Effect of Imatinib Mesylate on Neuroblastoma Tumorigenesis and Vascular Endothelial Growth Factor Expression

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Background: Alternative treatment options are needed for advanced neuroblastoma patients because their prognosis remains poor after intensive chemotherapy. Neuroblastoma cells express platelet-derived growth factor (PDGF), stem cell factor (SCF), and vascular endothelial growth factor (VEGF) and their respective receptors, PDGFR, c-Kit, and Flk-1. We therefore evaluated the effects of imatinib mesylate (imatinib), a selective inhibitor of the tyrosine kinase activities of c-Kit and PDGFR, on the growth of neuroblastoma cells in vivo and in vitro. Methods: We tested seven human neuroblastoma cell lines for their sensitivity to imatinib. Cell viability was assessed by trypan blue dye exclusion. Apoptosis was evaluated by nuclear staining, flow cytometry, and western blotting. Protein assays included immunoprecipitation, western blotting, enzyme-linked immunosorbent assays, and immunohistochemistry. mRNA expression was assessed by northern blotting. We used a xenograft model in SCID mice (10 mice per group) to evaluate the effects of imatinib oral therapy (50 or 100 mg/kg every 12 hours for 14 days) on neuroblastoma tumor growth. All statistical tests were two-sided. Results: All seven neuroblastoma cell lines treated with imatinib displayed concentration-dependent decreases in cell viability, which coincided with an induction of apoptosis, and with ligand-stimulated phosphorylation of c-Kit and PDGFR. The imatinib concentrations that caused 50% inhibition of growth and 50% inhibition of ligand-induced phosphorylation of these receptors were 9–13 μM and 0.1–0.5 μM, respectively. Expression of VEGF, but not phosphorylation of Flk-1, its receptor, was reduced in neuroblastoma cells treated with imatinib at 10 μM or higher. Mice treated with imatinib at 50 mg/kg or 100 mg/kg had statistically significantly smaller tumors than control mice treated with vehicle (mean tumor volume in mice treated with imatinib at 50 mg/kg = 1546 mm³, in control mice = 2954 mm³; difference = 1408 mm³, 95% confidence interval [CI] = 657 to 2159 mm³; P<.001; mean tumor volume in mice treated with imatinib at 100 mg/kg = 463 mm³; difference = 2491 mm³, 95% CI = 1740 to 3242 mm³; P<.001). Conclusions: Imatinib inhibited the growth of neuroblastoma cells in vitro and in vivo. This inhibition was associated with suppression of PDGFR and c-Kit phosphorylation and inhibition of VEGF expression.

Neuroblastoma is the most common solid extracranial tumor of early childhood (1). Several tumor characteristics, such as amplification of the N-myc oncogene and loss of heterozygosity at chromosome 1p, are statistically significant indicators of poor prognosis for neuroblastoma patients (3). Neuroblastoma patients with high-risk or disseminated disease continue to have a poor prognosis, even after they have undergone intensive chemotherapy and autologous bone marrow transplantation (2). Alternative treatments are therefore needed to improve the prognosis of neuroblastoma patients with high-risk disease.

Receptor tyrosine kinases have been proposed as potential targets for antitumor therapy. For example, imatinib mesylate (also known as ST1571 or Gleevec, and hereafter called imatinib) was initially shown to inhibit in vitro growth of Abl-transformed cells and Bcr-Abl–positive chronic myelogenous leukemia (CML) cells through selective inhibition of the Abl tyrosine kinase (4). Imatinib is also a highly selective inhibitor of the tyrosine kinase activities of c-Kit, the receptor for stem cell factor (SCF), and platelet-derived growth factor receptor (PDGFR) (5). Results of recent clinical studies (7) have shown that imatinib therapy is well tolerated and leads to remission in patients with Bcr-Abl–positive CML or c-Kit–positive gastrointestinal stromal tumor (GIST). Preclinical data suggest that imatinib has cytotoxic and cytostatic activities and effectively inhibits the growth of tumor cell lines that contain gain-of-function mutations in Abl and c-Kit as well as tumor cells that express autocrine growth loops (5). Imatinib has also been reported to inhibit the growth of glioblastoma, dermatofibrosarcoma protuberalens, and small-cell lung cancer, all of which may express the PDGF/PDGFR or SCF/c-Kit autocrine growth loops (8–10). Recently, we have shown that imatinib interferes with the in vitro and in vivo growth of Ewing’s sarcoma which, like neuroblastoma, is a tumor of peripheral neuroectodermal origin (11).

SCF and c-Kit mRNAs are expressed in neuroblastoma cell lines and in neuroblastoma tumor samples (12). The SCF/c-Kit autocrine growth loop is thought to be important for cell survival because neuroblastoma cells treated with an antibody to c-Kit undergo increased levels of apoptosis (13). Neuroblastoma cell lines also express functional PDGFR-α, PDGFR-β, and their respective ligands, PDGF-AA and PDGF-BB (14). These observations led us to hypothesize that imatinib might inhibit neuroblastoma cell growth and survival via inhibition of the SCF/c-Kit and PDGF/PDGFR autocrine growth loops.

Tyrosine kinase receptors also play a role in angiogenesis, an essential step for tumor growth and metastasis. Tumor cell growth and metastasis require perturbation of the local balance of pro-angiogenic and anti-angiogenic factors, which is referred to as...
to as the angiogenic switch (15). In addition, vascular endothelial growth factor (VEGF) plays a key role in tumor angiogenesis. Most neuroblastoma cell lines and tumors express VEGF (16,17), and the degree of VEGF expression and angiogenesis in neuroblastoma tumors is associated with both disease progression and poor prognosis (18). Results of recent studies (20) have shown that VEGF expressed by neuroblastoma cells contributes to the growth of endothelial cells in vitro and to angiogenesis in vivo. Blockade of VEGF function is associated with the suppression of neuroblastoma growth (21,22), suggesting the potential utility of VEGF-targeted therapies in neuroblastoma treatment.

Here, we examined the effects of imatinib on the growth of neuroblastoma cells in vitro and in vivo, on VEGF production, and on c-Kit and PDGFR phosphorylation.

**MATERIALS AND METHODS**

**Cell Culture and Reagents**

Seven human neuroblastoma cell lines, SMS-KCNR (23), NGP (24), SK-N-BE2 (25), LAN-1 (24), LAN-5 (24), SH-SY5Y (24), SK-N-AS (26), mouse NIH3T3 fibroblast cells (provided by J. Bolen, National Cancer Institute), and human CCL-243 cells, a Philadelphia chromosome–positive K562 CML cell line (American Type Culture Collection, Manassas, VA) were used in this study. The cells were cultured in RPMI-1640 medium and 10% fetal bovine serum as described previously in this study. The cells were cultured in RPMI-1640 medium and 10% fetal bovine serum as described previously (27). Imatinib was provided by Dr. Elisabeth Buchdunger (Novartis Pharmaceuticals, Basel, Switzerland). For growth factor stimulation assays, SMS-KCNR cells and SH-SY5Y cells (1 x 10^5) were plated on 60-mm dishes in medium containing 10% serum. The cells were shifted to 0.1% serum-containing medium for the indicated time and then incubated for 30 minutes with either 1 μM imatinib or media control. The cells were then incubated for 24 hours in medium containing 0.1% fetal bovine serum to which either human recombinant SCF at 50 ng/mL (Endogen, Woburn, MA) or human recombinant PDGFR-BB at 50 ng/mL (R&D Systems, Minneapolis, MN) and human recombinant VEGF (R&D Systems) or an equivalent volume of medium was added. Cells were stained with propidium iodide (PI) and subjected to fluorescence-activated cell sorting using a FACSscan apparatus and CellQuest software (BD Biosciences, San Jose, CA) to quantify the percentage of apoptotic cells (i.e., those in the sub-G1 fraction).

**Cell Viability and Cell Growth Assays**

We used the trypan blue dye exclusion assay to evaluate cell number and viability. Cells (1 x 10^5) were plated on 60-mm dishes and incubated with various concentrations of imatinib for 4 days. The cells were then trypsinized and stained with 0.2% trypan blue, and viable cells (i.e., those that excluded the dye) were counted under low-power microscopy. In some experiments, cell growth was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma Chemical Company, St. Louis, MO) after 4 days of imatinib treatment, as previously described (11).

**Apoptosis Assays**

We detected morphologic changes in the nuclear chromatin of cells undergoing apoptosis by staining with Hoechst 33342 (Molecular Probes, Eugene, OR). Neuroblastoma cells were grown in 96-well plates and cultured for 48 hours in the presence or absence of 15 μM imatinib. After treatment, the cells were incubated in the dark with Hoechst 33342 for 30 minutes at 37 °C and were then observed with a fluorescence microscope to detect morphologic changes in the nuclear chromatin of cells undergoing apoptosis. To assess cell cycle changes as well as the proportion of the cell population with less than a G1 content of DNA, cells were trypsinized, washed with ice-cold phosphate-buffered saline (PBS), and fixed in 70% ethanol. After another wash with PBS, cells were incubated with RNase at 100 μg/mL and PI at 50 μg/mL (Sigma Chemical Company). DNA–PI fluorescence was measured with a FACScan apparatus.

**Northern Blot Analysis**

Cells were harvested by trypsinization, and total RNA was extracted from 10^6 cells with the use of RNAeasy kit (Qiagen, Santa Clarita, CA). Northern blot analysis for VEGF RNA was performed according to a previously described protocol (27). Briefly, total RNA (25 μg) was resolved on 1% agarose–6% formaldehyde gels. The gels were stained with ethidium bromide at 2 mg/mL to allow inspection of the quantity and quality of the RNA. The RNA was transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) and then hybridized with a 32P-labeled VEGF or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

**Protein Assays**

Western blotting and immunoprecipitation were performed as described previously (28). Briefly, cells were harvested by trypsinization, resuspended in cold protein lysis buffer (0.5% Nonidet P-40, 50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA [pH 8.0], 10 mM NaF, 1 mM phenylmethylsulfonylfluoride, aprotinin at 1 μg/mL, leupeptin at 1 μg/mL, and 500 μM sodium orthovanadate), and incubated for 30 minutes on ice. Insoluble material was removed from the lysates by centrifugation at 10 000g for 15 minutes at 4 °C. For immunoprecipitation, we incubated 1 mg of protein lysate with 1 μg of an anti-PDGFR-α (Santa Cruz Biotechnology, Santa Cruz, CA), 1 μg of an anti-PDGFR-β antibody (Santa Cruz Biotechnology), 1 μg of an anti-c-Kit antibody (Lab Vision-NeoMarkers, Fremont, CA), or 1 mg of immunoglobulin G as a negative control (data not shown) overnight at 4 °C. We then added 20 μL of 10% (vol/vol) protein A–agarose beads (Santa Cruz Biotechnology) to each reaction and incubated the mixture, with gentle rocking, for 2 hours at 4 °C. The immune complexes were then collected by brief centrifugation (i.e., 500g for 2 minutes) and washed twice with lysis buffer followed by a single wash with water. Precipitates were resuspended in 20 μL of 2× Tris–glycine–sodium dodecyl sulfate (SDS) buffer (Invitrogen, Carlsbad, CA). The lysates and immunoprecipitates were separated by SDS–polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). Where indicated, the gels were electrophoresed under nonreducing conditions (i.e., the gel and running buffer did not contain SDS). The membranes were incubated in 5% powdered milk in Tris-buffered saline/Tween-20 (TBS–Tween; 20 mM Tris–HCl [pH 7.4], 150 mM NaCl, and 0.5% Tween-20) to block nonspecific antibody binding and then incubated at room temperature for 2 hours or at 4 °C overnight in...
the same blocking solution to which one of the following antibodies was added (at a 1:1000 dilution, except where indicated): anti-phospho-tyrosine (pTy) antibody, anti-PDGFR-α or anti-PDGFR-β antibody, anti-caspase-3 antibody, anti-VEGF antibody, and anti-Fk-1 antibody (1:500 dilution) (all from Santa Cruz Biotechnology); anti-c-Kit antibody (DAKO, Carpinteria, CA); anti-poly(ADP-ribose) polymerase (PARP) antibody (Pharmingen, San Diego, CA); anti-Akt antibody, anti-phospho-Akt antibody, anti-extracellular signal-regulated kinase (ERK)1/2 antibody, and anti-phospho-ERK1/2 antibody (all from Cell Signaling Technology, Beverly, MA); or anti-actin antibody (1:2000 dilution) (Oncogene Research Products, Darmstadt, Germany). The membranes were washed with TBS-Tween and incubated with horseradish peroxidase (HRP)—conjugated secondary antibodies (1:2000 dilution; Santa Cruz Biotechnology); anti-c-Kit antibody (DAKO, Carpinteria, CA); anti-poly(ADP-ribose) polymerase (PARP) antibody, anti-phospho-Akt antibody, anti-phospho-ERK1/2 antibody, and anti-phospho-Akt antibody (all from Cell Signaling Technology, Beverly, MA); or anti-actin antibody (1:2000 dilution) (Oncogene Research Products, Darmstadt, Germany). The membranes were washed with TBS-Tween and incubated with horseradish peroxidase (HRP)—conjugated secondary antibodies (1:2000 dilution; Santa Cruz Biotechnology) for 1 hour at room temperature. Bound antibodies were detected by chemiluminescence with the use of a Lumiglo detection system (Kirkgaard and Perry Laboratories, Gaithersburg, MD).

We measured the concentration of VEGF in conditioned medium obtained from 2 × 10⁷ cells that were incubated for 24 hours at 80% confluence in 12-well plates containing complete medium (27). The medium was collected and centrifuged at 1000g for 10 minutes, and the supernatants were stored at −80 °C. The concentration of VEGF protein was measured using enzyme-linked immunosorbent assay Quantikine kits (R&D Systems) with a mouse monoclonal antibody against human VEGF (R&D Systems), according to the manufacturer’s instructions.

Xenograft Tumor Study

Subconfluent SMS-KCNR cells were harvested by trypsinization and resuspended in Hank’s balanced salt solution at 4 × 10⁷ cells/mL. We injected 2 × 10⁶ cells in 50 μL of Hank’s balanced salt solution into the left gastrocnemii of 5-week-old female SCID mice (Taconic, Germantown, NY). One week after the injections, the mice (10 mice in each group) were randomly assigned to receive either imatinib at 50 mg/kg in PBS, imatinib at 100 mg/kg in PBS, or vehicle (PBS) alone every 12 hours for 14 days by oral gavage. We measured the dimensions of the resulting tumors at 2 weeks after the initiation of injections with the use of a digital caliper and determined tumor volume (V) by using the formula \( V = \pi/6 \times 2a \times b \), where a is the shorter diameter and b is the longer diameter, as described previously (29). All mice were killed by asphyxiation with CO₂, and their tumors were excised and immediately frozen at −80 °C. These xenograft studies were approved by the Animal Care and Use Committee of the National Cancer Institute, and all animal treatments, including their housing, were in accordance with institutional guidelines and as described in protocol number PB-023.

Statistical Analysis

A one-way analysis of variance (ANOVA) was performed on data for SMS-KCNR and SH-SY5Y cell lines treated with SCF, PDGF-BB, and all combinations of the two factors. Residuals were examined for normality and homogeneity of variance. Because the P value for the ANOVA F statistic was less than .001, we calculated the 95% confidence intervals (CIs) for the differences between the groups using the post-test Bonferroni multiple comparisons method to reduce the overall chance of a type I error (30). We analyzed xenograft tumor volume data by ANOVA with the use of Prism 3.0 software (GraphPad Software, San Diego, CA). Tumor volumes were compared using a post-test Bonferroni comparison of groups to reduce the overall chance of a type I error (30). We used Student’s unpaired t tests for the other statistical analyses. All statistical analyses were two-sided, and results were considered to be statistically significant at \( P < .05 \).

RESULTS

Effect of Imatinib on the Growth of Neuroblastoma Cell Lines

We studied the effects of imatinib on the growth of seven neuroblastoma cell lines: five cell lines [SMS-KCNR, NGP, SK-N-BE2, LAN-1, and LAN-5] had amplific N-myc genes and expressed N-Myc protein, one cell line (SH-SY5Y) expressed N-Myc protein but did not have an amplified N-myc gene, and one cell line (SK-N-AS) neither expressed N-Myc protein nor had an amplified N-myc gene (Table 1). We treated the neuroblastoma cell lines with 0–15 μM imatinib for 4 days and used trypan blue dye exclusion to determine cell viability at the end of the treatment period. All of the neuroblastoma cell lines treated with imatinib displayed a concentration-dependent decrease in cell viability (Fig. 1, A). The concentration of imatinib that was associated with 50% inhibition of growth of the neuroblastoma cell lines (biologic IC₅₀) ranged from 9 to 13 μM (Fig. 1, A). We also used the MTT assay to assess relative cell number and found that the biologic IC₅₀ for imatinib in the SMS-KCNR cell line was 10 μM, identical to the IC₅₀ in these cells as determined by using trypan blue dye exclusion, but higher than the IC₅₀ for imatinib in CCL-243 cells (IC₅₀ = 0.1 μM) (Fig. 1, A, inset graph). SMS-KCNR and SH-SY5Y cells treated with 10 μM imatinib displayed morphologic changes, such as becoming rounded, denser, more refractive, and detached, which were suggestive of the induction of cell death (Fig. 1, B). However, the growth of NIH3T3 cells was inhibited by only 25% when cultured in 10 μM imatinib (Fig. 1, A), and such treatment did not affect the morphologic appearance of NIH3T3 cells (Fig. 1, B).

Effect of Imatinib on Apoptosis in Neuroblastoma Cells

We next investigated one possible mechanism by which imatinib might inhibit the growth of neuroblastomas. Because

Table 1. Expression of c-Kit, platelet-derived growth factor receptor (PDGRF-α, and PDGRF-β) in neuroblastoma cell lines*
we previously showed that Ewing’s sarcoma cells treated with imatinib undergo apoptosis (IC₅₀ = 10–12 μM) (11), we evaluated the effect of imatinib on the induction of apoptosis in neuroblastoma cells. SMS-KCNR cell cultures treated for 48 hours with 15 μM imatinib (the concentration of imatinib that was associated with 80% inhibition of growth of the SMS-KCNR cells) and stained with Hoechst 33342 contained apoptotic cells that displayed nuclear fragmentation and condensation, whereas cells treated with 1 μM imatinib did not (Fig. 2, A). Similar results were found for the six other neuroblastoma cell lines treated with imatinib (data not shown).

Fang et al. (31) reported that imatinib-induced apoptosis in human leukemia cells is mediated by activation of caspases, cysteine proteases that are key mediators of apoptosis. Proteolytic cleavage of caspase-3 leads to its activation and ability to activate other caspases and degrade target proteins such as PARP. We therefore examined the effect of imatinib on caspase activation. SMS-KCNR cells displayed cleaved caspase-3 after 18 hours of treatment with 15 μM imatinib (Fig. 2, B). We also found that SMS-KCNR cells displayed cleavage of PARP, a substrate of caspase-3, after 24 hours of treatment with 15 μM imatinib (Fig. 2, C). These data suggest that imatinib induces apoptosis via activation of the caspase cascade.

**Effect of Imatinib on PDGFR and c-Kit Phosphorylation**

To address the pharmacologic effect of imatinib on PDGFR and c-Kit, we examined PDGFR and c-Kit expression in the seven neuroblastoma cell lines and the effect of imatinib on ligand-induced PDGFR and c-Kit phosphorylation in three representative cell lines. PDGFR-β and c-Kit were expressed in all seven neuroblastoma cell lines, whereas PDGFR-α was expressed only in SH-SY5Y cells (Table 1). In SMS-KCNR, SH-SY5Y, and SK-N-BE2 cells cultured in serum-free media, the basal level of PDGFR-β and c-Kit phosphorylation were, at most, barely detectable (Fig. 3, A). Cells pretreated with...
PDGF-BB (50 ng/mL) displayed an increase in PDGFR-β/phosphorylation, and cells pretreated with SCF (50 ng/mL) displayed an increase in c-Kit phosphorylation. Ligand-induced phosphorylation of PDGFR-β and c-Kit was inhibited in cells pretreated with imatinib (Fig. 3, A). The concentrations of imatinib that caused 50% inhibition of ligand-induced phosphorylation of these receptor tyrosine kinases (pharmacologic IC50) ranged from 0.1 to 0.5 μM (Fig. 3, B), consistent with results of a previous report (32). The pharmacologic IC50 of imatinib for ligand-treated cells cultured in media containing 10% serum was less than 1 μM (data not shown).

We and others have shown that the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways, which are activated by phosphorylation of receptor tyrosine kinases, play key roles in the growth and survival of neuroblastoma cells (34). To assess in more detail the consequences of imatinib inhibition of activation of a tyrosine kinase in a representative neuroblastoma cell line, we evaluated Akt and ERK1/2 phosphorylation. We found that SMS-KCNR cells treated with imatinib had less PDGF-BB–induced Akt and ERK1/2 phosphorylation than SMS-KCNR cells not treated with imatinib (Fig. 3, C). The pharmacologic IC50 of imatinib for inhibition of Akt and ERK1/2 phosphorylation in these cells was less than 1 μM (Fig. 3, C), similar to the pharmacologic IC50 of imatinib for inhibition of PDGFR autophosphorylation in these cells. Imatinib treatment, at similar concentrations, also inhibited SCF-induced Akt and ERK1/2 phosphorylation in SMS-KCNR cells (data not shown).

We next examined the effect of imatinib on the ability of PDGF and SCF to rescue SMS-KCNR and SH-SY5Y cells from serum starvation–induced apoptosis (Fig. 3, D). SMS-KCNR cells (solid bars) and SH-SY5Y cells (open bars) were plated on 60-mm dishes. The medium was changed to 0.1% serum-containing medium for 24 hours, and the cells were pre-incubated for 30 minutes with either 1 μM imatinib (+) or medium (−) followed by treatment for 24 hours in medium containing 0.1% fetal bovine serum either alone or supplemented with SCF (50 ng/mL) or PDGF-BB (50 ng/mL). Cells were stained with propidium iodide and subjected to fluorescence-activated cell sorter analysis to quantify the percentage of apoptotic cells (i.e., those in the sub-G1 fraction). Each experimental point was performed in duplicate, and the experiment was performed twice. Data are presented as the mean value (n = 4), and error bars represent 95% confidence intervals.

The concentrations of imatinib that caused 50% inhibition of ligand-induced phosphorylation of these receptor tyrosine kinases (pharmacologic IC50) ranged from 0.1 to 0.5 μM (Fig. 3, B), consistent with results of a previous report (32). The pharmacologic IC50 of imatinib for inhibition of Akt and ERK1/2 phosphorylation in these cells was less than 1 μM (Fig. 3, C), similar to the pharmacologic IC50 of imatinib for inhibition of PDGFR autophosphorylation in these cells. Imatinib treatment, at similar concentrations, also inhibited SCF-induced Akt and ERK1/2 phosphorylation in SMS-KCNR cells (data not shown).

We next examined the effect of imatinib on the ability of PDGF and SCF to rescue SMS-KCNR and SH-SY5Y cells from serum starvation–induced apoptosis (Fig. 3, D). SMS-KCNR cells cultured in serum-free medium had a statistically significantly higher percentage of apoptotic cells than SMS-KCNR cells cultured in medium containing 10% serum (50.2% versus 10.3%; difference = 39.9%, 95% CI = 33.9% to 46.0%; P<.001). Similarly, SH-SY5Y cells cultured in serum-free me-
media had a statistically significantly higher percentage of apoptotic cells than SH-SY5Y cells cultured in media containing 10% serum (45.6% versus 6.6%; difference = 39.0%, 95% CI = 32.7% to 45.3%; P < .001). SMS-KCNR cells cultured in the presence of SCF, PDGF-BB, or SCF and PDGF-BB combined had statistically significantly lower percentages of apoptotic cells than SMS-KCNR cells cultured in serum-free media (SCF versus serum-free media: 36.9% versus 50.2%; difference = 13.3%, 95% CI = 7.3% to 19.4%; P < .001; PDGF-BB versus serum-free media: 32.5% versus 50.2%; difference = 17.7%, 95% CI = 11.7% to 23.8%; P < .001; SCF and PDGF-BB versus serum-free media: 25.9% versus 50.2%; difference = 24.3%, 95% CI = 18.3% to 30.4%; P < .001). Similarly, SH-SY5Y cells cultured in the presence of SCF, PDGF-BB, or SCF and PDGF-BB combined had statistically significantly lower percentages of apoptotic cells than SH-SY5Y cells cultured in serum-free media (SCF versus serum-free media: 34.5% versus 45.6%; difference = 11.1%, 95% CI = 4.7% to 17.4%; P < .001; PDGF-BB versus serum-free media: 27.5% versus 45.6%; difference = 18.1%, 95% CI = 11.8% to 24.4%; P < .001; SCF and PDGF-BB versus serum-free media: 19.2% versus 45.6%; difference = 26.4%, 95% CI = 20.1% to 32.7%; P < .001). However, SMS-KCNR cells cultured in the presence of SCF, PDGF-BB, and 15 μM imatinib had a statistically significantly higher percentage of apoptotic cells than SMS-KCNR cells cultured in the presence of SCF and PDGF-BB (48.2% versus 25.9%; difference = 22.3%, 95% CI = 13.7% to 31%; P = .004) but did not have a higher percentage than SMS-KCNR cells cultured in serum-free media (48.2% versus 50.2%; difference = 2.0%, 95% CI = 1.3% to 2.7%; P = .39). Similarly, SH-SY5Y cells cultured in the presence of SCF, PDGF-BB, and 15 μM imatinib had a statistically significantly higher percentage of apoptotic cells compared with SH-SY5Y cells cultured in the presence of SCF and PDGF-BB (40.9% versus 19.2%; difference = 21.7%, 95% CI = 12.1% to 31.4%; P = .006) but did have a statistically significantly lower percentage of apoptotic cells than SH-SY5Y cells cultured in serum-free media (40.9% versus 46.5%; difference = 5.6%, 95% CI = 2.1% to 7.2%; P = .01) (Fig. 3, D). These results suggest that the signaling initiated by either PDGFR or c-Kit contributes to the survival of neuroblastoma cells and that imatinib blocks SCF- or PDGF-BB–induced survival of neuroblastoma cells.

**Effect of Imatinib on VEGF Expression in Neuroblastoma Cells**

Despite the ability of PDGFR and c-Kit to promote the survival of some neuroblastoma cells (Fig. 3, D), there was a difference between the biologic IC_{50} (9–13 μM) of imatinib and the pharmacologic IC_{50} (0.1–0.5 μM) of imatinib. The difference between the biologic IC_{50} (9–13 μM) and the pharmacologic IC_{50} (0.1–0.5 μM) of imatinib suggests the presence of additional mechanism(s) by which imatinib treatment might affect the growth and survival of neuroblastoma cells. Results of a recent study (35) suggest that imatinib inhibits VEGF-stimulated phosphorylation of Flnk-1 at an IC_{50} of 11 μM. In addition, VEGF and Flk-1 are co-expressed in many neuroblastoma cell lines and tumors (17), suggesting the possible presence of a VEGF/Flk-1 autocrine growth loop. For these reasons, we evaluated the effect of imatinib on Flk-1 phosphorylation and VEGF expression in several neuroblastoma cell lines that had readily detectable levels of Flk-1 expression. VEGF treatment of SMS-KCNR cells cultured in serum-free media induced autophosphorylation of Flk-1; however, imatinib did not inhibit VEGF-induced Flk-1 autophosphorylation (Fig. 4, A). We next assessed the effect of imatinib on VEGF mRNA and protein expression in three neuroblastoma cell lines. SMS-KCNR, SH-

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**Fig. 4. Effect of imatinib on vascular endothelial growth factor (VEGF) expression in neuroblastoma cells.** A) SMS-KCNR cells were pre-incubated for 30 minutes with 1 or 15 μM imatinib or medium (–), then incubated for 5 minutes in the presence (+) or absence (–) of 50 ng/mL VEGF. Protein lysates were prepared, and 1 μg of each lysate was immunoprecipitated (IP) with an anti-Flk-1 antibody and immunoblotted (IB) with an anti-phospho-tyrosine antibody (pTy) or an anti-Flk-1 antibody. B) SMS-KCNR, SH-SY5Y, and NGP cells were plated on 100-mm dishes in medium containing 10% fetal calf serum and treated with 15 μM imatinib for the indicated times. Total RNA was isolated, and 25 μg of each RNA sample was subjected to northern blot analysis to detect VEGF mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. C) SMS-KCNR cells (2 × 10^6) were plated on 100-mm dishes and incubated for 24 hours with the indicated concentrations of imatinib. Protein lysates were prepared, and 40 μg of each lysate was electrophoresed under nonreducing conditions and immunoblotted with an anti-VEGF antibody and an anti-actin antibody. D) Neuroblastoma cells (2 × 10^6) were plated on 12-well plates, treated for 24 hours with 15 μM imatinib (solid bars) or medium (shaded bars) in 10% serum-containing medium. The growth media were then collected from each well, and the concentration of VEGF in each medium was measured by an enzyme-linked immunosorbent assay. The number of viable cells in each well was determined by using the trypan blue dye exclusion assay.
SY5Y, and NGP cells treated with 15 μM imatinib had lower levels of VEGF mRNA and protein (Fig. 4, B and C, respectively) than untreated cells. The inhibitory effect of imatinib on VEGF protein expression in SMS-KCNR cells was concentration-dependent (Fig. 4, C), and the concentration of imatinib that was associated with a 50% reduction in VEGF was similar to the biologic IC₅₀ for imatinib in those cells. In addition, culture media collected from each of the seven neuroblastoma cell lines treated with 15 μM imatinib had lower concentrations of VEGF than culture media collected from the corresponding untreated cells (Fig. 4, D). Because VEGF has been shown to prevent neural cells from undergoing apoptosis (36), we examined whether VEGF promotes the growth and survival of neuroblastoma cells. SMS-KCNR and SH-SY5Y cells treated with VEGF still underwent serum starvation–induced apoptosis (data not shown). In addition, incubation of SMS-KCNR or SH-SY5Y cells with an anti-VEGF blocking antibody that would neutralize endogenous VEGF produced by cells did not inhibit cell growth (data not shown). These results suggest that it is unlikely that VEGF contributes to in vitro growth and survival of neuroblastoma cells.

**Effect of Imatinib on Neuroblastoma Tumor Growth In Vivo**

We used SMS-KCNR cells in a mouse gastrocnemius xenograft model to examine the in vivo therapeutic efficacy of imatinib because, in an initial study using this model (29), we found that 100% of mice injected with SMS-KCNR cells developed tumors. SMS-KCNR cells (2 × 10⁶) were injected into the gastrocnemius muscles of SCID mice. One week after injection, mice (10 per group) were randomly assigned to receive imatinib at 50 mg/kg, imatinib at 100 mg/kg, or vehicle alone (control), administered orally every 12 hours for 14 days. We observed no limiting toxicities during the imatinib treatment and detected no gross metastases in the livers, lungs, spleens, or kidneys of any mice (data not shown). Tumor volumes were measured at 14 days after initiation of injections. Mice treated with imatinib at 100 mg/kg developed smaller tumors than control mice (Fig. 5, A). The mean volumes of tumors in mice treated with vehicle alone, imatinib at 50 mg/kg, or imatinib at 100 mg/kg were 2954 mm³ (95% CI = 2527 to 3381 mm³), 1546 mm³ (95% CI = 1119 to 1973 mm³), and 463 mm³ (95% CI = 35 to 890 mm³), respectively. Mice treated with imatinib at either 50 mg/kg or 100 mg/kg had statistically significantly smaller tumors than control mice (difference in mean tumor volume between mice treated with imatinib at 50 mg/kg and control mice = 1408 mm³, 95% CI = 657 to 2159 mm³; *P* < .001; difference in mean tumor volume between mice treated with imatinib at 100 mg/kg and control mice = 2491 mm³, 95% CI = 1740 to 3242 mm³; *P* < .001) (Fig. 5, B). Mice treated with imatinib at 100 mg/kg had statistically significantly smaller tumors than mice treated with imatinib at 50 mg/kg (difference in mean tumor volume = 1083 mm³, 95% CI = 332 to 1834 mm³; *P* = .001; Fig. 5, B). No tumors developed in three of the 10 mice treated with the 100-mg/kg dose of imatinib.

We also examined the effect of imatinib on VEGF expression in neuroblastoma xenograft tumors by Western blot analysis of protein extracts prepared from excised tumors. The relative level of VEGF protein expressed in the tumor tissue was normalized to the relative level of actin expression in the samples, and the mean value for the ratios of four representative tumor samples from the control group or the group treated with imatinib at 100 mg/kg is expressed in relative densitometric units (RDU). Error bars correspond to 95% confidence intervals. *P* = .028, Student’s unpaired t test.
to the relative level of actin expressed in the same sample, and the mean values for the ratios of four representative tumor samples from the control group and the group treated with imatinib at 100 mg/kg were expressed in relative densitometric units (RDU) and graphed in the lower panel of Fig. 5, C. The relative levels of VEGF to actin in tumors from mice treated with imatinib at 100 mg/kg were statistically significantly lower than the relative levels of VEGF to actin in tumors from control mice (mean ratio of VEGF to actin for imatinib-treated mice = 2560 RDU; mean ratio of VEGF to actin for control mice = 8673 RDU; difference = 6113 RDU, 95% CI = 193 to 12 419 RDU; P = .028; Fig. 5, C).

**DISCUSSION**

Results of recent clinical studies (7) have suggested the promising therapeutic impact of imatinib in the treatment of CML and GIST. In the present study, we found that imatinib treatment was associated with the inhibition of *in vitro* growth of all neuroblastoma cell lines tested and of *in vivo* neuroblastoma tumor growth. The biologic IC$_{50}$ for imatinib in neuroblastoma cells was higher than that for imatinib in both the CML cell line used in this study and in other CML cell lines (36). The biologic IC$_{50}$s for imatinib in neuroblastoma cell lines are two- to three-fold higher than the serum imatinib concentration achieved in early clinical trials (4.6 μM) (6). However, it is not clear whether the maximally tolerated doses of imatinib were achieved in those trials. In addition to the known effects of imatinib on c-Abl, c-Kit, and PDGFR kinase activities (36), we propose that the inhibitory effect of imatinib on the *in vivo* growth of neuroblastoma cells may also be through the decreased expression of neuroblastoma-derived VEGF. We found that imatinib was cytotoxic in all seven neuroblastoma cell lines tested and that the cytotoxic effect was associated with the induction of apoptosis, mediated by PARP cleavage, which was evident by 24 hours after exposure to imatinib. All neuroblastoma cell lines tested expressed both PDGFR and c-Kit, and both receptors were functional because pretreatment with their respective ligands, PDGF and SCF, rescued neuroblastoma cells from serum starvation–induced apoptosis. Our finding that imatinib treatment was associated with the inhibition of PDGFR and c-Kit phosphorylation in these neuroblastoma cells at a pharmacologic IC$_{50}$ of less than 0.5 μM is consistent with results from previous studies (32). However, although the pharmacologic IC$_{50}$s of imatinib for PDGFR and c-Kit phosphorylation were in a similar range (<0.5 μM), approximately 20-fold higher concentrations of imatinib (9–13 μM) were required to induce apoptosis in neuroblastoma cells *in vitro*. This finding is consistent with our previously reported observation that the biologic IC$_{50}$ of imatinib in Ewing’s sarcoma cells (10–12 μM) (11) is much higher than the biologic IC$_{50}$s (1 μM) reported for CML and GIST cells (5).

The reason for the discrepancy between the pharmacologic IC$_{50}$ for imatinib and the biologic IC$_{50}$ for imatinib is unclear. It seems likely that an additional target or targets for imatinib-mediated inhibition of *in vitro* growth are expressed in neuroblastoma cells. There is precedent in the literature for such additional targets. Results of previous studies (10) suggest that imatinib inhibits epithelial growth factor–dependent growth of mouse epithelial BALB/MK cells and insulin-like growth factor–I–dependent growth of human small-cell lung cancer cells at concentrations similar to the biologic IC$_{50}$ for neuroblastoma cells, even though imatinib does not directly inhibit the insulin-like growth factor or epithelial growth factor receptors (4). Taken together, these observations suggest that imatinib might have other effects on growth factor–induced intracellular signaling pathways.

To examine the possible mechanism by which neuroblastoma cells are susceptible to imatinib-mediated apoptosis at 15 μM, we assessed phosphorylation of the downstream mediators Akt and ERK1/2, which play key roles in the growth and survival signaling pathway in neuroblastoma cells (34). We found that both PDGF and SCF were individually able to induce phosphorylation of Akt and ERK1/2 in neuroblastoma cells cultured in serum-starved conditions and that treatment with 1 μM imatinib inhibited this phosphorylation. However, imatinib treatment did not affect the basal levels of Akt and ERK1/2 phosphorylation in neuroblastoma cells cultured in media containing 10% serum. Therefore, in 10% serum-containing media, other growth factors may also activate the PI3K/Akt and MAPK pathways and, thus, contribute to the growth and survival of neuroblastoma cells. By contrast with the gain-of-function mutations in c-Abl and c-Kit, other serum factors may aid in the support of the unregulated growth and survival of neuroblastoma cells.

We found that treatment with imatinib was associated with the suppression of growth of the neuroblastoma xenograft tumors in a mouse model. Although we did not measure plasma levels of imatinib in the treated mice, a dosing regimen similar to ours resulted in an average plasma level of 11.8 μM imatinib (37), which is similar to the biologic IC$_{50}$ that we obtained for neuroblastoma cell lines *in vitro*. Our *in vitro* data showing that imatinib inhibits VEGF production in neuroblastoma cells suggest that imatinib may have anti-angiogenic activity *in vivo* in addition to its cytotoxic effects. VEGF is secreted by neuroblastoma cells (16–18) and, as such, is thought to play a role in the angiogenic environment of the growing neuroblastoma tumor. Results of *in vitro* studies have shown that the neuroblastoma cell–conditioned media-stimulated growth of endothelial cells can be inhibited by treatment with a monoclonal antibody to VEGF (20). In addition, results of recent animal studies (22) showed that blockade of VEGF function by an anti-VEGF monoclonal antibody or expression of an Flk-1 gene lacking the kinase domain, which interferes with Flk-1 signaling, was associated with the inhibition of angiogenesis and tumor growth in a neuroblastoma xenograft model, suggesting the important role of VEGF in development of neuroblastoma. Our *in vitro* data revealed that treatment with imatinib was associated with the inhibition of VEGF expression and secretion in neuroblastoma cells. Our *in vivo* data suggest that imatinib also inhibits VEGF expression in neuroblastoma xenografts. The decrease in VEGF expression was associated with suppression of *in vivo* tumor growth. These results suggest that imatinib may have anti-angiogenic potential via its effects on the expression of neuroblastoma cell–derived VEGF, which is known to stimulate proliferation of endothelial cells (21).

The potential anti-angiogenic role of imatinib is not likely to be limited to its effects on VEGF expression in tumor cells because imatinib may also inhibit the vascular formation activities of c-Kit and PDGFR (35) that are expressed on vascular endothelial cells. Results of a recent *in vivo* study showed that imatinib, especially in combination with paclitaxel, inhibits the growth of prostate cancer bone metastases and tumor angiogen-
esis by blocking PDGFR (38). Another study (39) reported that SCF secreted by colon tumor cells induces in vitro and in vivo angiogenesis. Therefore, it is possible that in a tumor environment imatinib could inhibit angiogenesis by inhibiting tumor cell-secreted PDGF- or SCF-stimulated phosphorylation of PDGFR and c-Kit on endothelial cells.

VEGF as a potential target for imatinib-associated apoptosis of neuroblastoma cells is intriguing for several reasons. First, PDGFR and c-Kit on endothelial cells. Cell ment imatinib could inhibit angiogenesis by inhibiting tumor angiogenesis. Therefore, it is possible that in a tumor environment decrease in VEGF expression may be associated with the inhibition of in vitro growth of neuroblastoma cells. Thus, although a not substantially promote the proliferation or survival of serum-starved neuroblastoma cells, we hypothesized that VEGF inhibition might be involved in imatinib inhibition of in vitro growth of neuroblastoma cells.

In summary, our results suggest that the mechanism by which imatinib suppresses the growth of neuroblastoma may include the inhibition of VEGF expression in addition to the inhibition of PDGFR and c-Kit phosphorylation. Our data showing that imatinib inhibited the in vivo growth of neuroblastoma cells suggest that imatinib should be considered for the treatment of neuroblastoma. Evidence for the potential of imatinib to decrease expression of VEGF in neuroblastoma cells also suggests that it may act against a broader range of tumors than initially predicted.

REFERENCES


54 ARTICLES


NOTES

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