK and Akt by WTKit, and ERK, Akt and STAT3 by V560GKit, which were all blocked by Imatinib. Imatinib also blocked the constitutive activation of Akt and STAT3 by V560GKit but had no affect on the constitutive activation of ERK, Akt, and STAT3 by D816VKit. Overall, these findings demonstrate the increased susceptibility of the Kit juxtamembrane mutant, V560G, and the resistance of the kinase domain mutant, D816V, to Imatinib compared with WTKit.

Introduction

c-Kit is a transmembrane receptor belonging to the type 3 subgroup of receptor tyrosine kinases that also includes PDGF-R,[3] colony-stimulating factor 1 receptor, and Flt-3 (1, 2). Members of this subclass are characterized by five immunoglobulin-like domains in the extracellular region and a split tyrosine kinase domain with separate ATP-binding and phosphotransferase regions. Binding of the dimeric Kit ligand, SCF, results in receptor dimerization and transphosphorylation of cytoplasmic tyrosine residues leading to intracellular signaling (3, 4). Activation of diverse signal transduction pathways can regulate proliferation, differentiation, survival, adhesion and chemotaxis. c-Kit is expressed in hematopoietic precursors, mast cells, germ cells, melanocytes, and interstitial cells of Cajal, which are the pacemaker cells of the gut (5–8). c-Kit is also expressed in some malignancies including acute myeloid leukemia (9, 10), mast cell tumors (11), GISTs (12), SCLC (13, 14), germ cell tumors (15), breast cancer (16–18), and neuroblastoma (19). Differential splicing of c-Kit results in four naturally occurring isoforms attributable to the inclusion or exclusion of the motif GNNK in the extracellular juxtamembrane domain and a serine in the interkinase domain (20–22).

Gain-of-function mutations in c-Kit were first described in the human mast cell line, HMC-1 (11); these cells have one WT *Kit* allele and one allele with two separate point mutations that result in amino acid substitutions, V560G within the intracellular juxtamembrane domain and D816V in the kinase region (23). Expression of either mutation, or the corresponding murine Kit mutants, V559G and D814V, in factor-dependent murine hematopoietic lines or murine bone marrow cells, resulted in constitutive receptor phosphorylation and kinase activity, growth-factor independence, and tumorigenicity in mice (24–29). Kinase domain mutations equivalent to D816V were also reported in murine mast cell line P-815 (D814Y) and rat cell line RBL-2H3 (D817V; Ref. 30, 31). The D816V mutation has been detected in the cells of patients with mastocytosis with associated hematological disorder,

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[3] The abbreviations used are: SCF, stem cell factor; GI_{50}, concentration of drug at which 50% inhibition of growth occurs; GIST, gastrointestinal stromal tumor; mGM-CSF, murine granulocyte macrophage colony-stimulating factor; SCLC, small cell lung carcinoma; WT, wild-type; MAb, monoclonal antibody; hu-SCF, human SCF; TBS, Tris-buffered saline; TBST, TBS/0.1% Tween 20; PI3-K, phosphatidylinositol 3′-kinase.

indolent systemic mastocytosis, atypical pediatric mastocytosis, and urticaria pigmentosa, which is mastocytosis confined to the skin (32–41). D816V, D816Y, and D816N mutations have been shown to occur in acute myelogenous leukemia (42–44). Both D816N and juxtamembrane mutations have also been reported in sinonasal lymphomas (45) and D816H has been detected in germ cell tumors (15).

Mutations of the Kit juxtamembrane region, first reported in HMC-1 (23), were subsequently found in the mouse mastocytoma line, FMA3 (46, 47), and in human GISTs, which are believed to arise from the interstitial cells of Cajal (12, 48). Juxtamembrane mutations were found to be present in >50% of GISTs and include base substitutions and/or deletions in amino acids 550–560 (12, 49). The presence of mutations correlated with a number of poor prognostic factors including malignancy and recurrence (49, 50). Germ-line juxtamembrane mutations in familial GISTs associated with cutaneous hyperpigmentation have been reported (51, 52).

Constitutive receptor phosphorylation of D816VKit and V560GKit could potentially alter the activation of signal transduction pathways. Cross-linking experiments suggested that the mechanisms of receptor activation might be distinct in the two mutant forms, because V560GKit caused the constitutive dimerization of the receptor, whereas D816VKit resulted in receptor activation in the absence of dimerization (24). However, subsequent experiments have suggested that D816V is constitutively dimerized but may occur by a different mechanism (53, 54). Constitutive activation of G560V may occur because of a conformational change of the juxtamembrane region, which is predicted to form an inhibitory α-helix in the WT receptor (55). A number of differences in signal transduction have been characterized in D816VKit, including enhanced degradation of Shp1, a phosphatase involved in the negative regulation of c-Kit activity (56, 57). In addition, PI3-K is constitutively recruited and activated in cells expressing D816VKit, which was shown to play a key role in growth factor independence and tumorigenesis (58). Another difference between WT and D816VKit is the constitutive activation of STAT3 by D816VKit, which is not observed in WTKit-expressing cells treated with SCF (59).

c-Kit kinase activity is a potential therapeutic target in conditions involving autocrine or paracrine SCF action or the constitutive activation of c-Kit through mutations. The kinase activity of Kit can be inhibited by Imatinib, a specific ATP-competitive kinase inhibitor that also inhibits c-Abl, c-Arg, BCR/ABL, Tel/Abl, v-Abl, and PDGF-R (60–62). Favorable results have been published for Phase II clinical trials for the treatment of Philadelphia-positive chronic myelogenous leukemia and acute lymphoblastic leukemia (63, 64). Early reports have indicated a good response rate in patients with GIST (65, 66). The molecular target for Imatinib treatment of GIST is Kit because >85% of GISTs express Kit (12, 48, 49, 67). Imatinib has been demonstrated to inhibit the growth of Kit-positive SCLC lines (68, 69). The megakaryocytic line Mo7e, which expresses WTKit, and the mast cell leukemia line HMC-1, which expresses constitutively active mutant Kit, were both inhibited by Imatinib in *in vitro* assays (70). However, there are two sublines of HMC-1. The original cell line, studied by Heinrich *et al.* (70), has only the juxtamembrane V560G mutation, whereas the cell line described by Furitsu *et al.* (23) has an additional D816V kinase domain mutation (32, 43). Recently, it was reported that the latter was resistant to Imatinib (71). Other mutants found in mastocytosis, *i.e.,* D816F and D816Y, were also shown to be resistant to Imatinib (71).

To compare the inhibitory effects of Imatinib on V560GKit and D816VKit to WTKit, expressed individually in the same cellular background, we used retroviral transduction of these forms of Kit into FDC-P1, factor-dependent murine early myeloid cells. Our data from growth, apoptosis, receptor phosphorylation and signaling assays indicated that the kinase domain mutant D816V was resistant to Imatinib compared with WTKit, in agreement with Ma *et al.* (71). Our data also demonstrate that the juxtamembrane mutant V560GKit was more sensitive than WTKit to inhibition by Imatinib, whereas naturally occurring isoforms have similar sensitivity. In the course of these studies, we observed signaling differences between V560GKit and WTKit activated by SCF. Differences in signaling between SCF-activated WTKit and constitutively active D816VKit and between Kit isoforms have previously been described (56, 58, 59, 72).

Overall, the finding that V560GKit was more sensitive to Imatinib than WTKit correlates with the good responses by patients with GISTs (66), which commonly contain juxtamembrane mutations (12). This study suggests that patient screening for Kit mutations is essential to identifying malignancies that are likely to be responsive or resistant to treatment with Imatinib.

Materials and Methods

c-Kit Expression Constructs. The WT human c-*Kit* cDNAs (22) encoding the GNNK+/S+, GNNK−/S+ or GNNK+/S− isoforms in Bluescript were cloned into pRUFneo to create pRUFneo(WT*Kit*) as described previously (27). The cDNA encoding D816V*Kit* was constructed by reverse transcription-PCR using RNA extracted from the HMC-1 cell line as a template to create pRUFneo(D816V*Kit*) as described previously (27). cDNA encoding V560G*Kit* was constructed by reverse transcription-PCR of RNA extracted from the HMC-1 cell line using primers 5′-CAAACCTGAACACCAGCAGT-3′(sense) and 5′-AGCTTCTGCATGATCTTCCT-3′ (antisense), with denaturation for 1 min at 94°C, annealing for 1 min at 56°C, and extension for 1 min at 72°C for 30 cycles, to obtain a 1-kb product. This fragment was digested with *Sna*BI and *Apa*I and directionally cloned into the corresponding sites in pRUFneo(WT*Kit*). The mutation of T to G at 1700 bp, which causes a Val to Gly substitution at amino acid residue 560 results in the introduction of a novel *Hph*I site. Hence, plasmid DNA from bacterial clones was extracted and screened for the presence of the *Hph*I site. DNA sequencing was used to confirm the presence of the mutation and to determine the GNNK isoform of the clones. Unless otherwise stated, FDC(WTKit), FDC(V560GKit), and FDC(D816VKit) lines used are GNNK−/S+, which is the predominant form of the receptor in human cells (22).

Generation of Virus-producing Cells. Ψ2 cells were transfected with pRUFneo, pRUFneo(WTKit), pRUFneo-(V560GKit), or pRUFneo(D816VKit) as described previously

(27). The transfectants were selected with 400 μg/ml G418 until untransfected control cells were killed. The transfected pools were labeled with MAb IDC3 (antihuman c-Kit; Ref. 73) and sorted using a FACStar^{PLUS} cell sorter (Becton Dickinson, Mountain View, CA) for cells positive for human Kit. The top 5% of cells were collected and expanded.

Generation of Infectants and Maintenance in Liquid Culture. All of the cell lines were derived from FDC-P1, of murine early-myeloid phenotype (74) and were maintained in DMEM containing 10% FCS and 40 units/ml mGM-CSF (produced in insect cells from baculovirus constructs kindly provided by Dr. Andrew Hapel, John Curtin School of Medical Research, Canberra, Australia). Infection of FDC-P1 was carried out by cocultivation of 5×10^4/ml FDC-P1 and 1.25×10^5/ml Ψ2 transfectants irradiated with 30 Gy. Infectants were selected with 1 mg/ml G418 until uninfected control cells were killed. FDC(WTKit) were selected and maintained in 100 ng/ml hu-SCF and FDC(G560Kit) and FDC(D816VKit) were selected and maintained in the absence of added factors.

Immunofluoresence Assay. Cells were harvested, washed in PBS/0.1% BSA/0.1% sodium azide (PBA) and resuspended in PBA/10% normal rabbit serum containing MAb IDC3 (anti-human c-Kit) or an isotype matched control. Cells were incubated on ice for 1 h, washed with PBA to remove unbound antibody, and then incubated with R-phycoerythrin-conjugated antimouse IgG (Southern Biotechnology Associates, Birmingham, AL) for 1 h on ice. Cells were washed to remove unbound antibody, fixed in fluorescence-activated cell sorting fixative (2% glucose, 0.02% sodium azide, and 1% formaldehyde in PBS) and analyzed on a profile II Flow Cytometer (Coulter) to detect immunofluorescence of the cells.

Cell Growth Assay. To evaluate cell growth, FDC lines were cultured at 1.2×10^5/ml in DMEM/10% FCS containing factors as indicated and various concentrations of Imatinib in 96-well trays in triplicate. After 48 h, live cells were quantitated using Cell Titer96 Aqueous cell proliferation assay (Promega, Madison, WI).

Annexin V Assay. FDC lines were cultured in the presence of Imatinib and 40 units/ml mmGM-CSF or 100 ng/ml hu-SCF as indicated for 24 h. Cells were then washed twice in PBS and resuspended at $0.5–2 \times 10^7$ cells/ml in binding buffer (Hanks/1% HEPES/2.5 mM $CaCl_2$) containing a 1/50 dilution of Annexin-V-FLUOS (Roche, Mannheim, Germany). After incubation for 15 min at room temperature in the dark, samples were diluted in binding buffer and analyzed immediately using a profile II Flow Cytometer (Coulter).

Immunoprecipitation and Western Blot Analysis. FDC lines were harvested and incubated in DMEM without FCS or growth factors in the presence of indicated concentrations of Imatinib for 2 h. Each immunoprecipitation used 2×10^7 FDC(WTKit) or FDC(V560GKit), or 4×10^7 FDC(D816VKit). Where stated in "Results" (Fig. 6), cells were pulsed at 37°C with 40 units/ml mGM-CSF for 15 min or hu-SCF at 100 ng/ml for 2 min (which were found in preliminary experiments to be the optimal concentrations and times for activation of signaling). Cells were lysed in 1 ml of ice-cold lysis buffer containing 1% NP40 in TSE (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA pH8.0) with Complete protease inhibitor cocktail (Boehringer Mannheim GmbH, Mannheim, Germany), 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 mM sodium fluoride, 5 mM sodium PP_i, 5 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). Cells were incubated on ice for 30 min with occasional vortexing and then centrifuged at 21,000 × *g* for 30 min at 4°C. Supernatants were removed to a fresh tube, and protein content was determined using BCA Protein assay reagent (Pierce, Rockford, IL). Immunoprecipitations were performed using 5 μg of anti-c-Kit MAb Kit4 (IgG2a) and 35 μl of protein A-Sepharose CL 4B (50% slurry; Pharmacia, Uppsala, Sweden). Sepharose pellets were washed in TSE with 1% NP40 and 5 mM sodium vanadate, then resuspended in reduced loading buffer, boiled for 2 min, and separated by SDS-PAGE. Gels were electroblotted to nitrocellulose membrane (Advantec MFS, Inc. Pleasanton, CA) and membranes were washed with TBS (pH 7.4). Phosphotyrosine blots were blocked with TBST and probed with a cocktail of antiphosphotyrosine antibodies, 4G10 (Upstate Biotechnology, Lake Placid NY) and PY20 (Transduction Laboratories, Lexington KY) diluted in TBST. After washes with TBST, membranes were incubated with antimouse immunoglobulin-alkaline phosphatase conjugate (Silenus, Mellbourne, Australia). Membranes probed for c-Kit were blocked with TBS containing 2.5% membrane-blocking agent (Amersham Life Science, Buckinghamshire, England) and probed with anti-human c-Kit MAb 1C1 (kindly provided by Dr. H-J. Buhring, University of Tübingen, Tübingen, Germany) diluted in blocking agent, washed in TBST, and incubated with antimouse immunoglobulin-alkaline phosphatase conjugate. Blots were incubated with ECF substrate (Amersham Pharmacia Biotech) and scanned at 570 nm using a FluorolImager 595 (Molecular Dynamics, Sunnyvale, CA). Quantitation of bands was carried out using ImageQuant Version 5.1 (Molecular Dynamics).

For Western blot analysis using whole cell lysates, aliquots containing 50 μg of protein were electrophoresed, transferred to nitrocellulose membranes, and blocked in TBS containing 2.5% membrane blocking agent. Membranes were incubated with anti-ERK1 and anti-ERK2, anti-STAT3, anti-phospho-STAT3 (Santa Cruz Biotechnology, Inc.), or with anti-Akt, anti-phospho-Akt, or anti-phospho-ERK1/2 (Cell Signaling Technology, Beverly, MA) diluted in blocking reagent. Membranes were then washed in TBST, probed with antimouse or antirabbit immunoglobulin-alkaline phosphatase conjugate (Silenus) and incubated with ECF substrate.

Results

Retroviral Transfer of WTKit, V560GKit, and D816VKit into mGM-CSF-dependent Cell Line FDC-P1. FDC-P1 cells were infected with retroviruses encoding human WTKit, V560GKit, or D816VKit. After selection in medium containing G418, FDC(WTKit) were selected for growth in hu-SCF, and FDC(V560GKit) and FDC(D816VKit) were selected for growth in the absence of added factors. Surface expression of human c-Kit was examined using immunofluorescence and flow cytometric analysis (Fig. 1). Results indicated that human Kit was expressed on the surface of FDC(WTKit), FDC(V560GKit), and FDC(D816VKit), but not FDC-P1. The relatively low levels of c-Kit on the surface of FDC(D816VKit)

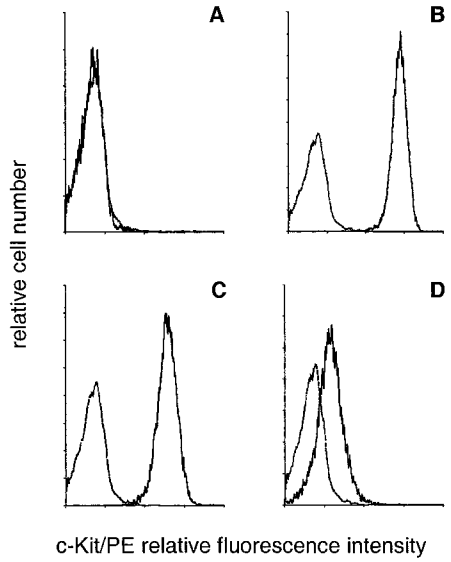

Fig. 1. Surface expression levels of c-Kit in FDC-P1 (*A*), FDC(WTKit) (*B*), FDC(V560GKit) (*C*), and FDC(D816VKit) (*D*), detected by immunofluorescence/flow cytometry with MAb IDC3 (antihuman c-Kit), are compared with an isotype-matched control MAb (*dashed line*).

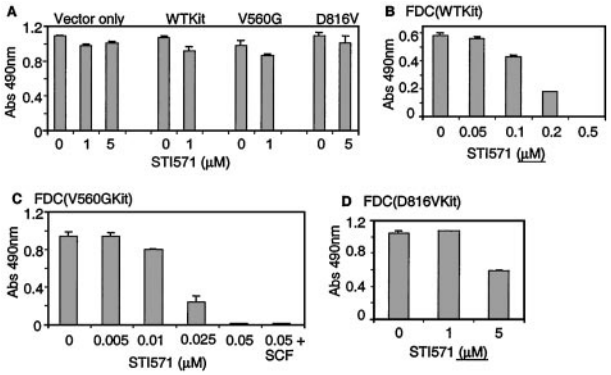

Fig. 2. Differential sensitivity of V560GKit and D816VKit to Imatinib. FDC expressing GNNK−/S+ WTKit, GNNK−/S+ V560GKit, and GNNK−/S+ D816VKit were cultured for 48 h in the presence of Imatinib (at the concentrations indicated on the *X* axis). Growth was assessed using a colorimetric assay for viable cell number. In *A*, cells were cultured in mGM-CSF. In *B*, WTKit were cultured in 100 ng/ml hu-SCF. V560GKit (*C*) and D816VKit (*D*) lines were cultured without added factors. *Abs*, absorbance.

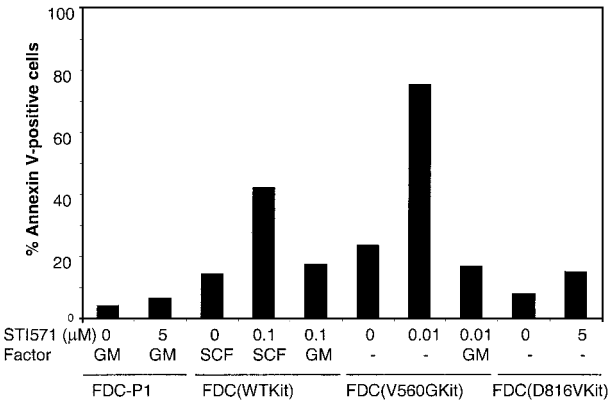

Fig. 3. Imatinib induces apoptosis in FDC(WTKit) and FDC(V560GKit). FDC-P1, FDC(WTKit), FDC(V560GKit), and FDC(D816VKit) were cultured in the presence of Imatinib and factors as indicated for 24 h. Apoptotic cells were detected by staining with Annexin-V and analysis by flow cytometry.

are consistent with previous results (24, 27) and may be caused by ligand-independent receptor turnover. Northern blot analysis indicated comparable expression levels of *kit* transcript in FDC(WTKit), FDC(V560GKit) and FDC(D816VKit; data not shown).

Imatinib Inhibits the Growth of FDC(V560GKit) More Potently Than FDC(WTKit) and Is Almost Ineffective against FDC(D816VKit). The effect of Imatinib on growth of FDC(WTKit), FDC(V560GKit), and FDC(D816VKit) was evaluated using a dye uptake assay that measures the number of live cells at the end of a 48-h culture period. When grown in mGM-CSF, the proliferation of all of the cell lines, including parental cells, was not greatly affected by the presence of Imatinib at concentrations of up to 5 μM (Fig. 2*A*), which indicated the absence of nonspecific effects at these doses. In contrast, the proliferation of FDC(WTKit) cultured with SCF was inhibited by Imatinib with a GI_{50} of 0.1–0.2 μM (Fig. 2*B*). The proliferation of FDC(V560GKit) in the absence of added factors was inhibited by Imatinib with an GI_{50} of 0.01–0.025 μM (Fig. 2*C*), which suggested that FDC(V560GKit) are much more sensitive to inhibition by Imatinib than are FDC(WTKit). This difference could not be explained by the presence of SCF in the media of FDC(WTKit) because the addition of SCF to FDC(V560GKit) did not restore growth of cells in 0.05 μM Imatinib (Fig. 2*C*). In contrast, the proliferation of FDC(D816VKit) was substantially less sensitive to inhibition by Imatinib than was FDC(WTKit) with a GI_{50} greater than 5 μM (Fig. 2*D*), which indicated that the D816V mutation could mediate resistance to Imatinib.

Imatinib Induces Apoptosis in FDC(WTKit) and FDC(V560GKit). To determine whether growth inhibition was caused by the induction of apoptosis, staining with Annexin-V was used (Fig. 3). After incubation of FDC-P1 in 5 μM Imatinib and mGM-CSF, the percentage of Annexin-V positive cells remained less than 7% of the total population. In contrast, incubation of FDC(WTKit) with 0.1 μM Imatinib and FDC(V560GKit) in 0.01 μM Imatinib resulted in 3-fold increases of Annexin-V-positive cells, which could be abrogated by the presence of mGM-CSF. Whereas incubation of FDC(D816VKit) in 5 μM Imatinib resulted in a slight increase in Annexin-V-positive cells, the percentage of apoptotic cells remained less than 15% of the total population. Similar results were obtained in a replicate experiment.

GNNK or S Isoforms of c-Kit Do Not Effect the Sensitivity to Inhibition by Imatinib. FDC lines were also used to determine whether c-Kit isoforms are differentially inhibited by Imatinib. Four naturally occurring isoforms of c-Kit result from alternative splicing events that cause the inclusion or exclusion of the GNNK motif in the extracellular juxtamembrane domain and a serine in the interkinase domain (20–22), designated here as GNNK+ or GNNK− and S+ or S−.

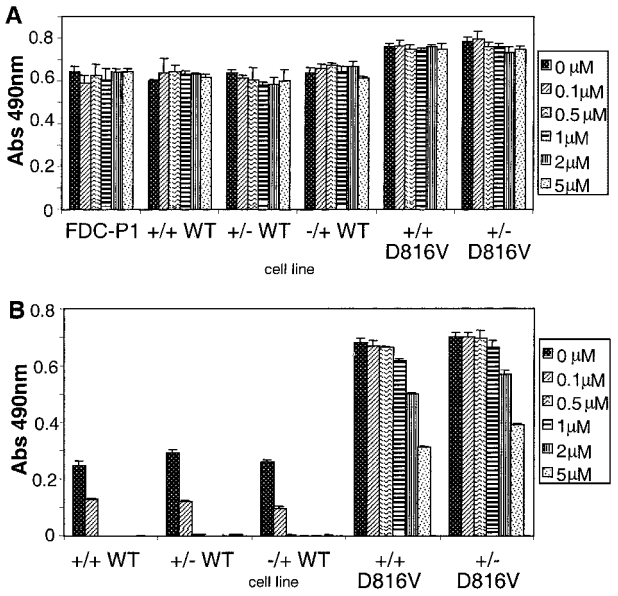

Fig. 4. GNNK+/− and S+/− isoforms do not differ in sensitivity to Imatinib. FDC expressing GNNK+/S+ WTKit, GNNK+/S- WTKit, GNNK−/S+ WTKit, GNNK+/S+ D816VKit, and GNNK−/S+ D816VKit were cultured in the presence of Imatinib for 48 h. Growth was assessed using a colorimetric assay for viable cell number. In *A*, cells were cultured in mGM-CSF. In *B*, WTKit lines were cultured in 100 ng/ml hu-SCF, and D816VKit lines were cultured in the absence of added factors. *Abs*, absorbance.

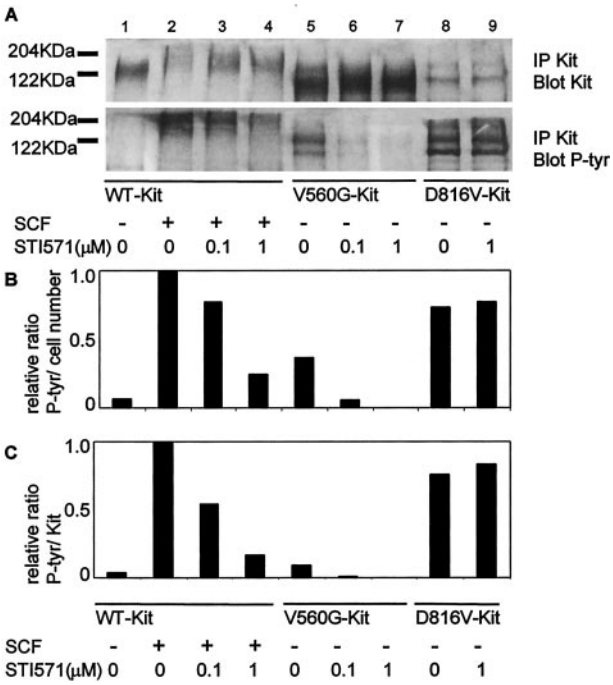

Fig. 5. Effect of Imatinib on phosphorylation of c-Kit. In *A*, FDC(WTKit), FDC(V560GKit), and FDC(D816VKit) were incubated in the absence of serum for 2 h with the indicated concentrations of Imatinib. FDC(WTKit) were then pulsed with 100 ng/ml SCF for 2 min. Cells were lysed and immunoprecipitation and Western blot analysis was performed as described in "Materials and Methods." *kDa*, molecular weight in thousands. In *B* and *C*, Kit and phosphotyrosine bands were quantitated using ImageQuant software. All of the values are relative to WTKit stimulated with SCF (*Lane 2*), which was assigned an arbitrary value of 1. The extent of phosphorylation is represented as phosphorylated Kit/cell number (*B*) and the ratio of phosphorylated Kit:total Kit for the same number of cells (*C*).

Proliferation assays were used to compare the effect of Imatinib on FDC(WTKit GNNK+/S+), FDC(WTKit GNNK−/S+), FDC(WTKit GNNK+/S−), FDC(D816VKit GNNK+/S+), and FDC(D816VKit GNNK−/S+). Proliferation in the presence of mGM-CSF was not inhibited by up to 5 μM Imatinib in any of the cell lines (Fig. 4*A*). Proliferation of WT-Kit lines FDC(WTKit GNNK+/S+), FDC(WTKit GNNK−/S+), and FDC(WTKit GNNK+/S−), cultured in SCF, were inhibited by Imatinib to a similar extent (Fig. 4*B*). Proliferation of FDC(D816VKit GNNK+/S+) and FDC(D816VKit GNNK−/S+), cultured in the absence of cytokines, were each inhibited by Imatinib to a similar extent with a $GI_{50} \geq 5$ μM. Therefore, these data indicate that GNNK and S isoforms of c-Kit do not differ in sensitivity to Imatinib.

Effect of Imatinib on Receptor Phosphorylation and the Activation of Downstream Signaling Pathways. To determine the effect of Imatinib on autophosphorylation of c-Kit, immunoprecipitation using anti-Kit antibody and Western blot for either Kit or phosphotyrosine was performed (Fig. 5*A*). WTKit was detected as a band of M_r 140,000, which increased in size after the addition of SCF, probably because of ubiquitination of the receptor rapidly after phosphorylation.[4] In contrast, G560VKit and D816VKit were detected as a major band at M_r 120,000 and a minor band at M_r 140,000, which suggests that these constitutively active Kit mutants are not fully glycosylated and may signal and be turned over without being transported to the cell surface. In the phosphotyrosine blot, smaller bands in V560GKit and D816VKit lanes may represent degraded Kit, because of a high rate of continuous turnover of the constitutively active forms.

Quantitation of phosphotyrosine levels revealed that Kit phosphorylation in FDC(WTKit) was inhibited by 22% in 0.1 μM Imatinib and 75% in 1 μM Imatinib (Figs. 5, *A* and *B*, *Lanes 2–4*). Phosphorylation of Kit in FDC(V560GKit) was reduced by 85% in the presence of 0.1 μM Imatinib and 99.5% in cells treated with 1 μM Imatinib (*Lanes 5–7*). In contrast, receptor phosphorylation in FDC(D816VKit) was unaffected by 1 μM Imatinib (*Lanes 8* and *9*). These results correlate well with growth assays, indicating that FDC(V560GKit) are more sensitive to Imatinib with an IC_{50} of <0.1 μM compared with FDC(WTKit) with an IC_{50} between 0.1 μM and 1 μM, whereas FDC(D816VKit) are resistant to inhibition by Imatinib with an IC_{50} of >1 μM.

Quantitation of total Kit and phosphorylated Kit was used to determine the relative extent of phosphorylation (Fig. 5*C*). WTKit was found to have relatively high levels of phosphorylation in the presence of SCF, compared with unstimulated cells. D816VKit displayed a high ratio of phosphorylated Kit:total Kit in the absence of factors. Immunoprecipitations using FDC(D816V)Kit were performed using twice the number of cells as the other lines because of low levels of

[4] S. Young, T. Cambareri, and L. Ashman, unpublished data.

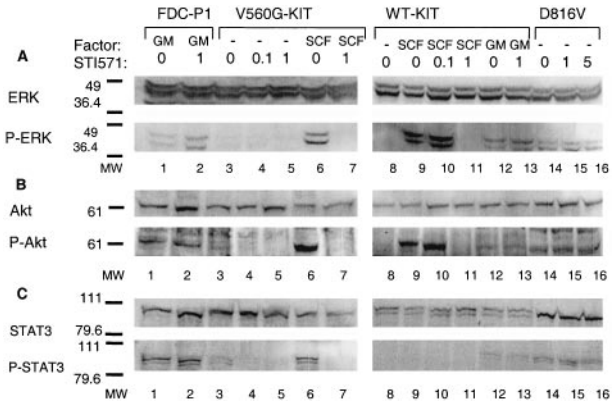

Fig. 6. Effect of Imatinib on the activation of downstream signaling pathways. FDC-P1, FDC(WTKit), FDC(V560GKit), and FDC(D816VKit) were incubated in the absence of serum for 2 h with the indicated concentrations of Imatinib. Cells were pulsed with hu-SCF for 2 min or mGM-CSF (*GM*) for 15 min. Cells were lysed and Western blot analysis was performed as described in "Materials and Methods." *MW*, molecular weight in thousands.

expression. After correction for cell number, the phosphorylated Kit:total Kit ratio of FDC(D816V)Kit was ~80% of that observed with FDC(WTKit) pulsed with SCF. V560GKit was shown to be expressed at comparatively high levels but the ratio of phosphorylated Kit:total Kit was relatively low. Because both FDC(D816VKit) and FDC(V560GKit) were selected for the ability to grow in the absence of factors, these data suggest that V560GKit must be expressed at high levels for constitutive activation to occur and for a threshold level of phosphorylation to be achieved.

Western blot analysis was used to determine the signaling differences between the Kit mutants and WTKit, as well as the effect of Imatinib on activation of downstream signaling pathways. The levels of ERK1/2 were not altered in any of the cell lines after treatment with Imatinib or growth factors (Fig. 6*A*). Phosphorylation of ERK1/2 was not detected in FDC(V560GKit) without the addition of growth factors (*Lanes 3–5*); however, the addition of SCF resulted in phosphorylation of ERK1/2, which was inhibited by preincubation with 1 μM Imatinib (*Lanes 6–7*). Likewise, the addition of SCF to FDC(WTKit) resulted in ERK phosphorylation, which could be blocked by the addition of 1 μM Imatinib (*Lanes 9–11*). FDC(D816VKit) contained a low level of constitutive ERK1/2 phosphorylation, which was resistant to inhibition by 5 μM Imatinib (*Lanes 14–16*). The addition of mGM-CSF to FDC-P1 and FDC(WTKit) resulted in the phosphorylation of ERK1/2, which, as expected, was not inhibited by preincubation with 1 μM Imatinib (*Lanes 1–2* and *12–13*).

Levels of total Akt in the cell lines were not altered by the 2-h treatment with Imatinib (Fig. 6*B*). mGM-CSF- pulsed FDC-P1 and -FDC(WTKit) exhibited low levels of phosphorylated Akt, which were not reduced by the presence of 1 μM Imatinib (*Lanes 1–2* and *12–13*). In FDC(V560GKit), only extremely low levels of phosphorylated Akt could be detected in cells without the addition of growth factors and Akt phosphorylation was ablated by 0.1 μM Imatinib (*Lanes 3–4*). The addition of SCF to FDC(V560GKit) resulted in much higher levels of Akt phosphorylation, which were completely inhibited by 1 μM Imatinib (*Lanes 6–7*). Similarly, the addition of SCF to FDC(WTKit) resulted in Akt phosphorylation, which was blocked completely by 1 μM Imatinib (*Lanes 9–11*). Phosphorylation of Akt occurred in FDC(D816VKit) in the absence of factors and was resistant to inhibition by 5 μM Imatinib (*Lanes 14–16*).

Levels of total STAT3 were unchanged after incubation with Imatinib (Fig. 6*C*). Phosphorylated STAT3 was detected in FDC-P1 and FDC(WTKit) pulsed with mGM-CSF and was not inhibited by 1 μM Imatinib (*Lanes 1–2* and *12–13*). Interestingly, a low level of constitutive phosphorylation of STAT3 was found in FDC(V560GKit), which was abrogated by incubation with 0.1 μM Imatinib (*Lanes 3–4*). The addition of SCF to FDC(V560GKit) resulted in increased levels of phosphorylated STAT3, which was blocked by 1 μM Imatinib (*Lanes 6–7*). Unlike FDC(V560GKit), the addition of SCF to FDC-(WTKit) did not cause phosphorylation of STAT3 (*Lane 9*), which indicated a difference in signal transduction. In FDC(D816VKit), STAT3 was constitutively activated at low levels and could not be inhibited by 5 μM Imatinib (*Lanes 14–16*).

Overall, these data indicate that the constitutive activation of ERK1/2, Akt, and STAT3 in FDC(D816V) is not inhibited by 5 μM Imatinib, which suggests that the D816V mutant is resistant to inhibition by Imatinib. However, SCF-induced phosphorylation of ERK1/2 and Akt in FDC(WTKit) and ERK1/2, Akt and STAT3 in FDC(V560GKit) were completely inhibited by 1 μM Imatinib. In addition, FDC(V560GKit), like FDC(D816VKit), were found to contain low levels of constitutively phosphorylated STAT3 and extremely low levels of phosphorylated Akt but in contrast to FDC(D816VKit), this phosphorylation was inhibited efficiently by Imatinib.

Discussion

The successful use of Imatinib for treatment of chronic myelogenous leukemia/acute lymphoblastic leukemia and the relatively low toxicity of the compound has prompted studies to expand the use of Imatinib to conditions associated with PDGF-R and c-Kit. Early trials revealed successful treatment of GIST, but there have been no reports for successful treatment of mastocytosis (65, 66, 75). To compare WTKit with Kit mutations that are commonly found in GIST or in mastocytosis, in the same cellular background, retroviral expression was used to produce murine factor-dependent early myeloid cells expressing WT and mutant human Kit; FDC(WTKit), FDC(V560GKit), and FDC(D816VKit). Growth assays in the presence of Imatinib indicated that FDC(V560GKit) were inhibited by Imatinib with a GI_{50} of 0.01–0.025 μM and, thus, were much more sensitive than FDC(WTKit) with a GI_{50} of 0.1–0.2 μM, whereas FDC(D816VKit) were relatively resistant to inhibition by Imatinib with a GI_{50} of >5 μM. As reported previously (76, 77), Imatinib induced apoptosis in sensitive cell lines.

The naturally occurring Kit isoforms (20–22) are of interest because splice variants characterized by the presence or absence of a GNNK sequence (GNNK+ or GNNK−) in the region extracellular to the transmembrane domain exhibit differences in the activation of signaling pathways (72). The

GNNK− isoform was shown to have a low level of constitutive activity (20) and NIH3T3 cells that expressed GNNK− but not GNNK+ were able to form tumors in mice (72). A second site of alternative splicing results in the presence or absence of a serine residue in the interkinase region of the receptor (22). Comparison of growth inhibition by Imatinib of FDC lines expressing GNNK+/− and S+/− isoforms of c-Kit indicated that the splice variants did not differ in their sensitivity to the inhibitory effects of Imatinib in growth assays.

D816VKit has been shown to confer an antiapoptotic phenotype and resistance to chemotherapeutic drugs (27, 42). Because Imatinib was shown to act at the level of cell survival, it was possible that the resistance of FDC(D816VKit) cells was a consequence of the antiapoptotic phenotype. Therefore, the effects of Imatinib on Kit phosphorylation and downstream signaling were examined. Receptor phosphorylation in the presence of Imatinib was analyzed using immunoprecipitation and Western blotting. Autophosphorylation of FDC(WTKit) and FDC(V560GKit) was inhibited by 0.1 μM Imatinib. Receptor phosphorylation of FDC(V560GKit) was inhibited to a greater extent than that of FDC(WTKit), which supported the results of the growth assays that the juxtamembrane mutant is more sensitive to Imatinib (with a lower IC_{50}) compared with FDC(WTKit). In contrast, receptor phosphorylation in FDC(D816VKit) was not inhibited by 1 μM Imatinib, indicating resistance. Thus, the effects of Imatinib on cell growth paralleled the effects on receptor phosphorylation.

V560GKit was shown to be expressed at relatively high levels but the ratio of phosphorylated Kit:total Kit was low compared with results obtained for D816VKit or WTKit after a SCF pulse. Because FDC(D816VKit) and FDC(V560GKit) were both selected for factor-independent growth, this suggests that V560GKit must be highly expressed for threshold levels of receptor phosphorylation and downstream signaling to be achieved. Whether the difference in receptor phosphorylation, between FDC(WTKit) stimulated with SCF and the constitutive activation of FDC(V560GKit), is attributable to a different extent of receptor phosphorylation or to differences in the proportion of receptor molecules that are activated is not known. The relative inefficiency of constitutive activation of V560GKit may at least partially explain the sensitivity of this mutant to Imatinib.

We and others have previously demonstrated differences in signaling between D816VKit and WTKit (56, 58, 59). Such differences were confirmed here, and, in addition, differences between V560GKit and WTKit were also observed. Although ERK1/2 phosphorylation was strongly activated by WTKit stimulated with SCF, it was not constitutively activated by V560GKit and was only weakly activated by D816VKit. Previous studies have shown that the STAT3 and the PI3-K pathway were required for the tumorigenic potential of D816VKit mutants (58, 78). STAT-3, which is not activated by SCF-induced WTKit, was constitutively activated by V560G and D816V Kit mutants. Akt, which acts downstream of PI3-K, has been associated with cell survival in response to SCF (79) and was constitutively activated by V560GKit and by D816VKit, although at much lower levels than by SCF-activated WTKit and V560GKit.

Imatinib inhibited activation of ERK and Akt in FDC(WTKit) by SCF stimulation but not by activation with GM-SCF. These results are in agreement with previous studies investigating the effect of Imatinib on a SCF-pulsed SCLC line and the leukemic line, Mo7e (62, 68, 69). SCF stimulation of FDC(V560GKit) resulted in the activation of ERK, Akt, and STAT3, which were all inhibited by Imatinib. In addition, Imatinib was able to inhibit the low levels of constitutive activation of STAT3 and very low amounts of phosphorylated Akt seen in FDC(V560GKit). In contrast, constitutive activation of ERK, Akt, and STAT3 by D816VKit was not inhibited by 5 μM Imatinib. These data demonstrate that SCF-induced signal transduction in FDC(WTKit) and FDC(V560GKit) and constitutive signaling in FDC(V560GKit) are inhibited by Imatinib, whereas constitutive activation of signaling pathways in FDC(D816VKit) cannot be blocked by Imatinib.

Overall, the signaling experiments indicate that the differences in sensitivity of the Kit mutants are a direct consequence of the ability or inability of Imatinib to inhibit Kit phosphorylation and possibly a result of altered interaction of the drug with the ATP-binding pocket of Kit. Additional studies will be required to determine the structural basis of proposed differences in Imatinib binding.

The inhibition of V560GKit and WTKit by Imatinib indicates a potential use in the treatment of several malignancies. Early reports of GIST clinical trails have shown considerable efficacy (65, 66). Juxtamembrane mutations in GIST are relatively common (12). Our finding that V560GKit is actually more sensitive to Imatinib than WTKit is correlates with the success in early clinical trails. Other potential applications of Imatinib are conditions that involve autocrine stimulation of c-Kit such as SCLC (13, 14). Imatinib was found to inhibit cell growth, receptor phosphorylation, generation of reactive oxygen species, and cell motility in c-Kit-positive SCLC lines (68, 69). Coexpression of c-Kit and SCF can also occur in neuroblastoma, melanoma, non-SCLC, and breast cancer, and these cell lines were growth inhibited by c-Kit antisense nucleotides or blocking antibodies (17, 19). Screening of patients will be useful for identifying c-Kit-positive neoplasms that may respond to treatment with Imatinib. Mutations in the juxtamembrane region have also been detected in sinonasal lymphomas and mastocytosis (11, 45). Because these conditions are also associated with kinase domain mutations, sequencing of cDNA prepared from patient samples could be used to identify candidates for therapy with Imatinib.

Additional studies are required to determine whether other known mutant forms of c-Kit are inhibited by Imatinib. Recently, K642EKit, detected in GIST, was shown to be inhibited by Imatinib (80). Additional kinase domain mutations have been found at codons 839 (37) and 820 (81) in mastocytosis and at codon 825 in sinonasal lymphoma (45). A mutation at codon 52, in the SCF-binding domain, has been detected in myeloproliferative disorders (82), and the duplication of codons 501 and 502 in the extracellular juxtamembrane domain has been found in GIST (83).

In summary, the results presented here indicate that a juxtamembrane region mutant V560GKit is inhibited by Imatinib to a greater extent than WTKit, whereas the kinase

domain mutant D816VKit is resistant to inhibition by Imatinib. These results suggest that the effective dose for treatment of malignancies in which Kit is a main target will differ depending on the presence and type of any inherent mutation. This study emphasizes that patient screening for Kit mutations is essential to identifying malignancies and even individual patients who are likely to be responsive or resistant to treatment with Imatinib.

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