Effect of Imatinib mesylate on platelet aggregation and fibrinogen binding to isolated platelets

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Objectives: We have investigated the relationship between imatinib mesylate and platelets. One of the complications of imatinib mesylate is thrombocytopenia. Patients with chronic myelogenous leukemia (CML) also show abnormal platelet functions. In this study we aimed to test the effect of imatinib mesylate on in vitro agonist induced platelet aggregation, to platelets and GpIIb/IIIa fibrinogen binding.

Background: Platelet functions such as aggregation and fibrinogen binding to platelets are abnormal in chronic myelogenous leukemia. Imatinib mesylate is a novel molecularly targeted therapy of chronic myelogenous leukemia.

Methods: Different concentrations of commercial imatinib mesylate effect on in vitro ADP and epinephrine induced platelet aggregation were examined. Agonist induced platelet aggregation in PRP was followed on Chronolog-Lumi aggregometer with and without imatinib mesylate. For binding studies, platelets were obtained through platelet apheresis. FITC-labelled fibrinogen (Fg-FITC) binding to isolated platelets and purified GpIIb/IIIa complex with and without imatinib mesylate was followed with FACScan Becton-Dickinson flow cytometer.

Results: Imatinib mesylate caused inhibition of ADP and epinephrine induced platelet aggregation, concentration dependently. Also, imatinib mesylate inhibited fibrinogen binding to isolated platelets but had no effect on fibrinogen binding to GpIIb/IIIa complex coated micro beads.

Conclusion: Imatinib mesylate has a platelet antiaggregation effect this aggregation inhibition might be partially due to its inhibition on fibrinogen binding to platelets via inhibition of phosphatidylinositol 3-kinase dependent pathway that regulates the fibrinogen receptor GpIIb/IIIa.

Key words: Platelet fibrinogen binding, platelet aggregation, imatinib mesylate


Introduction

Imatinib mesylate (GleevecTM, Glivec, formerly ST1571; Novartis pharmaceuticals) is an inhibitor of the Bcr-Abl tyrosine kinase that has a central role in the pathogenesis of chronic myelogenous leukemia (CML). It is generally well tolerated during the treatment of the disease but it can lead to some adverse effects such as oedema, myalgia and abnormality in liver function tests. The most notable side effects are neutropenia and thrombocytopenia.
sel walls and subendothelial connective tissues thereby forming the initial platelet plug. Activation of platelets causes the exposure of phospholipase (PL) and the membrane GpIIb/IIIa on the platelet surface, providing a platform upon which the members of the coagulation cascade can assemble. When platelets activated with different agonists and especially when activated by tumor cells angiogenesis is initiated or promoted. The mediators of this specific intracellular communication are surface adhesion molecules and β3 integrins in particular. Integrins of tumor cells and platelets are engaged and bridged by soluble RGD-containing matrix molecules of the circulation, vitronectin, fibrinogen and fibronectin. Interaction of antineoplastics with platelets might play an important role. Therefore, targeting platelets and GpIIb/IIIa with an alternative approach is of interest.

Materials and Methods

Commercial Gleevec (Novartis, Turkey), fibrinogen (Sigma), fluoroisothiocyanate (FITC) (Sigma), fetal calf serum (FCS) (Sigma), polystyrene micro beads (Bangs Lab.) arginin-glycine-arginin (Calbiochem), dimethyl sulfoxime (DMSO) (Sigma) and other chemicals were reagent grade from Sigma.

Platelet aggregation assay

Platelet aggregation was followed on a Chronolog Lumi Aggregometer by addition of inducers on PRP. Commercial imatinib mesylate was kindly supplied from Novartis (Istanbul-Turkey). Human platelets were isolated from freshly drawn citrated (3.8% sodium citrate) blood of healthy volunteers who had not taken any medication for at least 10 days before sampling. Samples were centrifuged at 800 rpm for 10 minutes at room temperature, to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was obtained by centrifugation of PRP at 1.500 rpm for 20 minutes to calibrate the aggregometer. PRP was stimulated in the aggregometer cuvette at 37°C with ADP (10 µM) and epinephrine (10 µM). Imatinib mesylate to a final concentrations of 1.37, 3.4, 6.8, 13.6 µM were added to PRP and incubated for 10 minutes before the addition of ADP and epinephrine. Aggregation was recorded on Lumi-Aggregometer as shown on Figure 1 and Figure 2 Max. amplitude of primary and secondary aggregation curves are measured in cm. Percent inhibition (Table 1) of aggregation was calculated by comparing the Max. amplitudes to samples with imatinib mesylate that of without imatinib mesylate.

Fibrinogen binding assay

Conjugation of fibrinogen with fluorescence isothiocyanate (FITC). Fibrinogen was conjugated with fluorescence isothiocyanate (FITC) solution containing 1 mg fibrinogen, 10 µl FITC (in 1 mg/ml DMSO), 900 µl NaHCO₃/NaCO₃ (pH 9.6) and was incubated with slight mixing overnight at 4°C.

 Feng-FITC binding to platelets: For binding experiments, platelets were obtained from normal human subjects who have not ingested any medications for at least 10 days by platelet apheresis. Platelet suspension was obtained by centrifugation for 10 minutes at 1.500 rpm at room temperature. Fetal calf serum (FCS) solution (1 ml) was added to supernatant and centrifuged for 10 minutes at 3.000 rpm at room temperature.

Table 1
Percent inhibition of platelet aggregation by imatinib mesylate

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Imatinib mesylate (µM)</th>
<th>Aggregation Inhibition</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Epinephrine (10 µM)</td>
<td></td>
<td>0.00</td>
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<tr>
<td>(n: 7)</td>
<td>1.37</td>
<td>13.05</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>34.14**</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>57.18**</td>
</tr>
<tr>
<td></td>
<td>13.4</td>
<td>87.16**</td>
</tr>
<tr>
<td>ADP (10 µM)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>(n: 7)</td>
<td>1.37</td>
<td>19.37</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>54.22*</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>78.37**</td>
</tr>
<tr>
<td></td>
<td>13.4</td>
<td>85.80**</td>
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</table>

*Statistically significant compared to the samples without imatinib mesylate

Supernatant was removed and pellet was suspended in FCS solution. Platelets in FCS were incubated with different imatinib mesylate concentrations (10-150 µg/ml) for 30 minutes at room temperature. Imatinib mesylate concentration of 150 µg/ml gave maximum inhibition of fibrinogen binding as shown in Figure 3 different concentrations of Fg-FITC from 1 mg/ml Fg-FITC stock solution were added to each tube, again incubated for 30 minutes at room temperature, in dark. Samples were completed to 1 ml with FCS and centrifuged at 10,000 rpm for 5 minutes at room temperature and fluorescence was measured in two hours by flow cytometry. Percent bound ligand was determined with and without imatinib mesylate at different concentrations of Fg-FITC (Figure 3).10

**Fg-FITC binding to GpIIb/IIIa coated microbeads:** Purified GpIIb/IIIa complex was coated with micro beads as described.10-12 GpIIb/IIIa complex coated micro beads were incubated with FITC-labelled fibrinogen in various concentrations and binding were evaluated by flow cytometry.

**Flow cytometry:** A Becton Dickinson FACScan analyzer was used to quantify fluorescence (excitation wavelength: 488 nm and emission wavelength: 530 nm) at the single-cell level, and data were analyzed using Cellquest version 3.3 (Becton Dickinson) software. In each sample, the mean fluorescence intensity of the analyzed cells was determined after gating the cell population by forward and side light scatter, signals were recorded on a dot plot. A total of 60,000 events were acquired and debris were excluded by prior gating, thereby limited cell populations usually contained 10,000 cells. Fluorescence in those cells produced the percentage of ligand bound by platelets, and signals were recorded on a frequency histogram. Results were expressed as percentage of bound ligand representing gate percent.
Statistical analysis

The results of platelet aggregation experiments (n: 7) and of flow cytometric platelet fibrinogen binding assays (n: 10) were expressed as mean ± SD. Statistical analyses were performed with Student’s t-test for paired data where P<0.05 value considered significant.

Results

This report presents two methods for investigating the effect of imatinib mesylate on platelet functions. Agonist induced platelet aggregation was followed on Chronolog Lumi-aggregometer. Different concentrations of imatinib mesylate from commercial imatinib mesylate capsules (Novartis, 100 mg) were prepared and antiaggregating effect were examined on aggregometer. Imatinib mesylate at 1.37, 3.4, 6.8, 13.6 mM final concentrations inhibited ADP (10 mM) and epinephrine (10 mM) induced primary and secondary aggregation, concentration dependently (Figure 1, Figure 2 and Table 1). Imatinib mesylate at increasing concentrations were added to platelet rich plasma (PRP) and incubated just before the addition of epinephrine and ADP. A dose dependent decrease of agonist induced platelet aggregation was observed, according to seven independent experiments, imatinib mesylate at different concentrations decreased ADP induced platelet aggregation (Figure 1, Table 1). Furthermore, similar concentrations of imatinib mesylate also caused a significant decrease of epinephrine induced platelet aggregation (Figure 2, Table 1).

Binding of fluorescence isothiocyanate (FITC) labeled fibrinogen to isolated platelets and to GpIIb/IIIa coated micro beads was followed in FACScan Becton-Dickinson flow cytometer. Sample solutions were daily prepared and analyzed within 2 hours after preparation. Imatinib mesylate inhibited...
Fg-FITC binding to isolated platelets, concentration dependently (n: 10) (Figure 3). Between up to 150 mg/ml concentrations imatinib mesylated had effect on Fg-FITC binding to GpIIb/IIIa coated micro beads. The results show that imatinib mesylate inhibited the ADP and epinephrine induced platelet aggregation in PRP and Fg-FITC binding to isolated platelets, but not to GpIIb/IIIa complex coated micro beads.

**Discussion**

Platelet functions such as aggregation are often abnormal in chronic myelogenous leukemia (CML). Imatinib mesylate is a novel molecularly targeted therapy of CML. Platelet integrin GpIIb/IIIa complex is the member of platelet adhesive fibrinogen receptors that upon activation with different agonists mediates platelet aggregation by binding of fibrinogen. Since these agonists are related to platelet adhesion and aggregation, the GpIIb/IIIa receptor complex plays an important role in the process of platelet activation and thrombosis. Receptor number of GpIIb/IIIa complex can be overexpressed or depressed in CML disease. Imatinib mesylate’s inhibition of fibrinogen binding to platelets found in this work might be related to its effect on fibrinogen binding to GpIIb/IIIa. Previous studies indicated that platelet activation via the collagen receptor GPVI was not altered in platelets from CML patients. The effect of thrombopoietin (TPO) on the platelet aggregation from 17 patients with chronic myelogenous leukemia in the chronic phase (CML-CP) was examined and detected that TPO by itself dose-dependently induced the aggregation of platelets from patients with CML-CP but it did not have this effect on normal or other myeloproliferative disordered patient’s platelets like polycytemia vera, essential thrombocytemia, myelofibrosis or with CML-CP in cytogenetical complete remission. It was also shown that TPO modulated the degree of the primary wave of aggregation and the lag phase, but not the slope of the secondary wave of aggregation with ADP. TPO also induced binding of fibrinogen to platelets in a comparable degree observed with ADP. It is reported that the platelet integrin alpha IIb beta 3 (GpIIb/IIIa) is regulated by the integrin linked kinase (ILK) in a PI3-kinase dependent pathway. 

The PI3-kinase inhibitors and Abl kinase inhibitor imatinib mesylate suppresses TPO induced aggregation of CML-CP platelets. Bcr-Abl tyrosine kinase inhibitor are involved the constitutive activation of PI3-kinase in CML-CP platelets. As shown in this work imatinib mesylate’s inhibitory effect on platelet aggregation is in accordance with its inhibitory effect on fibrinogen binding to platelets. On the other hand fibrinogen binding to GpIIb/IIIa complex coated micro beads was not inhibited by imatinib mesylate. This result shows that imatinib mesylate’s antiaggregation effect and fibrinogen binding inhibitory effect are due to its inhibition of PI3-kinase dependent pathway activation leading to inhibition of GpIIb/IIIa complex expression on the platelet surface to make available fibrinogen binding sites. GpVI of CML patients provide a tendency to platelet aggregation when imatinib mesylate was administered we think it essential for them to undergo platelet aggregation test.

**References**


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