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Imatinib Mesylate Inhibits Glucose Uptake in Gastrointestinal Stromal Tumor Cells by Downregulation of the Glucose Transporters Recruitment to the Plasma Membrane

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Abstract: Imatinib mesylate, the inhibitor of the KIT protein tyrosine kinase that is constitutively activated in Gastrointestinal Stromal Tumors (GISTs), has been established as the first highly effective drug in the treatment of patients with advanced GISTs. Recent studies suggest that changes in the glucose metabolism could be an additional mechanism of the anti-proliferative action of imatinib. The aim of this study was to investigate the effect on glucose flux and metabolism in a human GIST882 cell line after exposure to imatinib. Imatinib induced a concentration-dependent inhibition of cell proliferation in GIST882 cells (IC_{50}, 0.030 ± 0.006 μM). By ^{18}F-FDG uptake measurements, after 24 h exposure to the drug at concentrations of 0.03 μM and 0.3 μM, the glucose uptake decreased by ~25% and ~95%, respectively. Moreover, after a 3-h treatment at the concentration of 0.3 μM of imatinib the decrease in glucose-uptake was already more than 50%. After 24-h of treatment with 0.3 μM imatinib, the measurements of the hexokinase and glucose-6-phosphate dehydrogenase activity revealed a 30% and 37% decrease, respectively. Western blotting disclosed mainly expression of glucose transporter GLUT-2 in GIST cells. Exposure of GIST cells to imatinib resulted in the decline of the GLUT-2 receptor recruitment to cell membrane, which paralleled with the elevated amount of the total KIT protein. These findings suggest that a rapid decline in glucose uptake following imatinib treatment in GIST cells is dependent on glucose transporter impaired anchorage to the plasma membrane, with the subsequent recruitment of KIT protein.

Key words: Imatinib mesylate, GIST, KIT, GLUT-2, glucose uptake

INTRODUCTION

The antineoplastic drug imatinib mesylate, a derivative of 2-phenylaminopyrimidine, also known as Glivec®, Gleevec® or STI-571, was initially developed as a competitive inhibitor of BCR-ABL, a fusion protein and constitutively active protein tyrosine kinase that was identified as the primary cause of Philadelphia chromosome positive Chronic Myelogenous Leukemia (CML)[1]. However additional investigations have shown that imatinib also inhibits other protein tyrosine kinases such as the Platelet-derived Growth Factor Receptors (PDGFRs) and KIT (stem cell factor receptor), which also play a key role in tumor development[2].

More recently, multicenter clinical trials have proved that imatinib is highly effective for the treatment of inoperable and/or metastatic Gastrointestinal Stromal Tumors (GISTs)[3-5]. GISTs are the most common mesenchymal tumors in the gastrointestinal tract. These tumors are characterized by the ubiquitous expression of the protein tyrosine kinase KIT, the receptor for Stem Cell Factor (SCF)[6-8]. KIT receptor is known to be essential for the development and function of several cell types including the interstitial cells of Cajal that regulate the gut peristalsis and most probably are the cells from which GISTs originate[9]. The majority of GISTs harbor KIT somatic gain-of function mutations, most commonly in the highly conserved juxtamembrane region encoded by exon 11. These mutations are associated with a constitutive activation of the receptor, which promotes proliferation and anti-apoptotic signaling in the absence of the ligand[10-13].

The molecular determinants of GISTs response to imatinib treatment are of current interest. Glucose is the primary source of carbon for de novo synthesis of nucleic acids, lipids and amino acids and is the major

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source for energy production. Recent studies suggested that changes in the glucose flux and metabolism could account for the anti-proliferative action of imatinib in BCR-ABL positive cells. Thus, exposure of K562 leukemia cells to imatinib resulted in a decreased activity of two key enzymes of glucose metabolism, namely hexokinase and glucose-6-phosphate dehydrogenase, as well as a decreased glucose uptake[14, 15].

To our knowledge metabolic changes in glucose metabolism and expression of glucose transporters in GISTs have not been investigated so far. The aim of this study was to investigate the effect and underlying mechanisms of glucose uptake in GIST cells treated with imatinib.

MATERIALS AND METHODS

Chemicals and reagents: Imatinib mesylate was a kind gift of Novartis Pharmaceutical (Basel, Switzerland). DMEM/F12 with L-glutamine and RPMI 1640 cell culture media, Foetal Bovine Serum (FBS), penicillin (5000 U mL^{-1}) /streptomycin (5000 µg mL^{-1}) solution and trypsin (0.25%) were purchased from Invitrogen (Merelbeke, Belgium). Rabbit antibodies to human GLUT-1 (AB1341), GLUT-2 (AB1342), GLUT-3 (AB1345) and GLUT-4 (AB1346) were purchased from Chemicon International (Ternecula, USA). Rabbit antibody to human KIT (anti-CD117, A4507) and secondary goat anti-rabbit antibody was from DAKO (Glostrup, Denmark). Sulforhodamine B (SRB), Triton X-100, NADP, ATP, glucose, glucose 6-phosphate dehydrogenase and protease inhibitor cocktail were purchased from Sigma Aldrich (St. Louis, USA). For protein determination a Bio-Rad reagent was used according to the manufacturer's specifications.

Cell lines and cell culture: The GIST882 cell line was a kind gift from Dr. Jonathan Fletcher. GIST882 cells were grown in culture flasks in DMEM/F12 medium supplemented with 10% Foetal Bovine Serum (FBS), 100 U mL^{-1} Penicillin and 0.1 mg mL^{-1} streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. GIST GDG1 is an imatinib resistant GIST cell line derived from a progressive GIST patient and was grown in the same medium as GIST882. MCF-7, a human breast cancer cell line, was grown in RPMI 1640 also supplemented with 10% FBS and Penicillin/Streptomycin.

Cell survival after treatment with imatinib: GIST882 cells were harvested by trypsinisation from exponentially growing cultures and subcultured in 96-well plates. Optimal seeding density to ensure exponentional growth was 20000 cells $well^{-1}$. At 24 h following sub-culturing (day 1) increasing concentrations of imatinib (from 0.001 to 5 µM) or DMSO vehicle control were added to the medium. After 72 h of treatment (day 4), imatinib-containing medium was removed and replaced with fresh medium. On day 7, the cell survival was determined by the Sulforhodamine B (SRB) colorimetric assay as described[16, 17]. Experiments were performed in quadruplicates. The survival rates were calculated by dividing mean Optical Density (OD) of the treated cells by the mean OD of control cells x 100%.

Determination of 2-[F-18] Fluor-2-deoxy-D-Glucose (^{18}F-FDG) uptake: ^{18}F-FDG up-take was determined at two imatinib concentrations, 0.03 µM and 0.3 µM, respectively. Approximately 10^6 GIST882 cells were seeded in 60-mm Petri-dishes and treated for 24 h with either imatinib or DMSO vehicle as control. One hour prior to the ^{18}F-FDG-uptake experiment, the medium was removed and replaced with Phosphate Buffered Saline (PBS). Four MBq ^{18}F-FDG in PBS was added to each Petri dish and incubation was performed at 37°C for 5, 10, 30 and 60 min, respectively. After incubation, the medium was removed, cells were detached mechanically, transferred to Eppendorf tubes and homogenized in lysis buffer (150 mM NaCl, 0.5% Triton X-100, protease inhibitor cocktail. Aliquots were taken for either radioactivity measurements (Wallac gamma counter) or protein determination. ^{18}F-FDG-uptake was expressed as percentage Injected Dose (ID) per 10 mg protein.

Cell lysis for protein concentration, Western analysis or enzymatic activities: Cells were washed twice with ice-cold PBS and lysed in a cold buffer containing 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and a protease inhibitor cocktail. Lysates were rocked for 30 min at +4°C and then centrifuged at 21,000 x g for 30 min at +4°C. Supernatants were removed to a fresh tube and the protein content was determined using protein assay reagent (Bio-Rad).

Western analysis: To identify what type of glucose transporter is expressed in GIST882 and GIST GDG1 cells, Western analysis for GLUT-1, GLUT-2, GLUT-3 and GLUT-4 proteins were performed. As a positive control for GLUT-1, we used red blood cells. For GLUT-2 the breast cancer cell line MCF-7, which is known to express GLUT-2 transporter[18] and also human liver tissue were used. For GLUT-3 and GLUT-4 positive control specimens, human astrocytoma tissue and normal heart tissue were utilized.

Thirty µg of protein were separated on SDS-polyacrylamide gels and electroblotted onto PVDF membranes (0.45 µm). Membranes were blocked in PBST (0.05% Tween-20 in PBS) containing 5% nonfat dried milk and incubated sequentially with anti-human GLUT-1 (dilution 1:5000), GLUT-2 (dilution 1:1000), GLUT-3 (dilution 1:5000) or GLUT-4 (dilution 1:2500) and anti-human KIT (dilution 1:500) antibodies, diluted

in PBST. Membranes were then washed in PBST, probed with anti-rabbit immunoglobulin-HRP conjugate (DAKO) and incubated with ECL substrate (Pierce).

Determination of the hexokinase and glucose-6-phosphate dehydrogenase activity: Both enzyme activities were measured on total cell lysates at 37°C. The hexokinase (EC 2.7.1.1) was assayed in a system coupled with glucose-6-phosphate dehydrogenase. The assay mixture contained 40 mM Tris (pH 7.6), 4 mM glucose, 2 mM ATP, 8 mM $MgCl_2$, 2 mM NADP and 1.3 μg mL^{-1} glucose-6-phosphate dehydrogenase in a total volume of 250 μl. NADPH was measured spectrophotometrically at 340 nm. The assay mixture for glucose-6-phosphate dehydrogenase (EC 1.1.1.44) was similar to that for the hexokinase assay except that glucose has been replaced by glucose-6-phosphate and no exogenous glucose-6-phosphate dehydrogenase was added. For each hexokinase or glucose-6-phosphate dehydrogenase assay a blank reaction was performed to account for the contribution of endogenous glucose-6-phosphate and NADPH to the absorption at 340 nm.

Cell surface biotinylation GLUT-2 allocation to the plasma membrane was assessed by a biotinylation method as described previously with minor modifications[19]. Briefly, cells were grown to confluence in a 6-well plate, then washed three times with PBS (pH. 8.0) and 0.5 mg mL^{-1} EZ-Link-sulfo-NHS-SS-biotin (Pierce, Rockford, USA) was added to the cells. After 30 min, the reaction was terminated by washing the cells with ice-cold quenching buffer (100 mM glycine in PBS, pH 7.4). The cells were then lysed in 1 mL of lysis buffer containing protease-inhibitors. The lysates were then vortexed, incubated on ice for 30 min and centrifuged at 10000 x g for 30 min at 4°C. An aliquot of the supernatant was used for protein measurements. Supernatants were incubated overnight with 25 μl of streptavidin-agarose beads (Pierce, Rockford, USA) at 4°C. The following day the beads were washed twice with 1 mL of lysis buffer. Proteins bound to the streptavidin-agarose beads were eluted in 20 μl of Laemmli sample buffer containing 4% β-mercapto-ethanol and heated at 95°C for 10 min. Samples were subjected to SDS-PAGE, electrophoretically transferred to PVDF membranes and Western blot analysis was performed as described above.

Data analysis and statistics: All experiments were performed at in triplicate or quadruplicate and repeated at least twice. Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's *t* test for unpaired samples. A value of $P<0.05$ was taken as significant.

RESULTS

Cell proliferation: The effect of various concentrations of imatinib on the proliferation of GIST882 cells as determined by the colorimetric SRB-assay is shown in Fig. 1. Imatinib inhibited the proliferation of GIST882 cells in a dose-dependent manner with an IC_{50} = 0.030 ± 0.006 μM. The maximal level of inhibition of proliferation was 70%. For further glucose uptake experiments we used imatinib concentrations of 0.03 μM (IC_{50}) and 0.3 μM.

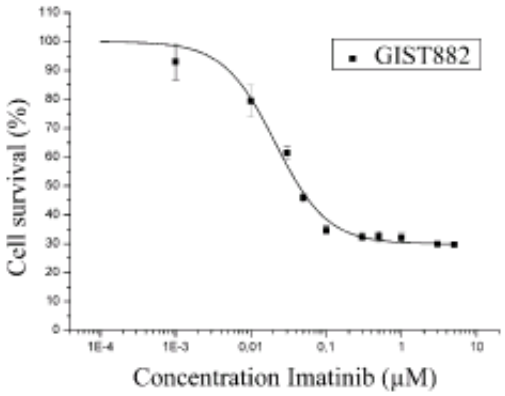
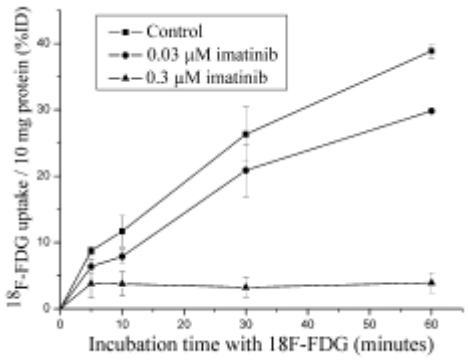


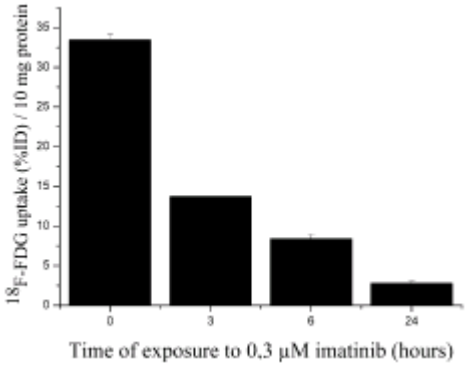
Fig. 1: Inhibition of GIST882 cell proliferation by imatinib. Adherent cells, proliferating in 96-well plates (2.10^4 cells $well^{-1}$), were incubated with increasing concentrations of imatinib for a period of 6 days. Cell proliferation was determined by the SRB assay. Values are expressed as a percentage of the control (untreated) cells. The data shown are the mean from three independent experiments, each with quadruplicate wells

Glucose uptake: We used the radioactive glucose analogue 2-[F-18] Fluor-2-deoxy-D-Glucose (^{18}F-FDG) to assess the glucose up-take by GIST882 cells following treatment with imatinib (Fig. 2a). After 24 h of treatment, the ^{18}F-FDG up-take decreased by some 25% in the presence of 0.03 μM imatinib (n=3, $P<0.01$) and was negligible at 0.3 μM imatinib (n=3, $P<0.001$). In addition, the inhibition of ^{18}F-FDG up-take at 0.3 μM imatinib was time-dependent (Fig. 2b), with a decrease in ^{18}F-FDG uptake of more than 50% after 3 h of exposure.

Hexokinase and glucose-6-phosphate dehydrogenase (G6PDH) activity: The levels of hexokinase and G6PDH activity in GIST882 cells treated with 0.3 μM imatinib for 24 h are given in Fig. 3. After imatinib treatment hexokinase and G6PDH activity were decreased by about 30% (n=9, $P<0.01$) and 37% (n=9, $P<0.01$), respectively.



(a)



(b)

Fig. 2a: Time course of ^{18}F-FDG accumulation in GIST882 cells. ^{18}F-FDG uptake is expressed as % Injected Dose (ID)/10 mg protein. Cells were pre-treated for 24 h with vehicle (controls), 0.03 µM or 0.3 µM imatinib. Values are mean ± standard error of the mean (SEM) of two independent determinations within the same experiment. Three additional similar experiments yielded identical results.
2b: ^{18}F-FDG uptake in GIST882 cells. Pre-treatment with 0.3 µM imatinib was performed for the indicated period. Incubation with ^{18}F-FDG was performed for 1h. ^{18}F-FDG-uptake is expressed as % ID/10 mg total cell protein

GLUT-2 is the main glucose transporter in GIST882 cells: Of the four glucose transporters GLUT-2 was identified as the major transporter in GIST882 cells, migrating as a major band of 64 kDa (Fig. 4). In GIST882 cells, GLUT-4 appeared as a very weak band of 45 kDa and GLUT-1 en -3 could not be detected. Similarly to GIST882 cells, GLUT-2 was the major protein expressed in imatinib resistant GIST-GDG1 cells. Although GLUT-4 and GLUT-3 was also detected, their level of expression was relatively less significant.

The effect of imatinib on GLUT-2 expression: We further focused on the GLUT-2 transporter since it's the

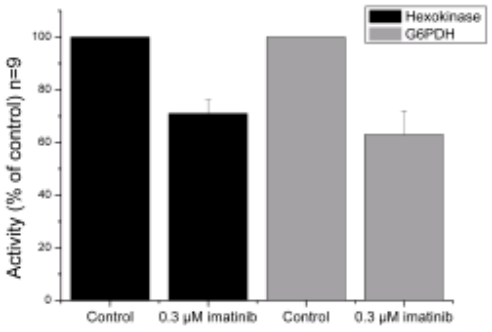


Fig. 3: GIST882 cells show a significant decrease in hexokinase and glucose-6-phosphate dehydrogenase activity after treatment with 0.3 µM imatinib by about 30 and 37%, respectively

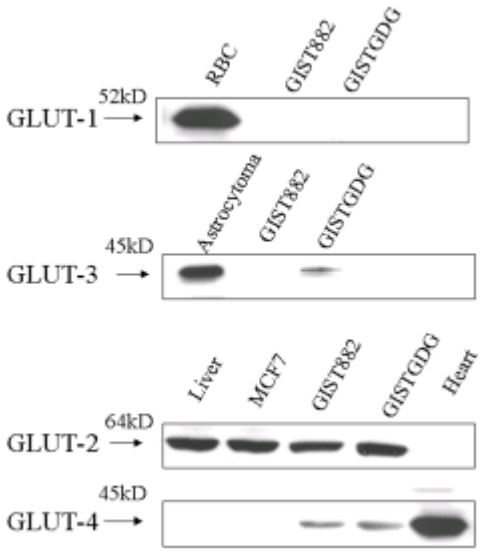


Fig. 4: Identification of GLUT transporters in GIST882 and GIST-GDG1 cell lines. After incubation with GLUT-2 antibody, the same membrane was stripped and re-incubated with GLUT-4 antibody. Similarly to GIST882 cells, GLUT-2 was the major protein expressed in GIST-GDG1 cells. Although GLUT-4 and GLUT-3 was also detected, their level of expression was relatively less significant. Human red blood cells (RBC) were used as a positive control for GLUT-1, MCF-7 cells and liver tissue for GLUT-2, astrocytoma tissue for GLUT-3 and heart tissue for GLUT-4

main glucose transporter present in both GIST cell lines as detected by Western blot.

To test the effect of imatinib on GLUT-2 expression we first performed Western analysis in GIST882 cells treated with 0.3 µM imatinib for 24 h as well as in control cells (Fig. 5b). Imatinib did not affect

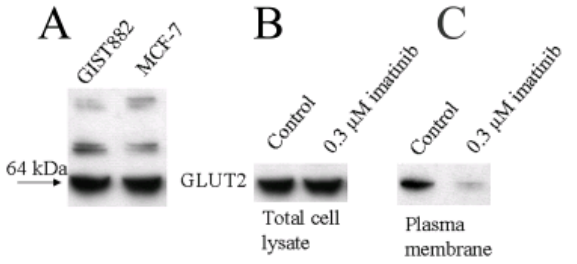


Fig. 5a: Western blot analysis identifies GLUT-2 as the major transporter present in GIST882 cells. MCF-7 cells were used as a positive control.
5b: Detection of GLUT-2 in total cell lysates after 24 h of incubation with 0.3 μM imatinib. The same amount of proteins was loaded in each lane. The total cell lysates revealed no change in GLUT-2 levels in control GIST882 cells compared with treated cells.
5c: Plasma membranes were labelled with biotin, recovered with streptavidin beads and examined by Western blotting for alterations in GLUT-2 protein. The isolated biotinylated proteins are representative for surface expressed GLUT-2. After imatinib treatment, the amount of GLUT-2 in plasma membranes decreased significantly

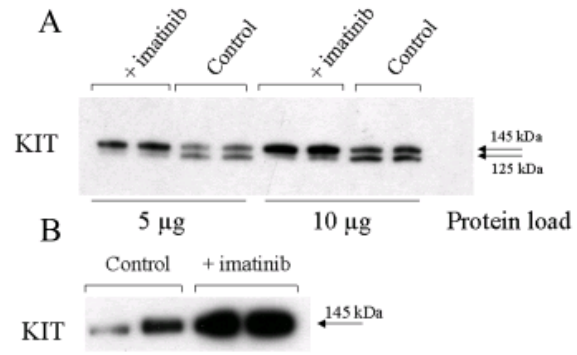


Fig. 6a: Whole cell lysates were analyzed for KIT expression by Western analysis. An increase of the 145-kDa form of KIT was detected after a 24 h treatment with 0.3 μM imatinib.
6b: Plasma membrane fractions were analyzed and only the mature 145-kDa form of KIT is visible. There is a significant increase in the total mature KIT protein form after a 24 h treatment with 0.3 μM imatinib

the level of GLUT-2. We further considered the possibility of impaired translocation of GLUT-2 receptor to the plasma membrane in cells treated with imatinib. To test this hypothesis we performed a cell-surface biotinylation experiment (Fig. 5c). After 24 h of exposure to imatinib 0.3 μM, plasma membrane proteins were labelled with a non-permeable biotin-analogue and bound to streptavidin beads for recovery of purified plasma membranes. Western analysis revealed a significant decrease of GLUT-2 protein levels after treatment with imatinib.

Imatinib effect on KIT expression: Whole cell lysates were analysed by Western blot for the expression of the KIT receptor (Fig. 6). Cells were either untreated or exposed to 0.3 μM imatinib for 24 h. In the untreated cells, anti-KIT antibodies identified a duplet of 125 and 145 kDa in size. The latter may represent a more mature, fully glycosylated form of KIT. Following imatinib treatment, an increase in the level of KIT 145-kDa forms was noted in whole cell lysates, with a corresponding decrease in the level of the 125-kDa form (Fig. 6a). The increase of the former form was much more significant when the plasma membrane fraction rather than whole cell lysates were analyzed (Fig. 6b).

DISCUSSION

Imatinib mesylate specifically inhibits the KIT protein, a protein tyrosine kinase that is constitutively activated in GISTs through gain-of-function mutations and that is of critical importance in GIST pathogenesis[10, 20].

In the current study we used the GIST882 cell line to characterize novel molecular effects of imatinib. We established that a 6-day imatinib treatment inhibited the proliferation of GIST cells with an IC_{50} of 0.030 μM. Previous reports showed that over a 5-day period, GIST882 proliferation was consistently inhibited by imatinib concentration of at least 0.1 μM[21]. Thus, GIST882 cells display a high sensitivity to imatinib treatment. In comparison, for the BCR-ABL positive CML-derived K562 cells, an IC_{50} of 0.69 μM was reported[14]. The growth of other cancer cell lines was previously found to be inhibited by even higher imatinib concentrations, varying from 2 μM to 31.5 μM[22].

To assess the glucose uptake by GIST882 cells following treatment with imatinib, the radioactive glucose analog 2-[F-18] Fluor-2-deoxy-D-Glucose (^{18}F-FDG) was used. FDG is transported across the cell membrane by GLUTs and is phosphorylated similarly to glucose. However, in contrast to glucose-6-phosphate, phosphorylated FDG is metabolically trapped, except in the liver, where glucose-6-phosphatase is present in large amounts. GISTs are FDG-avid, highlighting their hypermetabolic state[23]. We found a significant decrease in the ^{18}F-FDG uptake after treatment with 0.03 μM imatinib for 24 h, this uptake being negligible at 0.3 μM of the drug. Moreover, after 3h exposure to imatinib at a concentration of 0.3 μM, the decrease in ^{18}F-FDG uptake was more than 50%. Our study suggests a rapid decline in glucose uptake following imatinib treatment

in a GIST cell line. This is consistent with early functional changes in glucose metabolism previously identified in GIST patients through imagining by Positron Emission Tomography (PET) employing ^{18}F-FDG[23,24]. By contrast, glucose metabolism declines only gradually during a cytotoxic treatment. More importantly, the early functional changes in GIST tumor metabolism correlate closely with the response to imatinib. A rapid and almost complete shutdown of the glucose metabolism observed soon after the start of imatinib treatment may be due to a drug effect on the glucose metabolism, through inhibition of key glycolytic enzymes and may not only represent a direct antineoplastic effect. Tumor cells are known to be highly glycolytic and overexpression of hexokinase activity, a key enzyme in glucose metabolism, has been documented in various cancers including the gastrointestinal ones. We determined the level of hexokinase activity in GIST882 cells treated with 0.3 μM imatinib for 24 h. Glucose uptake is almost abolished under these conditions; however we found a decrease in hexokinase activity of only 30%. Similar results were previously obtained in K562 leukemia cells[14]. Since such a decrease in hexokinase activity alone cannot account for almost complete inhibition of glucose uptake, we further focused on the influence of imatinib on the transport of glucose across the plasma membrane. The K562 cells, like most malignant cells, express the glucose transporter GLUT-1. In contrast, in the present study we showed that GIST882 cells primarily express GLUT-2, a hitherto not reported finding. GLUT-2 is a low affinity and high capacity glucose transporter, mainly expressed in hepatocytes, pancreatic β-cells, renal proximal tubules and the basolateral membrane of enterocytes[25]. GLUT-4 was also detected in GIST882 cells, but in much smaller amounts than GLUT-2. As a comparison we evaluated the presence of GLUT transporters in an imatinib resistant cell line GIST GDG1 and found the presence of not only GLUT-2 and GLUT-4 but also GLUT-3, which could signify that GIST cells can express different glucose-transporters depending on their glucose requirements.

We found no difference in the GLUT-2 protein level when whole lysates from control cells (not treated) or imatinib-treated cells where assayed by Western blotting. To further study the influence of imatinib on GLUT-2 allocation, we performed Western analysis of cell surface proteins following their biotinylation in intact cells. A marked decrease of the amount of GLUT-2 in plasma membranes was found in the treated cells compared with the untreated ones. Translocation from an intracellular pool to the plasma membrane has been widely described for GLUT-1 and GLUT-4[25,26]. Moreover, in hematopoeietic M07e cells, a role for KIT tyrosine kinase on glucose transport activation through GLUT-1 translocation, has been suggested[27]. Recent studies in enterocytes have shown that GLUT-2 can also traffic from the basolateral membrane to the brush border membrane in response to high luminal glucose loads, the presence of glucagon-like peptide 2 or in response to AMP-activated protein kinase activation[28-30].

Expression of KIT is considered to be the best defining feature of GISTs[9]. It is seen in about 95% of these tumors, regardless of their histological appearance and biologic behavior. By immunohistochemical staining, GIST cells commonly express strong diffuse cytoplasmic KIT positivity, but may also show a 'dot like' pattern (so-called 'Golgi pattern') within the cytoplasm. GIST882 cells do express a constitutively phosphorylated KIT allele, bearing hemizygous K642E mutation in the tyrosine kinase domain[21]. In GIST882 cells, anti-KIT antibody identifies two proteins, 125 kDa and 145 kDa in size, respectively[4]. To our knowledge, the relationship between these forms of proteins is unknown, but the larger size protein may represent a more mature, glycosylated form of KIT. We found a marked increase in the 145-kDa form at the plasma membrane in the imatinib-treated cells compared to untreated ones. Such a finding is unexpected but this phenomenon may represent a positive feedback mechanism of GIST cells in response to KIT tyrosine kinase blocking by imatinib.

CONCLUSION

In conclusion, we have identified GLUT-2 as the major glucose transporter in GIST882 cells. Its impaired recruitment to the plasma membrane combined with the decreased hexokinase activity may account for the rapid decline in glucose utilization shortly after imatinib treatment. We have also found an increased KIT level at the plasma membrane in imatinib-treated cells compared to the untreated ones. This is important in the light of a recent study showing that pre-treatment serum KIT levels were elevated in patients with GISTs, but decreased to 69% following a one-month imatinib treatment[31]. Our study demonstrates that imatinib increases KIT expression at the plasma membrane. This together with increased serum SCF may account for the increased SCF-induced cell signalling following imatinib withdrawal.

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