Effect of SR121566A, a Potent GP IIb-IIIa Antagonist, on the HIT Serum/heparin-Induced Platelet Mediated Activation of Human Endothelial Cells

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Summary

Heparin-induced thrombocytopenia (HIT) is a common adverse effect of heparin therapy that carries a risk of serious thrombotic events. This condition is caused by platelet aggregation, which is mediated by anti-heparin/platelet factor 4 antibodies. Sera from patients with HIT in the presence of platelets, induced the expression of E-selectin, VCAM, ICAM-1 and tissue factor and the release of IL1β, IL6, TNFα and PAI-1 by human umbilical vein endothelial cells (HUVECs) in vitro and initiated platelet adhesion to activated HUVECs. These effects which occurred in a time-dependent manner were significant in the first 1-2 h of incubation and reached a maximum after 6 to 9 h. The GP IIb-IIIa receptor antagonist SR121566A which has been shown to block platelet aggregation induced by a wide variety of agonists including HIT serum/heparin, reduced in a dose-dependent manner the HIT serum/heparin-induced, platelet mediated expression and release of the above mentioned proteins. The IC50 for inhibition of HIT serum/heparin-induced platelet dependent HUVEC activation by SR121566A was approximately 10-20 nM. ADP, but not serotonin release, also heparin-induced platelet dependent HUVEC activation by SR121566A. The IC50 for inhibition of HIT serum/heparin, reduced in a dose-dependent manner the HIT serum/heparin-induced, platelet mediated expression and release of the above mentioned proteins. These data described that GP IIb-IIIa inhibitors can potently block platelet aggregation induced by HIT serum/heparin and suggested that they can be used for the treatment of patients with HIT or as prophylaxis for HIT in those who are likely to produce a HIT response.

In our study, we sought to determine whether heparin-dependent antibodies in the serum of patients with HIT initiate platelet-endothelial cell interactions that trigger procoagulant/proadhesive reactions predisposing the patients to thrombosis. We then examined the effect of SR121566A, a potent and selective GP IIb-IIIa inhibitor (11) which has been already shown to strongly reduce HIT serum/heparin-induced platelet aggregation in vitro (9, 10), on HIT serum-induced, platelet mediated activation of HUVECs.

Introduction

Heparin-induced thrombocytopenia (HIT) is a complex clinical syndrome in which individuals sensitized to heparin are susceptible to potentially life-threatening thrombocytopenia and paradoxical venous or arterial thrombosis (1). The mechanism for the thrombocytopenia is believed to be immunological as demonstrated by the presence of a heparin-dependent platelet antibody in the plasma of these patients (2). It has been postulated that platelet activation is caused by a heparin/anti-heparin IgG complex which activates platelets via their Fc receptors (CD32) (3, 4). More recently, it has been shown that this antibody reacts with platelet factor 4 (PF4) complexed with heparin in solution or with glycosaminoglycan molecules on the surface of human umbilical vein endothelial cells (HUVECs) (5). HUVECs, known to express heparin-like glycosaminoglycans on their surface, were recognized by antibodies purified from the plasma of HIT patients in the presence of PF4 alone (5); this reaction was inhibited by excess heparin, but not by anti-FcγRII, an antigen which has been shown not to be expressed at the surface of these cells (6). Stimulation of the production of tissue factor at the surface of endothelial cells by serum samples from HIT patients suggested that immune injury to endothelial cells may have a role in the development of thrombosis in some patients after heparin therapy (7). To date, attempts to treat HIT with platelet function inhibiting agents such as aspirin, dipiridamole and iloprost have met with variable success and it is only recently that agents that bind to the GP IIb-IIIa receptor have been shown to inhibit platelet aggregation in the presence of heparin and heparin-associated antibodies (8-10). These data described that GP IIb-IIIa inhibitors can potently block in vitro platelet activation and aggregation induced by HIT serum/heparin and suggested that they can be used for the treatment of patients with HIT or as prophylaxis for HIT in those who are likely to produce a HIT response.

Materials and Methods

Materials

Heparin (172 IU/mg, sodium salt from porcine intestinal mucosa), endothelial cell growth supplement, endotoxin, o-phenylenediamine dihydrochloride, apyrase and bovine serum albumin (Fraction V) were purchased from Sigma chemical Co (St Quentin Fallavier, France). SR121566A and SR46349 were from Sanofi Recherche (Toulouse, France). Human recombinant tissue thromboplastin (Inovin®; Dade) was from Baxter Diagnostics Inc. (Deerfield, IL, USA). PPSB, a concentrate of coagulation factors VII, X, II and V was from Pharmacia (Stockholm, Sweden). The monoclonal antibody to Fc receptors (IV.3) was from Medarex (Annandale, NJ). Fetal calf serum, ATP-S and all tissue culture reagents were from Boehringer Mannheim (Meylan, France).

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Serum Samples
Eighteen sera were obtained from patients at Loyola University Medical Center clinically diagnosed with HIT. HIT was identified in patients whose platelet count was less than 100,000 or who experienced a 50% decrease in platelet count for no apparent reason other than heparin administration (12). Normal human sera were collected from healthy volunteers not receiving heparin or other agents known to affect the coagulation system. These sera were aliquoted and stored at –80°C until use. Prior to use in any HIT assay, the sera were heated at 56°C for 1 h and centrifuged to remove any residual thrombin activity. Sera were designated as positive or negative based on their ability to promote platelet aggregation in a HIT aggregation system (10) and with regard to the so-called “SRA” assay (50°C-serotonin release for HIT-activated human platelets) and the titer of the anti-heparin/PF4 antibodies was determined using the HIPA ELISA assay (Diagnostica Stago, Asnières, France).

Preparation of the IgG Fraction
The IgG fraction of sera from 5 patients with HIT was purified by affinity chromatography using a protein G column (Pharmacia, Stockholm, Sweden) and was eluted as recommended by the manufacturer. The eluted fraction was dialyzed against 5 l of saline for 16 h. The volume of the dialysate was reduced to the original serum volume by a microconcentrator (Centricon 30, Amicon). As controls, 5 sera from healthy donors were prepared in a similar manner.

Preparation of Human Platelets
Human blood (25 ml) was collected by venipuncture from normal healthy volunteers who had not taken any medication for at least 10 days and transferred into a tube containing 5 ml ACD as an anticoagulant. Platelet rich plasma (PRP) was obtained by centrifuging the blood sample at 170 g for 20 min. at 20°C. Centrifugation of the PRP yielded a platelet pellet that was suspended in 30 ml of buffer A: 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 5 mM MgCl₂. The platelets were washed twice in buffer B by further centrifugation (2500 × g, 10 min, 4°C) and finally suspended in buffer B: 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM EDTA, 5 mM MgCl₂, 0.25% BSA (w/v) to give a final concentration of 2 × 10⁸ cells/ml.

Determination of Tissue Factor Activity on the Cells
HUVEC (passage 3-10) (Clonetics, Tebu, Le Perray, France) were cultured in 96-well culture plates as described above. Cells were then incubated 24 h at 37°C with serum from HIT patients (final concentration: 5%) or purified HIT-IgG (5 µg/ml), heparin (0.5 IU/ml) and platelets (10⁸ cells/ml) in the presence of saline or increasing concentrations of SR121566A. The medium was removed and wells were washed twice with 1 ml of phosphate buffered saline and incubated for 45 min at 37°C with 250 µl of M-199 containing PBS (0.44 U/ml FVII) and 100 µg/ml of substrate S2222. The optical density (OD) was measured at 405 nm. The tissue factor activity was obtained from a standard curve (log [OD₅₄₀/min] vs. log [U/ml]) using serial dilutions of human recombinant tissue thromboplastin in M-199 assayed as described above. Undiluted thromboplastin was arbitrarily assigned a value of 1 U/ml. The tissue factor activity was normalized to the cell counts from the same well and expressed as ng of tissue factor/10⁶ cells.

Induction and Detection of Cell Surface ICAM-1, E-selectin and VCAM
The expression of ICAM-1, E-selectin and VCAM was determined by ELISA. HUVEC (2–5 × 10⁵ cells/well) were seeded in 96-well tissue culture plates 48 h before the experiment performed in RPMI 1640 supplemented with 5% fetal calf serum and antibiotics. After incubation for 24 h at 37°C with serum from HIT patients (final concentration: 5%) or purified HIT-IgG (5 µg/ml), heparin (0.5 IU/ml) and platelets (10⁸ cells/ml) in the presence of saline or increasing concentrations of SR121566A, ICAM-1, E-selectin and VCAM expression was determined using specific monoclonal antibodies followed by goat anti-mouse conjugated peroxidase antibody (British Biotechnology Ltd., UK) and o-phenylenediamine dihydrochloride as substrate. The plates were read on a microtiter plate reader at 492 nm. All data were given as the mean ICAM-1, E-selectin and VCAM concentration ± SD (n = 6) calculated on the basis of a standard curve determined in parallel.

Release of IL-1β, IL6, PAI-1 and TNFα by HUVEC
HUVEC were cultured in 96-well culture plates as described above. Cells were then incubated 24 h at 37°C with serum from HIT patients (final concentration: 5%) or purified HIT-IgG (5 µg/ml), heparin (0.5 IU/ml) and platelets (10⁸ cells/ml) in the presence of saline or increasing concentrations of SR121566A. IL-1β, IL6, PAI-1 and TNFα levels in the supernatant were determined using ELISA kits from R&D systems (UK). Results shown are means ± SD (n = 6).

Fig. 1 Effect of serum from HIT patients on the expression of cell surface proteins and cytokines on HUVECs. Serum prepared from blood taken from HIT patients (final concentration: 5%) was incubated for the indicated periods of time with HUVEC (2.5 × 10⁵ cells) in the presence of 0.5 IU/ml heparin and saline (empty symbols) or washed human platelets (10⁸ platelets/ml) (full symbols). The expression (A) of E-selectin (○, ○ ICAM-1 (●, □), VCAM (△, △) and tissue factor (▲, ▲) and the release (B) of IL-1β (●, □), IL6 (▲, △) PAI-1 (○, ○) and TNFα (▲, ▲) was determined as described under “Materials and Methods”. Results are expressed as mean ± SD (n = 6).

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Platelet Adhesion to HUVECs

Washed human platelets (4 × 10^9 cells/ml) prepared as described above were incubated for 15 min at 37°C with 1 mCi 111 Indium chloride (114 mCi/mMole) (Amersham, Les Ulis, France) and tropolone (30 μg/ml). Labelled platelets were then washed once in buffer B to remove unbound radioactivity, and finally resuspended in the same buffer. Under these conditions, 95% of the counts were found to be associated with the platelet fraction, and the buffer radioactivity did not exceed 5%, even following 3 h incubation.

In order to test the effect of HIT serum/heparin on platelet adhesion to endothelial cells, HUVEC, were cultured in 96-well culture plates as described above. Cells were then incubated 18h at 37°C with serum from HIT patients (final concentration: 5%) or purified HIT-IgG (5 μg/ml), heparin (0.5 IU/ml), [111Indium]-labelled platelets (10^5 cells/ml) in the presence of saline or increasing concentrations of SR121566A. After 60 min at 37°C, unbound platelets were aspirated and the endothelial cells were washed extensively with buffer B. The HUVEC-bound platelets were then solubilized with 1N NaOH (1 ml/well) and the radioactivity of the lysate was measured in a gamma counter (Kontron, Model MR 480).

Statistical Analysis of Data

All data are expressed as mean ± 1 SD. Grouped data were analysed for comparison by the control group using the Mann-Whitney test. The level of significance was chosen as p < 0.05.

Results

HIT Serum/heparin-induced Expression of Membrane Proteins and Cytokines

Exposure of confluent HUVEC to sera prepared from blood obtained from patients clinically diagnosed with HIT, heparin (0.5 IU/ml) and washed human platelets (10^8 cells/ml) resulted in a significant increase of the expression of E-selectin, ICAM-1, VCAM and tissue factor at the cell surface (Fig. 1A). Under the same experimental conditions, treatment of HUVEC with these sera led to the release of IL-1β, IL6, PAI-1 and TNFα into the culture medium (Fig. 1B). These effects which occurred in a time-dependent manner were significant in the first 1-2 h of incubation and reached a maximum after 6 to 9 h. Platelets or heparin alone did not cause the expression and release of these proteins and, in the absence of washed human platelets, HUVEC activation was strongly reduced (Fig. 1). At the concentration tested, (50 μl/ml culture medium), the effect represented 70-80% of the effect of endotoxin (1 μg/ml). When a purified HIT-IgG was tested at a concentration of 5 μg/ml, a similar effect was observed but no activity was found for a purified IgG prepared from the serum of healthy donors (not shown).

Inhibition of HIT Serum/heparin-induced HUVEC Activation by SR121566A

Addition of SR121566A, a selective inhibitor of fibrinogen to the activated GP IIb-IIIa complex in the presence of HIT serum, heparin (0.5 IU/ml) and washed human platelets resulted in a concentration-dependent decrease in the amount of expressed E-selectin, ICAM-1, VCAM and tissue factor at the surface of HUVEC as well as a dose-dependent inhibition of the release of IL1β, IL6, PAI-1 and TNFα (Figs. 2A, 2B). A 50% inhibition of HIT serum/heparin-induced HUVEC activation was observed at inhibitor concentrations of approximately 10-20 nM. Increasing the concentration to 300 nM completely prevented the expression and release of these proteins (Fig. 2).

Importance of ADP and Serotonin in HIT Serum/heparin-induced HUVEC Activation

Apyrase, which converts ADP to AMP, and ATPγS which blocks the aggregating effect of ADP on platelets, were used to examine the
The importance of serotonin in the cascade of reactions leading to platelet activation by HIT serum/heparin has been evaluated using SR46349, a potent and selective 5-HT2 inhibitor on platelets (14). As shown in Fig. 3, SR46349 (1 μM) did not affect the expression of adhesive/coagulation proteins and the release of cytokines by HIT serum/heparin platelet-activated HUVECs.

Effect of HIT Serum/heparin-induced HUVEC Activation on Platelet Adhesion

As shown in Fig. 4, an 18-h incubation with HIT positive serum in the presence of heparin (0.5 IU/ml) resulted in a significant increase of the adhesion of 111In-labelled platelets. This represented a 9-fold increase (P > 0.001) compared to the adhesion of platelets in the presence of normal serum. Addition of SR121566A (1 μM), simultaneously with HIT serum/heparin almost totally inhibited platelet adhesion and the subsequent aggregation to activated HUVECs (95% inhibition; P < 0.001) whereas the addition of apyrase (400 U/ml) or ATPγS (50 μM) only slightly affected (37% and 24% respectively; P > 0.05) 111In-labelled platelet adhesion to HUVECs (Fig. 4).

Discussion

HIT is thought to be mediated by immunoglobulins that activate platelets in the presence of pharmacologic concentrations of heparin, but the molecular basis if this relatively common and often serious complication of heparin therapy has not been completely established. Several studies have suggested that antibodies against the heparin/PF4 complex may be the cause of this syndrome (5). These antibodies presumably bind the heparin/PF4 complex via the Fab portion of the antibody and cause platelet activation via binding to the FcγRII receptors on the platelet surface, though direct evidence of immune complex binding to platelets has not been presented (5, 15). Cross-linking the platelet FcγRII receptor has been shown to result in the activation of phospholipase C, a common mechanism involved in the expression of the activated GP IIb-IIIa complex at the platelet surface (16). Inhibitors of the GP IIb-IIIa complex have been shown to block the aggregation response induced by a variety of agonists including ADP, arachidonic acid, collagen, thrombin, thrombin receptor activating peptide... and we and others have shown that GP IIb-IIIa inhibitors are also capable of blocking the in vitro activation/aggregation response of platelets to HIT serum/heparin (8-10).

Since it has been previously shown that sera from patients with HIT and thrombosis contain antibodies that react with endothelial cells (7), we sought to determine whether heparin-dependent antibodies in the serum of patients with HIT initiate platelet-endothelial cell interactions that trigger procoagulant/proadhesive reactions predisposing the patients to thrombosis. We then examined the effect of SR121566A, a potent and selective GP IIb-IIIa inhibitor (11) which has been already shown to strongly reduce HIT serum/heparin-induced platelet aggregation in vitro (9,10), on HIT serum-induced, platelet mediated activation of HUVECs.

Our results indicate that, in addition to platelet-activating effects, serum from patients with HIT, in the presence of heparin and washed human platelets, induced at the surface of cultured HUVECs, the expression of several proteins involved in inflammatory cell recruitment (E-selectin, VCAM, ICAM-1) and in the elaboration of a procoagulant activity of the endothelium (tissue factor). HIT sera, in the presence of heparin also influenced the release of inflammatory cytokines such as IL1β, IL6 or TNFα as well as PAI-1, a potent inhibitor of
fibrinolysis. This effect of HIT serum/heparin seemed not to be due to a direct effect of the anti-heparin/PF4 antibodies with the glycosaminoglycans present at the surface of endothelial cells as suggested earlier (7). Indeed, in the absence of platelets, endothelial cells, incubated with serum from HIT patients elaborated very low levels of adhesion/coagulation proteins and cytokines. Since the addition of exogenous PF4 did not increase this response, it is therefore most improbable that heparan sulfate, which has been shown to be present at the surface of cultured HUVECs (17), may compose part of the antigenic site responsible for the activation of these cells by anti-heparin/PF4 antibodies present in HIT sera. Direct interaction of anti-heparin/PF4 antibodies with FcγRII receptors on HUVECs has also to be excluded since such a protein has been shown not to be expressed at the surface of these cells (6). This was indeed confirmed by the lack of effect of a 1-h pre-incubation of HUVECs with 100 μg/ml of the monoclonal antibody (IV.3) to Fc receptors (5% inhibition, p > 0.05). At this concentration, when co-incubated with platelets in the presence of serum from HIT patients, the antibody totally inhibited platelet-induced HVEC activation (100% inhibition, p < 0.001). The presence of platelets seemed therefore to be an absolute requirement for HIT serum/heparin to induce the activation of HUVECs. This was ascertained by the fact that SR121566A, a potent and selective inhibitor of GP IIb-IIIa (11), strongly inhibited the HIT serum/heparin-induced activation of HUVECs in the presence of platelets only. In that respect, the ability of SR121566A to decrease platelet-mediated activation of HUVECs can be ascribed to its ability to strongly decrease HIT serum/heparin-induced platelet activation/aggregation which occurred in the same range of doses (9) but also, as reported by Jeske et al., to reduce the release of the content of activated platelet granules (10) which occurs following activation of platelets with HIT serum/heparin. Indeed, platelet granules have been shown to contain several elements, among them PF4, serotonin and ADP which have been shown to activate endothelial cells and in particular to induce the expression of tissue factor at the surface of HUVECs (18).

Along these lines, another interesting observation of our work is that ADP may also be a link in the mediation of platelet aggregation induced by HIT serum/heparin since removal of ADP by apyrase or inhibition of the effect of ADP at the level of its receptor by ATP Y strongly interfered with HIT serum/heparin-induced aggregation (2, 9) and platelet-mediated HVEC activation. Similar results have been reported by De Gaetano et al. (19) with an immunoglobulin preparation of a patient with idiopathic thrombocytopenic purpura.

Since SR121566A has been shown to reduce HIT serum/heparin-induced serotonin release by HIT-activated platelets (9, 10), and serotonin has been shown to activate HUVECs (20), we studied the effect of SR46349, a potent 5-HT2 receptor antagonist (14). This compound did not affect HIT serum/heparin-induced, platelet-dependent HVEC activation, showing that serotonin can be excluded as playing a role in the cascade of events leading to HVEC activation following platelet activation (and release) in response to anti-PF4/heparin antibodies. It is noteworthy that this compound did not affect the HIT serum/heparin-induced platelet aggregation in vitro (not shown).

In an attempt to gain further insight into the mechanisms by which SR121566A and ADP blockers affect HIT serum/heparin-induced, platelet-dependent activation of HUVECs, we determined their effects on platelet adhesion to activated endothelial cells. SR121566A, as a result of its potent antiaggregating effects but also by interfering at the level of the platelet-endothelium interactions which has been found to be mainly mediated by GP Ib-IIIa (21), strongly inhibited platelet adhesion to HUVECs. Apyrase and ATP Y were much less potent inhibitors probably because they only affected the amplification process of platelet activation and did not interfere with platelet adhesion itself. These differences in efficacy on platelet adhesion may explain the differences observed for these compounds to affect HIT-induced platelet aggregation (2, 9, 10) and HVEC activation.

These observations, therefore, show that platelet-dependent immune injury of endothelial cells may play an important role in the seemingly paradoxical occurrence of thrombocytopenia and thrombosis in some patients treated with heparin. Moreover, these new results extend our observations and other's showing that GP Ib-IIIa inhibitors and ADP blockers, by effectively inhibiting HIT serum/heparin-induced platelet aggregation, can be of clinical interest for the treatment of patients with HIT or as prophylaxis for HIT in those who are likely to produce a HIT response.

References


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Vitamin K Deficiency Bleeding (VKDB) in infancy has a high incidence of intracranial bleeding. Vitamin K given intramuscularly at birth is the most effective way of prophylaxis, but since it was suspected to increase the risk of childhood cancer it was replaced in many countries by oral Vitamine K with consequent increase in VKDB. To solve this dilemma internationally renowned experts presented their controversial standpoints. The facts reported serve as base for evaluation of the ideal prophylaxis.