

Intravenous immunoglobulin inhibits anti-glycoprotein IIb-induced platelet apoptosis in a murine model of immune thrombocytopenia

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Summary

We have previously shown that injection of anti-glycoprotein (GP) IIb induces murine immune thrombocytopenia (ITP) and that intravenous immunoglobulin (IVIg) ameliorates ITP. We hypothesise that murine ITP may be associated with platelet apoptosis, which is upregulated by anti-GPIIb and downregulated by IVIg. The current study demonstrated that anti-GPIIb injection induced three critical apoptosis manifestations in platelets: (i) mitochondrial inner transmembrane potential ($\Delta\Psi_m$) depolarisation; (ii) caspase-3 activation; and (iii) phosphatidylserine (PS) exposure. IVIg administration inhibited caspase-3 activation and PS exposure, but not $\Delta\Psi_m$ -depolarisation, in anti-GPIIb-treated platelets, demonstrating that IVIg ameliorates thrombocytopenia concomitantly with inhibiting late, but not early mechanisms of platelet apoptosis.

Keywords: murine model, immune thrombocytopenia, anti-glycoprotein IIb antibody, intravenous immunoglobulin, platelet apoptosis.

Received 3 November 2005; accepted for publication 2 December 2005

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While intravenous immunoglobulin (IVIg) is used to treat immune thrombocytopenia (ITP) and other autoimmune diseases, its mechanism of action remain unclear. Several theories have been proposed, including the ability of IVIg to block the reticuloendothelial system (RES), an effect on the inhibitory Fc γ RIIB receptor, neutralisation by anti-idiotypic antibodies and modulation of cytokine cascades (Lazarus & Crow, 2003). An effect of IVIg on apoptosis of nucleated cells has been demonstrated both *in vivo* and *in vitro*; IVIg manifests anti-apoptotic or pro-apoptotic action depending on the targeted cell type (Prasad *et al*, 1998; Viard *et al*, 1998).

Over the last decade, apoptosis of anucleated cytoplasts and platelets has been recognised (Vanags *et al*, 1997; Wolf *et al*, 1999; Brown *et al*, 2000; Leytin & Freedman, 2003; Leytin *et al*, 2004). Platelet apoptosis has been documented in different

models using well-defined apoptosis markers of nucleated cells (Budihardjo *et al*, 1999; Kroemer & Reed, 2000) such as depolarisation of the mitochondrial inner transmembrane potential ($\Delta\Psi_m$), Bcl-2 family proteins expression, caspase-3 activation and phosphatidylserine (PS) exposure (Leytin & Freedman, 2003). These data demonstrate that platelets contain the essential components for cytoplasmic apoptosis.

Many antiplatelet antibodies, notably anti-glycoprotein (GP) IIb/IIIa and anti-GPIb, induce ITP in humans and mice (Nieswandt *et al*, 2000). Some of these antibodies induce thrombocytopenia by targeting platelets through Fc receptors in the RES, while others act by Fc-independent mechanisms (Nieswandt *et al*, 2000). We have previously shown that profound thrombocytopenia induced in mice by injection of antiplatelet antibodies can be ameliorated by IVIg treatment

(Crow *et al*, 2001; Song *et al*, 2003; Siragam *et al*, 2005). Piguet and Vesin (2002) reported that antiplatelet antibodies induced thrombocytopenia in murine models; this was associated with activation of platelet caspases and prevented by treatment with a pan-caspase inhibitor ZVAD-fmk. In contrast, injection of antagonistic anti-apoptotic antibodies increased platelet count and lifespan due to decreased caspase activation (Piguet & Vesin, 2002). Taken together, these data suggest a possible causal role of platelet apoptosis in the development of thrombocytopenia, which can be induced by pro-apoptotic antibodies and prevented by apoptosis inhibitors and anti-apoptotic antibodies.

The present study tested the hypothesis that injection of anti-GPIIb antibody will induce apoptosis in mouse platelets and that treatment with IVIg will inhibit platelet apoptosis thus potentially ameliorating ITP.

Materials and methods

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and treated as described in Fig 1. Blood from saphenous veins was collected into capillary tubes and 100 μ l of blood was diluted in 900 μ l of buffer A [145 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgCl₂, 5.6 mmol/l glucose, 0.1% bovine serum albumin (BSA), 10 mmol/l HEPES, pH 7.4], containing 0.42% sodium citrate.

The platelet count was determined in blood diluted (1:10) with buffer A, containing sodium citrate using a Coulter LH750 Analyzer (Beckman-Coulter, Miami, FL, USA). Platelet-rich plasma (PRP) was prepared by centrifugation of the 1:10 diluted blood for 5 min at 209 g for determining platelet apoptosis by flow cytometry (Leytin *et al*, 2004).

Depolarisation of $\Delta\Psi_m$ was determined using the cell-penetrating lipophilic cationic fluorochrome JC-1 (Molecular Probes, Eugene, OR, USA) which accumulates in the mitochondrial matrix, driven by $\Delta\Psi_m$ (Kroemer & Reed, 2000), and expressed as an increase of green-to-red (G/R) fluorescent ratio reflecting the decrease in content of JC-1 aggregates when the inner mitochondrial membrane becomes depolarised (Leytin *et al*, 2004). The JC-1 stock solution [2 mg/ml in dimethylsulphoxide (DMSO); Sigma Chemical Co., St Louis, MO, USA) was diluted with buffer B [phosphate-buffered saline (PBS; Sigma) supplemented with 1 mmol/l MgCl₂, 5.6 mmol/l glucose, 0.1% BSA, 10 mmol/l HEPES, pH 7.4] to a final concentration of 1 μ g/ml, mixed by vortex, incubated in the dark for 15 min at room temperature and centrifuged for 15 min at 5996 g in 1.5 ml eppendorf tubes in an Eppendorf 5415C microfuge (Eppendorf, Westbury, NY, USA). The supernatants were harvested, combined and used immediately in the $\Delta\Psi_m$ depolarisation assay as the JC-1 working solution. Aliquots (40 μ l) of PRP were incubated in the dark for 45 min at 37°C with 460 μ l of JC-1 working solution and samples were acquired on a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA).

Caspase-3 activation was measured by the cell-penetrating carboxyfluorescein-labelled fluoromethyl ketone tetrapeptide (FAM-DEVD-FMK; Chemicon International, Temecula, CA, USA) that specifically and covalently binds to active caspase-3 (Piguet & Vesin, 2002; Leytin *et al*, 2004). FAM-DEVD-FMK was dissolved in 50 μ l of DMSO to create a 150x stock solution, according to the manufacturer's recommendation, diluted (1:24) with PBS, pH 7.4, mixed by vortex and used immediately in caspase-3 activation assay. Aliquots (40 μ l) of PRP were incubated in the dark for 1 h at 37°C with 10 μ l of the 1:24 diluted FAM-DEVD-FMK solution, 450 μ l of buffer A was added and samples were analysed by flow cytometry.

Phosphatidylserine exposure on the platelet surface was determined by dual colour assay using concurrent staining of platelets with fluorescein isothiocyanate (FITC)-conjugated annexin V and phycoerythrin (PE)-conjugated hamster anti-mouse CD61 monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA, USA). Aliquots (30 μ l) of PRP, further diluted (1:6) with buffer A, were mixed with 10 μ l of buffer A containing 8 mmol/l of CaCl₂ and incubated in the dark for 15 min at room temperature with a mixture of 2.5 μ l annexin V-FITC and 2.5 μ l anti-CD61-PE. Buffer A (300 μ l) containing 2 mmol/l of CaCl₂ was then added and samples were analysed by flow cytometry using fluorescence-2 threshold to acquire platelet (CD61)-positive events (Leytin *et al*, 2004).

Results and discussion

Figure 1A shows that injection of anti-mouse GPIIb antibody induced marked thrombocytopenia in C57BL/6 mice, resulting in a drop of peripheral platelet count to $23.4 \pm 1.9\%$ of the mean value in untreated animals ($P < 0.0001$). Administration of non-immune IgG (used as a negative control for anti-GPIIb) did not induce thrombocytopenia. However, injection of 50 mg IVIg per mouse (equivalent to a dose of 2 g/kg body weight, used for ITP treatment in humans) significantly ameliorated anti-GPIIb-induced thrombocytopenia ($P < 0.0001$).

After injection of anti-GPIIb, platelets showed significantly higher caspase-3 activation (Fig 1B, $P = 0.02$), PS exposure (Fig 1C, $P = 0.04$) and $\Delta\Psi_m$ depolarisation (Fig 2, $P = 0.02$). Treatment with IVIg ameliorated the anti-GPIIb-induced caspase-3 activation ($P = 0.04$) and PS exposure ($P = 0.03$) in the platelets (Fig 1B and C), but did not inhibit $\Delta\Psi_m$ depolarisation (Fig 2, $P = 0.42$).

Two main pathways have been shown to lead to apoptosis in nucleated cells. The first, is the extrinsic pathway, which is initiated via the cell-surface death receptors, while the second is the intrinsic pathway, which is triggered by disruption of mitochondrial integrity including $\Delta\Psi_m$ depolarisation. Activation of executioner caspase-3 and PS exposure are downstream of the extrinsic death signalling and intrinsic mitochondrial disintegration pathways (Budihardjo *et al*, 1999; Kroemer & Reed, 2000; Leytin & Freedman, 2003). Aberrant exposure of PS residues on the outer plasma

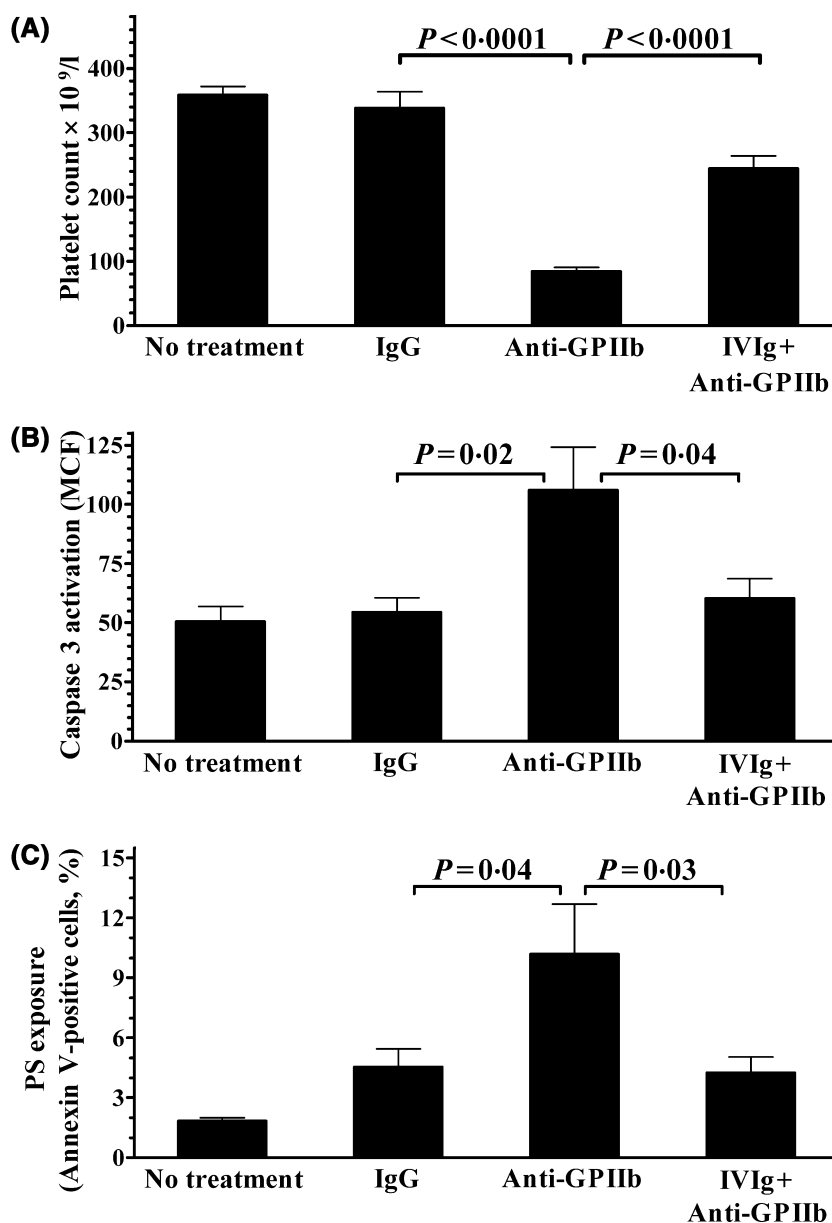


Fig 1. Administration of intravenous immunoglobulin (IVIg) ameliorates anti-glycoprotein (GP) IIb-induced immune thrombocytopenia (A), and inhibits caspase-3 activation (B) and phosphatidylserine (PS) exposure (C) in mouse platelets. In the anti-GPIIb-treatment group, thrombocytopenia was induced on day 1 by intraperitoneal injection of 2 μ g rat anti-mouse GPIIb (MWReg30, IgG1; PharMingen, Mississauga, ON, Canada) in 200 μ l of phosphate-buffered saline (pH 7.2). In the control IgG-treatment group, 2 μ g of non-immune rat IgG was injected on day 1. In the IVIg + anti-GPIIb-treatment group, mice were injected intraperitoneally on day 0 with 500 μ l of 10% IVIg (50 mg/mice, 2 g/kg; Gamimune 10%; Bayer, Elkhart, IN, USA), followed by injection of 2 μ g anti-GPIIb on day 1. Platelet enumerations and apoptosis tests were assayed on day 2. In the 'no treatment' group, mice were bled before administration of IgG, anti-GPIIb or IVIg + anti-GPIIb, and platelet count and apoptosis were analysed on day 0. Platelet caspase-3 activation was quantified as the mean channel fluorescence (MCF) of FAM-DEVD-FMK-labelled platelets. Platelet PS exposure was determined by annexin V-binding assay as the percentage of annexin V-positive cells. Results of three independent experiments, each using two mice per group, are presented. Hence, mean \pm SEM for six mice per group are shown and *P*-values between indicated groups were calculated by unpaired *t*-test; for 'no treatment' versus IgG-treatment groups, *P* = 0.51 (A), *P* = 0.67 (B) and *P* = 0.04 (C).

membrane leaflet of nucleated cells allows for the phagocytic recognition and elimination of apoptotic cells (Budihardjo *et al*, 1999; Kroemer & Reed, 2000; Leytin & Freedman, 2003). In human platelets stimulated with calcium ionophore A23187, caspase-3 activation and PS exposure are also

downstream of $\Delta\Psi_m$ depolarisation (Leytin *et al*, 2004). The data presented in this study indicate that IVIg does not affect upstream breakdown of $\Delta\Psi_m$ in platelets (Fig 2), but does inhibit downstream apoptotic events, caspase-3 activation (Fig 1B) and PS exposure (Fig 1C).

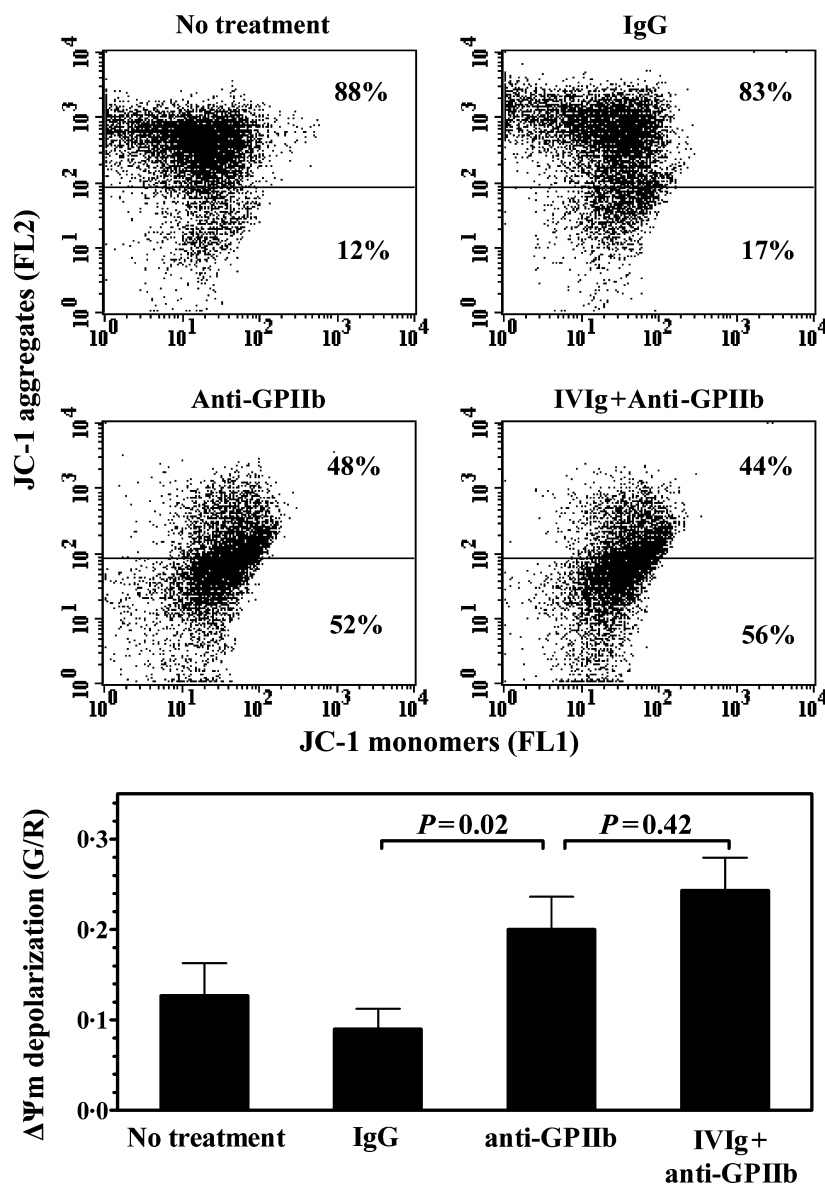


Fig 2. Administration of anti-glycoprotein (GP) IIb induces depolarisation of mitochondrial inner transmembrane potential ($\Delta\Psi_m$) in mouse platelets, but intravenous immunoglobulin (IVIg) does not inhibit $\Delta\Psi_m$ depolarisation. Mice were either untreated or injected with non-immune IgG, anti-GPIIb or IVIg + anti-GPIIb as described in Fig 1. Platelet $\Delta\Psi_m$ depolarisation was determined using the JC-1 fluorescent probe. In the upper and middle panels, representative flow cytometric dot plots are shown and percentages of platelets containing high or low levels of JC-1 aggregates presented. In the lower panel, depolarisation of platelet $\Delta\Psi_m$ was quantified as the green-to-red (G/R) fluorescence ratio. Results of three independent experiments, each using two mice per group, are presented. Hence, mean \pm SEM for six mice per group are shown and *P*-values between indicated groups were calculated by unpaired *t*-test; for 'no treatment' versus IgG-treatment groups, *P* = 0.41.

Nieswandt *et al* (2000) demonstrated that the specificity of the target antigen recognised by antiplatelet antibodies determined the mechanism of phagocytic clearance of platelets in murine ITP. They have shown that ITP is induced by injection of intact anti-GPIIb/IIIa monoclonal antibodies, but not F(ab)₂ fragments, indicating Fc-dependent mechanism of platelet phagocytosis provoked by anti-GPIIb/IIIa. In contrast, ITP induced by anti-GPIb α monoclonal antibodies is Fc-independent. Although several mechanisms have been proposed to explain the success of IVIg therapeutic treatment in ITP, the

most accepted mechanism is the competitive inhibition of Fc receptors on phagocytic cells (reviewed by Lazarus & Crow, 2003). In a murine ITP model, we have shown that IVIg does not inhibit the binding of monoclonal anti-GPIIb to platelets both *in vitro* and *in vivo* (Crow *et al*, 2001). Taken together, these data suggest that: (i) platelet apoptosis induced by anti-GPIIb is an Fc-receptor-mediated process, i.e. a process dependent on the interactions of Fc fragments of anti-GPIIb antibodies with Fc receptors on phagocytic cells; and (ii) the suppressive effect of IVIg on anti-GPIIb-induced downstream

platelet apoptotic events is the result of inhibition of Fc fragment–Fc receptor interactions, rather than inhibition of anti-GPIIb binding to platelets.

In summary, we have shown that *in vivo* administration of an antibody against GPIIb is associated with apoptotic changes in mouse platelets characterised by the dissipation of mitochondrial transmembrane potential, activation of caspase-3 and exposure of PS. However, IVIg treatment significantly inhibited platelet caspase-3 activation and PS exposure. While the extent of the role played by apoptosis in the pathogenesis of ITP remains unknown, these results suggest a novel mechanism of IVIg action involving anti-apoptotic effects in platelets that may contribute to the amelioration of ITP.

Acknowledgements

This work was supported by a grant from the Bayer–Canadian Blood Services–Héma-Québec–CIHR Partnership Fund.

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