An *In vivo* Tumor Model Exploiting Metabolic Response as a Biomarker for Targeted Drug Development

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In *in vivo* models that recapitulate oncogene-dependent tumor genesis will greatly facilitate development of molecularly targeted anticancer therapies. We have developed a model based on activating mutations in c-KIT in gastrointestinal stromal tumors (GISTs). This model comprises murine tumors of FDC-P1 cell lines expressing c-KIT mutations that render the tumors either responsive (V560G) or resistant (D816V) to the small-molecule c-KIT inhibitor, imatinib. Clinically, GIST response to imatinib is associated with rapid reduction in fluorodeoxyglucose (FDG) uptake on positron emission tomography (PET), preceding changes in conventional response criteria by several weeks. Using the FDC-P1 model in small animal PET, FDG uptake into tumors expressing the c-KIT V560G mutation was significantly reduced as early as 4 hours after imatinib treatment. In contrast, no change in FDG uptake was observed in resistant c-KIT D816V-expressing tumors after 48 hours of imatinib treatment. Consistent with the PET results, expression of the glucose transporter, GLUT1, was significantly reduced in V560G tumors at 4 hours, preceding changes in markers of proliferation by several hours. *In vitro*, imatinib treatment of V560G cells resulted in a reduction of glucose transporter numbers at the cell surface and decreased glucose uptake well before changes in cell viability. Notably, decreased ambient glucose concentrations enhanced the cytotoxic effect of imatinib. Taken together, these data account for the rapidity and significance of the PET response to imatinib and suggest that metabolic effects may contribute to imatinib cytotoxicity. Further, the FDC-P1 model represents a very useful paradigm for molecularly targeted drug development. (Cancer Res 2005; 65(21): 9633-6)

Introduction

Gastrointestinal stromal tumors (GISTs) develop from interstitial cells of Cajal in the gastrointestinal tract and typically express activating mutations in the c-KIT oncogene leading to tumor cell growth, proliferation, and survival (1). Although GISTs are refractory to standard chemotherapy and are often associated with poor prognosis, recent studies have shown striking results following treatment of GIST with imatinib, a small-molecule inhibitor of the c-KIT kinase (2).

Although tumor response to treatment is generally determined using conventional anatomic imaging techniques, such as computed tomography (CT) and magnetic resonance imaging, volume changes in GIST in response to imatinib treatment occur late and are often only detected several months following commencement of treatment. Therefore, the utility of such techniques in assessing response to molecularly targeted agents, such as imatinib, is increasingly in question. Several recent studies have suggested metabolic imaging using fluorodeoxyglucose-based positron emission tomography (FDG-PET) as an early predictor of GIST response to imatinib treatment (3). Remarkably, FDG-PET uptake into GIST is significantly reduced within 48 hours of commencement of imatinib treatment. However, an important problem that remains is the emergence of imatinib resistance, typically associated with new mutations in c-KIT and the reestablishment of FDG uptake (4, 5). Addressing this issue presents a major challenge to new drug development. Here, we describe a novel *in vivo* tumor model that exploits the relationship between drug efficacy and metabolic response on PET that is also very likely to have general utility for development of molecularly targeted cancer therapeutics.

Materials and Methods

**Materials.** Imatinib mesylate was kindly supplied by Novartis (Basel, Switzerland). FDG was purchased from Cyclotek (Melbourne, Victoria, Australia).

**Cells.** FDC-P1 cells expressing c-KIT mutations V560G and D816V were as described previously (6).

**Glucose uptake studies.** 2-Deoxyglucose (2DOG) uptake was measured by the zero-trans method (6). Transporter affinity and number at the cell surface were analyzed using a Lineweaver-Burk plot as described previously (7).

**Glucose deprivation studies.** Cells were washed thrice in glucose-free RPMI 1640 and cultured in glucose-free RPMI 1640 supplemented with 2.5% fetal bovine serum ± 11 mmol/L glucose and 5 µmol/L imatinib for 8 hours. Cell cycle analysis was then done as described below.

**Cellular DNA content analysis.** Cells (1 × 10⁶) were washed, fixed with 70% ice-cold ethanol, and stored overnight at 4°C. Cells were pelleted and suspended in 1 mL propidium iodide (50 µg/mL) and RNase A (100 Kunitz units/mL) in PBS containing 1 mg/mL glucose and left at room temperature for 30 minutes. Samples were analyzed in a Becton Dickinson (San Jose, CA) FACScan flow cytometer, and cell cycle analysis was carried out using the ModFit LT version 1.0 cell cycle analysis software (Verity Software House, Topsham, ME).

**Animal tumor growth.** Female DBA/2 mice (Animal Resources Centre, Canning Vale, Western Australia, Australia) were inoculated s.c. on both flanks with 5 × 10⁶ FDC-P1 cells expressing c-KIT mutations V560G or D816V cells in 1:1 PBS/Matrigel (Becton Dickinson). Once the tumors had reached a volume of ~200 mm³, the mice were randomized into two groups of seven to eight animals (day 0). Imatinib was given p.o. at 100 mg/kg in H₂O b.i.d. beginning day 1.
Fluorodeoxyglucose-based positron emission tomography imaging. The mice were fasted for 3 hours, anesthetized (2.5% isoflurane in 1:1 O2 and air delivered at a flow rate of 200 mL/min), and then injected via the tail vein with 300 μCi FDG. Anesthesia was maintained for a further 20 minutes before the animals were allowed recover. Then, 1.5 hours after tracer injection, the mice were again anesthetized and scanned for 5 minutes on a Phillips (Cleveland, OH) A-PET prototype small animal PET scanner. PET images were reconstructed using the three-dimensional RAMLA algorithm (8, 9) and displayed using standard image software available on the scanner workstation. Tracer uptake was measured using the region-of-interest (ROI) software. Briefly, ROIs were manually placed around the tumor and a background region on transaxial slice images. The background ROI was chosen to represent FDG present within the blood pool and mediastinal nontumor tissue, excluding regions of increased uptake, such as heart or brown fat. Tumor-to-background FDG uptake ratios were calculated by dividing the maximum pixel intensity within a tumor ROI by the average pixel intensity within the background ROI. Mice were scanned on days 0, 1 (V560G only), and 2. Tumors in responding mice were too small to generate quantifiable images after day 2.

Immunohistochemistry. Bromodeoxyuridine (BrdUrd; 100 mg/kg i.p.) was given 90 minutes before sacrifice. The tumors were then excised, fixed in formalin, and analyzed for BrdUrd (Becton Dickinson; clone B44) and GLUT1 (DAKO, Glostrup, Denmark) by immunohistochemistry. Bound antibody was detected using the polymer-linked detection system (EnVision+, DAKO) with DAB+ visualization. For each tumor section, six high-power (>400) fields were photographed and the cells were scored as either positively stained (irrespective of staining intensity) or unstained. A total of 18 high-power images (six fields per section, three tumors per group) were scored for each group per time point.

Results and Discussion

Rapid reduction of uptake of fluorodeoxyglucose in a human gastrointestinal stromal tumor treated with imatinib. An increasing body of clinical experience supports an important role for FDG-PET in the early assessment of response of GIST to imatinib that predicts subsequent response on standard CT criteria. Consistent with these data, we have observed dramatic changes in FDG-PET responses after imatinib treatment of GIST patients. As seen in Fig. 1A, a striking reduction in tumoral uptake of FDG was observed 24 hours following imatinib treatment compared with baseline. This correlated with rapid symptomatic improvement and with subsequent partial response on CT. To investigate the mechanism underlying this dramatic metabolic response to imatinib, we sought to develop and characterize an in vivo tumor model in which this effect was recapitulated.

Imatinib-induced reduction in uptake of fluorodeoxyglucose in vivo precedes cell cycle arrest. The model uses FDC-P1 murine hemopoietic cell lines expressing either the imatinib-sensitive c-KIT V560G intracellular juxtamembrane domain mutation or the imatinib-resistant c-KIT D816V kinase domain mutation (10). c-KIT juxtamembrane mutations, such as V560G, are commonly observed in GISTs, whereas the D816V mutation is often observed in mastocytosis (5).

S.c. implantation of these cell lines into syngeneic DBA/2J mice resulted in a 100% take rate with rapid growth as discrete tumor masses. Using a dedicated small animal PET scanner, we then characterized changes in FDG uptake in response to imatinib treatment in both V560G- and D816V-expressing tumors. D816V tumor growth was unaffected by imatinib treatment. Metabolic responses to imatinib were characterized by FDG-PET. Both the V560G- and D816V-expressing tumors were FDG avid at baseline, consistent with GIST in the clinical setting (Fig. 1B). Imaging of V560G tumor-bearing mice following imatinib treatment revealed a substantial reduction in FDG uptake compared with untreated mice, detectable as early as 4 hours after imatinib dosing (Table 1; 71% of control; P = 0.002) and more marked at 24 hours (44% of control; P < 0.001). FDG uptake into D816V tumors was not reduced following imatinib treatment.

To probe the mechanism by which imatinib treatment affects FDG uptake, we examined the effect of imatinib on tumor expression of the ubiquitous glucose transporter, GLUT1, as well as uptake of BrdUrd, a marker of cellular proliferation in tumors. Whereas both V560G and D816V tumors exhibit high baseline expression of GLUT1 (Fig. 1C), total and membrane-associated GLUT1 staining were rapidly and significantly reduced in V560G tumors at both 4 hours (92% of untreated; P = 0.03) and 24 hours (53% of untreated; P < 0.01) after imatinib dosing (Table 2). This was confirmed by real-time PCR analysis of GLUT1 transcript levels, which were reduced by 63% at 4 hours compared with baseline (data not shown). In contrast, significant changes in BrdUrd incorporation were not detected in
these tumors until 24 hours of treatment (Table 2). Together, these data suggest that changes in glycolytic metabolism preceded effects on proliferation during the tumor response to imatinib.

Imatinib-induced reduction in uptake of fluorodeoxyglucose in vitro precedes cell cycle arrest and apoptosis. To examine in greater detail the effect of imatinib on in the FDC-P1 tumor model, we used biochemical assays of metabolism and cytotoxicity. As seen in vivo, exposure of V560G cells in vitro to imatinib resulted in extremely rapid inhibition of 2DOG uptake, with a 50% reduction observed at 2 hours (Fig. 2A), whereas changes in cell viability or cell cycle status were not observed until 8 hours of drug exposure (Fig. 2B). No effect of imatinib on glucose uptake or cell viability was observed in the D816V cells (Fig. 2A and B). In addition to GLUT1, a large number of cell membrane transporter isofoms have been identified (11) and several of these are efficient transporters for glucose. To determine the effect of imatinib on overall transporter levels and activity, a kinetic analysis of 2DOG was done in V560G cells (Fig. 2C). Not only were transporter numbers (V\textsubscript{max}) reduced in cells treated with imatinib (control 36.4 ± 1.8 nmol/10\textsuperscript{6} cells/min and imatinib 28.3 ± 3.1 nmol/10\textsuperscript{6} cells/min), affinity for glucose (K\textsubscript{m}) was also affected (control 1.2 ± 0.1 mmol/L and imatinib 1.7 ± 0.2 mmol/L). These findings indicate that decreased glucose uptake in the imatinib-treated V560G cells is due to reduction in both number and activity of glucose transporters at the cell surface. We further investigated directly the effect of glucose levels on imatinib-induced apoptosis (Fig. 2D), revealing that V560G cells cultured in low-glucose conditions (0.25 mmol/L) are twice as susceptible to the apoptotic effects of imatinib as cells cultured in high-glucose conditions (56% versus 23% cell death at 8 hours). No apoptosis was seen in cells cultured in low-glucose conditions in the absence of imatinib. These data show that limiting ambient glucose availability increases the cytotoxic effects of imatinib in V560G tumor cells, suggesting that metabolic changes may contribute directly to the cytotoxicity of imatinib in GIST.

Warburg (12) first hypothesized that glucose metabolism may play a key role in carcinogenesis. This was based on observations that cancer cells manifested impaired aerobic metabolism of glucose (oxidative phosphorylation). Loss of oxidative phosphorylation, a mitochondrial process, greatly reduces the number of ATP generated from each moleule of glucose, necessitating increased consumption of glucose. The recent realization of the importance of mitochondrial function in mediating apoptosis (13) has led to the reassessment of a causal relationship among impaired mitochondrial function, impaired oxidative phosphorylation, and apoptosis (see ref. 14). Tumor cells carrying KIT mutations, as in GIST, have constitutively activated pathways that lead to rapid uptake of glucose (as evidenced by high FDG uptake in PET) and increased cell survival. Reducing glucose concentrations in our experiments most likely reduces ATP stores to barely sustaining levels, and by inhibiting c-KIT, imatinib blocks the major pathway required to produce energy to survive. Consistent with this, the oncogene AKT (a target of c-KIT signaling) regulates both metabolic function and cell survival in cancer (15), whereas enforced expression of the glucose transporter GLUT1 and the metabolic enzyme hexokinase can prevent apoptosis induced by growth factor withdrawal (16). Our data showing down-regulation of glucose transport preceding apoptosis in the V560G GIST model are consistent with the key role for glucose metabolism in survival of c-KIT mutant GIST.

More practically, our data specifically account for the rapidity of PET responses in treatment of GIST. PET is likely to be increasingly important for assessment of cancer therapeutics, given the lack of sensitivity of standard RECIST criteria in evaluating clinically meaningful treatment outcome in molecularly targeted therapeutics. This has implications for early-phase clinical trial design, for which a rapid, sensitive measure of response may accelerate the identification of effective novel therapies and thereby contribute to reduction in time and cost of anticancer drug development. The challenge is how to therapeutically exploit tumor dependence on metabolic processes. Cancer microenvironments often exist at the margins of nutrient availability, a fact recently used with clinical benefit in a trial of the hypoxic radiosensitizer, tirapazamine (17). Furthermore, several novel therapeutic strategies targeting glucose avidity have recently been developed (18, 19), and the clinical utility of these measures will become clear with time.

Together with the role of glucose metabolism in the GIST response to imatinib, we describe the generation of a readily manipulated, in vivo model to rapidly monitor efficacy of agents targeting specific gene mutations in cancer cells. As resistant mutations emerge in response to molecularly targeted therapeutics, the ability to rapidly screen novel agents in vivo with models in which clinically relevant mutations can be readily generated will be vital. Our model incorporates rapid assessment of biological contexts relevant to cancer therapeutics, including pharmacokinetic and pharmacodynamic variables, as well as

### Table 1. FDG uptake ratio in FDC-P1 tumors expressing V560G or D816V c-KIT mutations

<table>
<thead>
<tr>
<th>c-KIT mutation</th>
<th>Time (h)</th>
<th>Control</th>
<th>Imatinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>V560G</td>
<td>4</td>
<td>4.2 ± 0.3*</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.4 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>D816V</td>
<td>24</td>
<td>6.3 ± 0.6</td>
<td>8.3 ± 0.6</td>
</tr>
</tbody>
</table>

\*Mean ± SE FDG uptake (n = 6). FDG uptake was measured at different times in the same animals using ROI software on the PET. Tumor-to-background FDG uptake ratios were calculated by dividing the maximum pixel intensity within a tumor ROI by the average pixel intensity within a background ROI. Note that a value of 1 represents complete abolition of tumor-related signal (i.e., tumor = background).

### Table 2. Quantitative immunohistochemical analyzes in V560G tumors

<table>
<thead>
<tr>
<th></th>
<th>4 h</th>
<th>24 h</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Imatinib</td>
</tr>
<tr>
<td>GLUT1</td>
<td>84 ± 2\†</td>
<td>77 ± 3.0</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>26 ± 2.3</td>
<td>26 ± 4.1</td>
</tr>
</tbody>
</table>

\*Student’s t test.

\† Mean ± SD percentage positive staining cells determined from ~2,000 cells per slide scored at each time and treatment point (n = 3).

\‡ Not significant.
yielding information about biological mechanism and tumour-host interactions. We believe that this model will prove to be a useful tool for future studies in GIST as well as other cancers for which oncogene mutations play significant roles.

Acknowledgments

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References

10. Frost MJ, Ferrao PT, Hughes TP, Ashman LK. Juxtamembrane mutant V560GKit is more sensitive to imatinib (STI571) compared with wild-type c-kit where- as the kinase domain mutant D816VKit is resistant. Mol Cancer Ther 2002;1:1115–24.
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