The GPIIbIIIa antagonist drugs eptifibatide and tirofiban do not induce activation of apoptosis executioner caspase-3 in resting platelets but inhibit caspase-3 activation in platelets stimulated with thrombin or calcium ionophore A23187

Platelet surface receptor, glycoprotein (GP) IIbIIIa (integrin αIIbβ3), mediates platelet aggregation and plays a key role in hemostasis and thrombosis.[1,2]

Numerous GPIIbIIIa antagonists have been designed and tested as inhibitors of platelet aggregation.[3] Two of these antagonists, eptifibatide (Integrilin) and tirofiban (Aggrastat), are approved by the US Food and Drug Administration (FDA) for clinical use for preventing and treating thrombotic complications in patients undergoing percutaneous coronary intervention and in patients with acute coronary syndromes.[4] It has been reported, however, that some GPIIbIIIa antagonists, such as orbofiban and xemilofiban, promote apoptosis in cardiomyocytes by activation of the apoptosis executioner caspase-3,[5] raising the possibility that platelets also may be susceptible to pro-apoptotic effects of Integrilin and Aggrastat.

Over the past decade it has been well-documented by us and others that apoptosis occurs not only in nucleated cells but also in anucleated platelets stimulated with thrombin, calcium ionophores, very high shear stresses and platelet storage (see references in [6-8]). It has been further reported that platelet activation and apoptosis may be induced by different mechanisms and/or require different levels of triggering stumuli.[9,10] Recently, we have shown that injection of anti-GPIIb antibody induced caspase-3 activation in mouse platelets,[11] suggesting that direct GPIIbIIIa-mediated pro-apoptotic signaling is able to trigger caspase-3 activation within platelets.

The current study aimed to examine, for the first time, the effect of Integrilin and Aggrastat on caspase-3 activation in human platelets. We studied the effects of Integrilin and Aggrastat on caspase-3 activation in resting platelets which express GPIIbIIIa receptors in their non-active (*closed*) conformation, and in platelets stimulated with thrombin and calcium ionophore A23187, which induce transition of GPIIbIIIa receptors into active (*open*) conformation.

Table 1A shows that treatment of resting platelets with Integrilin and Aggrastat did not affect caspase-3 activation ($p>0.05$). In contrast, a 2.3-2.7-fold increase in caspase-3 activation was observed in platelets after thrombin and A23187 stimulation (Table 1B and C, $p<0.01$). However, when platelets were preincubated with Integrilin and Aggrastat before agonist treatment, these drugs significantly inhibited agonist-induced caspase-3 activation (Figure 1 and Table 1B and C, $p<0.05$).

The fact that Integrilin and Aggrastat do not promote caspase-3 activation in unstimulated platelets (Table 1A) suggests that these GPIIbIIIa antagonists do not induce transmission of pro-apoptotic transmembrane signals inside platelets through inactive GPIIbIIIa integrin. They also do not activate caspase-3 directly by interacting with Arg-Gly-Asp (RGD)-binding motif of pro-caspase-3, as was reported for cell-penetrating GPIIbIIIa antagonists, orbofiban and xemilofiban, in rat cardiomyocytes,[5] indicating that the chemically different Integrilin and Aggrastat do not penetrate human platelets.

The inhibitory effect of Integrilin and Aggrastat on thrombin- and A23187-induced caspase-3 activation (Table 1 B and C) suggests a role for GPIIbIIIa integrin in caspase-3 activation induced by these platelet agonists.

Table 1. Effect of GPIIbIIIa antagonists, eptifibatide (Integrilin) and tirofiban (Aggrastat), on caspase-3 activation in resting human platelets and platelets stimulated with thrombin and calcium ionophore A23187.

Treatment of platelets	Caspase-3 activation (MCF)	Inhibition by GPIIbIIIa antagonists (%)
A. Resting platelets		
Control buffer	65.4±5.2	
Integrilin	62.6±5.5^{NS}	NS
Aggrastat	63.9±4.3^{NS}	NS
B. Thrombin-stimulated platelets		
Thrombin	173.7±21.5**	
Integrilin + Thrombin	117.0±9.3*	46.3±7.4
Aggrastat + Thrombin	121.6±12.2*	44.1±8.4
C. A23187-stimulated platelets		
A23187	153.3±19.2**	
Integrilin + A23187	105.4±8.5*	47.5±9.7
Aggrastat + A23187	107.6±9.9*	49.8±8.2

*Resting platelets were treated with control buffer, 0.48 μM Integrilin or 0.48 μM Aggrastat (A), and stimulated platelets were treated with 1 U/mL thrombin (B) and 10 μM A23187 (C), or preincubated with Integrilin or Aggrastat before treatment with thrombin or A23187 (B,C). Caspase-3 activation was determined by flow cytometry as the mean channel fluorescence (MCF) of the cell-penetrating FAM-DEVD-FMK probe (*see Online Supplementary Appendix). *Means ± SEM are presented for seven experiments in each platelet group. p-values were calculated by one-way ANOVA between: (A) Integrilin- and Aggrastat-treated platelet groups vs. buffer-treated platelet group (NS: not significant, p>0.05,), and (B,C) thrombin- and A23187-treated groups vs. buffer-treated group (**p<0.01), Integrilin/Aggrastat + thrombin- vs. thrombin-treated groups (*p<0.05) and Integrilin/Aggrastat + A23187- vs. A23187-treated groups (*p<0.05). Percentage of inhibition by GPIIbIIIa antagonists of caspase-3 activation in thrombin- and A23187-stimulated platelets was calculated as described in the* Online Supplementary Appendix.

In the absence of Integrilin and Aggrastat, caspase-3 activation induced by thrombin and A23187 is primarily triggered by pro-apoptotic signal transduction through proteinase-activated receptors (PAR)[7,12] and by calcium mobilization/overloading,[8] respectively; GPIIbIIIa integrins are then secondarily activated by inside-out signaling, bind plasma- and/or platelet-derived fibrinogen and/or von Willebrand factor (VWF)[1,2] and transmit pro-apoptotic outside-in signaling further promoting thrombin- or A23187-induced caspase-3 activation. In the presence of Integrilin or Aggrastat, on the other hand, the GPIIbIIIa antagonist inhibits caspase-3 activation induced by thrombin or A23187, attenuating transmission of pro-apoptotic outside-in signaling through active GPIIbIIIa by inhibiting fibrinogen/VWF binding and/or preventing conversion of GPIIbIIIa to a fully active conformation.

We have previously shown that injection of anti-GPIIb antibody is able to trigger caspase-3 activation in mouse platelets *in vivo*,[11] indicating that GPIIbIIIa integrin may be directly involved in pro-apoptotic signal transduction inside platelets. The data obtained in the current study suggest that GPIIbIIIa may be also involved in positive and negative modulation of platelet apoptosis triggered by thrombin- and calcium ionophore-mediated pathways, when apoptosis is potentiated by sending pro-apoptotic signals through active GPIIbIIIa, and inhibited by binding of the fibrinogen-mimetic therapeutics, Integrilin and Aggrastat.

In conclusion, we have shown a novel platelet-directed activity of two clinically used GPIIbIIIa antagonist drugs, eptifibatide (Integrilin) and tirofiban (Aggrastat), with

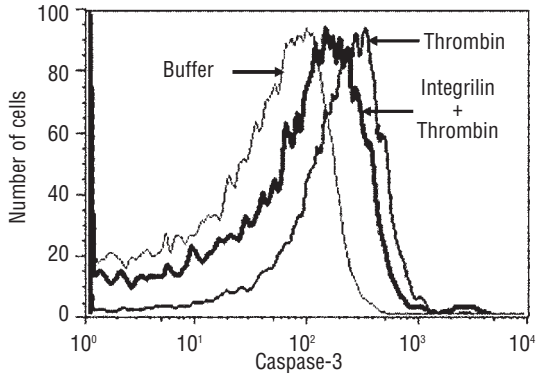

Figure 1. Flow cytometric histograms illustrating inhibitory effect of eptifibatide (Integrilin) on thrombin-induced caspase-3 activation in human platelets. Platelets were treated with control buffer and 1 U/mL thrombin or preincubated with 0.48 μM Integrilin before treatment with thrombin and caspase-3 activation was determined by the cell-penetrating carboxyfluorescein-labeled probe FAM-DEVD-FMK, which covalently binds to active caspase-3 (***Online Supplementary Appendix***). Note that treatment of platelets with thrombin induces caspase-3 activation in comparison with platelets treated with control buffer, whereas preincubation with Integrilin markedly attenuates thrombin-induced caspase-3 activation.

ability to inhibit apoptosis executioner caspase-3 induced by potent platelet agonists, thrombin and A23187, and the absence of adverse pro-apoptotic effects on resting platelets. Taken together with earlier reported data,[11] the current study indicates that, besides their well-known participation in platelet activation and aggregation, GPIIbIIIa receptors are involved in the modulation of platelet apoptosis. This GPIIbIIIa-mediated mechanism of apoptosis modulation may be very efficient given the extremely large number of GPIIbIIIa copies (≈80,000[2]) on the platelet surface.

Valery Leytin,[1-3] Asuman Mutlu,[1] Sergiy Mykhaylov,[1] David J. Allen,[1] Armen V. Gyulkhandanyan,[1] and John Freedman[1-3]

[1]Division of Transfusion Medicine, Department of Laboratory Medicine, The Keenan Research Centre in the Li Ka Shing Knowledge Institute of St. Michael's Hospital, Toronto; [2]Toronto Platelet Immunobiology Group, Toronto; [3]Departments of Laboratory Medicine and Pathobiology, and Medicine, University of Toronto, Toronto, ON, Canada

The online version of this article contains a supplementary appendix.

Key words: eptifibatide, tirofiban, GPIIbIIIa antagonists, caspase-3, platelet apoptosis.

Funding: This work was supported by a grant from the Heart and Stroke Foundation of Ontario, Canada.

Acknowledgments: authors are grateful to Dr. Elena Lyubimov for helpful discussion.

Correspondence: Valery Leytin, Transfusion Medicine, St. Michael's Hospital, 30 Bond St, Toronto, ON, M5B 1W8 Canada. E-mail: leytinv@smh.toronto.on.ca

Citation: Leytin V, Mutlu A, Mykhaylov S, Allen DJ, Gyulkhandanyan AV, and Freedman J. The GPIIbIIIa antagonist drugs eptifibatide and tirofiban do not induce activation of apoptosis executioner caspase-3 in resting platelets but inhibit caspase-3 activation in platelets stimulated with thrombin or calcium ionophore A23187. Haematologica 2009; 94:1783-1784. doi: 10.3324/haematol.2009.012708

References

1. Plow EF, Pesho MM, Ma Y-Q. Integrin aIIbβ3. In: Michelson AD, ed. Platelets. Amsterdam: Academic Press 2007. p.165-78.
2. Prévost N, Shattil SJ. Outside-in signaling by integrin aIIbβ3. In: Michelson AD, ed. Platelets. Amsterdam: Academic Press 2007. p.347-57.
3. Scarborough RM, Gretler DD. Platelet glycoprotein IIb-IIIa antagonists as prototypical integrin blockers: Novel parenteral and potential oral antithrombotic agents. J Med Chem 2000;43:3453-73.
4. Agah R, Plow EF, Topol EJ. aIIbβ3 (GPIIbIIIa) antagonists. In: Michelson AD, ed. Platelets. Amsterdam: Academic Press 2007. p.1145-63.
5. Adderley SR, Fitzgerald DJ. Glycoprotein IIb/IIIa antagonists induce apoptosis in rat cardiomyocytes by caspase-3 activation. J Biol Chem 2000;275:5760-6.
6. Leytin V, Freedman J. Platelet apoptosis in stored platelet concentrates and other models. Transfus Apheresis Sci 2003;28:285-95.
7. Leytin V, Allen DJ, Mykhaylov S, Lyubimov E, Freedman J. Thrombin-triggered platelet apoptosis. J Thromb Haemost 2006;4:2656-63.
8. Leytin V, Allen DJ, Mutlu A, Gyulkhandanyan AV, Mykhaylov S, Freedman J. Mitochondrial control of platelet apoptosis: Effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. Lab Invest 2009;89:374-84.
9. Leytin V, Allen DJ, Lyubimov EV, Freedman J. Higher thrombin concentrations are required to induce platelet apoptosis than to induce platelet activation. Br J Haematol 2007;136:762-4.
10. Leytin V, Allen DJ, Mutlu A, Mykhaylov S, Lyubimov E, Freedman J. Platelet activation and apoptosis are different phenomena: evidence from the sequential dynamics and the magnitude of responses during platelet storage. Br J Haematol 2008;142:494-7.
11. Leytin V, Mykhaylov S, Starkey AF, Allen DJ, Lau H, Ni H, et al. Intravenous immunoglobulin inhibits anti-GPIIb-induced platelet apoptosis in a murine model of ITP. Br J Haematol 2006;133:78-82.
12. Flynn AN, Buret AG. Proteinase-activated receptor 1 (PAR-1) and cell apoptosis. Apoptosis 2004;9:729-37.