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Low Density Lipoprotein Apheresis Reduces PF4 on the Surface of Platelets: a Possible Protective Mechanism Against HITT

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Abstract

Background—Heparin-induced thrombocytopenia and thrombosis (HITT) is characterized by thrombocytopenia due to the formation of antibodies against heparin:platelet factor 4 (PF4) complexes. Despite the exposure to heparin during treatment and predisposition of patients with atherosclerosis to HITT, HITT in patients undergoing LDL apheresis is rare. We investigated the possibility that LDL apheresis decreases PF4 on platelet surfaces and/or plasma HITT antibody levels, either of which would disfavor HITT.

Study Design and Methods—We enrolled 25 patients undergoing LDL apheresis at the Hospital of the University of Pennsylvania. Blood samples were drawn before and after treatment. Plasma samples were drawn proximal and distal to the LA-15 treatment column. PF4, HITT antibodies, heparin levels and P-selectin were measured.

Results—No patient had clinical symptoms of HITT. The LA-15 column was found to efficiently remove PF4. PF4 levels in peripheral blood plasma did not change significantly after LDL apheresis. However, platelet surface PF4 significantly decreased after treatment. HITT antibodies were found in only 2 patients and were nonfunctional. Platelet surface P-selectin did not change during treatment.

Conclusion—We have demonstrated that LDL apheresis via dextran sulfate adsorption removes plasma PF4 and reduces the amount of PF4 on the surface of circulating platelets. Reduced surface PF4 may decrease antibody formation and/or recognition by HITT antibodies. These data provide a potential explanation for the near lack of HITT in hypercholesterolemic patients undergoing LDL apheresis. They also suggest the possibility that LDL-apheresis using dextran sulfate adsorption may have therapeutic value in the treatment of HITT.

Keywords

LDL apheresis; HITT; PF4

Introduction

Heparin-induced thrombocytopenia and thrombosis (HITT) is a transient autoimmune disorder characterized by a >40% drop in the platelet count 5 to 10 days after starting heparin therapy ¹. HITT occurs in about 1-5% of patients receiving heparin for more than a week ². Of these patients, venous and/or arterial thrombosis occurs in approximately one

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third to one half of patients ^{1,3,4}. The mortality rate is ~20% and ~10% of patients require limb amputations or suffer other major morbidity ⁵⁻⁷.

The pathogenesis of HIT is characterized by the following three-step process: 1) at a therapeutic concentration of unfractionated heparin (UFH) ranging from 0.1 to 1.0 U/ml, heparin administration induces a release of platelet factor 4 (PF4) from glycosaminoglycans of the endothelial layer and from platelets; 2) PF4 binds to heparin and the complex becomes immunogenic; 3) the anti-heparin/PF4 antibody complex interacts with platelet FcγRIIa leading to platelet activation, thrombin generation and procoagulant microparticle generation. PF4 released from activated platelets can then form additional complexes with heparin and facilitate the generation of additional antibodies inducing platelet activation and thrombosis ^{5,6}.

PF4 is a platelet-specific chemokine released in large amounts by activated platelets that binds heparin with high affinity and that exists as a tetramer at concentrations found at sites of platelet activation ⁸. Reilly et al demonstrated, through the development of a transgenic mouse model of HIT, that heparin, PF4, anti-PF4-heparin antibody and platelet FcγRIIa are necessary and sufficient to recapitulate the salient features of HIT in vivo ⁹. Our lab has shown that UFH and tetrameric PF4 form ultralarge (>670kDa) complexes (ULC) only over a narrow molar range with an optimal ratio of heparin to PF4 of approximately 1:1 ¹⁰. Mutation studies showed that formation of ULCs required the presence of PF4 tetramers ¹⁰. Changes in the molar ratio of heparin to PF4 by as little as 40% reduces the proportion of ULCs that is formed with a simultaneous increase in the proportion of smaller complexes ¹⁰. Increasing or decreasing the concentrations of heparin relative to PF4 lead to a loss of antigenicity ¹¹. The antigenicity of the complex depends on the molar ratio of the reactants as well as on the length, chemical composition and structure of the glycosaminoglycan itself ¹².

Heparin is the anticoagulant used in all lipid apheresis systems in the United States. LDL apheresis is most commonly used for patient with familial hypercholesterolemia and is an important treatment option for those patients with severe hypercholesterolemia who do not achieve adequate LDL cholesterol reduction with standard approaches such as dietary modification and statins ¹³.

Several different types of LDL apheresis systems have been developed. Heparin-induced extracorporeal LDL/fibrinogen precipitation (HELP) (Braun, Melsungen, Germany) was first reported in 1983. Plasma and blood cells are separated by a hollow fiber plasma separator and the precipitates are removed by filtration through a polycarbonate filter ¹⁴⁻¹⁶. In 1987, Mabuchi et al ¹⁷ reported on dextran sulfate. This method of LDL removal is commonly performed using the Liposorber® LA-15 system (Kaneka, Osaka, Japan) that utilizes two LDL adsorption columns containing dextran sulfate-cellulose beads that remove apo-B containing lipoproteins ¹⁷⁻¹⁹. In 1993, Bosch et al ²⁰ described a low-density lipoprotein hemoperfusion (direct adsorption of lipoproteins, DALI: Fresenius, St. Wendel, Germany) system. The DALI system is compatible with human whole blood and uses polyacrylate-coated polyacrylamide columns that do not require regeneration for the adsorption of cholesterol, LDL, Lp(a) and triglyceride ²¹.

Patients undergoing LDL apheresis receive treatments as frequent as once a week. Despite the exposure to large amounts of heparin and the predisposition of patients with vascular diseases such as atherosclerosis to HIT, the incidence of HIT among this patient population is very rare. A search of the literature (Pubmed) revealed one case report of HIT in a 50 year old male with a history of hypercholesterolemia, hyperlipoproteinemia, diabetes and premature CHD, who underwent HELP-LDL apheresis treatment weekly for

more than 100 treatments²². That HITT is not more common in this high risk patient population was somewhat unexpected. We, therefore, investigated the possibility that the LDL apheresis procedure decreases PF4 adsorbed to cell surfaces, which is the pathologic target for HITT antibodies, and/or plasma HITT antibody levels, either of which would disfavor HITT.

Materials and Methods

Study population and design

The study population included 25 patients receiving lipoprotein apheresis treatment at the Apheresis and Infusion Clinic at the Hospital of the University of Pennsylvania from October 1999 to August 2010. All patients were >18 years old with a history of familial hypercholesterolemia, medication intolerance or high levels of Lp(a). This study was approved by the University of Pennsylvania IRB, and written informed consent was obtained from the study participants. Inclusion criteria included age greater than 18 years, receiving LDL apheresis for clinical indications at the time of enrollment and ability to read, understand and sign the informed consent. Exclusion criteria included age less than 18 years, no desire to participate in the study and pregnant females.

LDL apheresis was carried out according to standard procedures using the Liposorber[®] System (Kaneka Pharma America LLC, New York City, NY) which uses a heparin anticoagulant. Although heparin dosing varies somewhat for each patient, a typical heparin loading dose was 30-40U/kg followed by 3000-4000 units/hour for a typical total heparin exposure of 8000-12000 units per treatment. Approximately 15-20 ml of blood was drawn into 3-4 blue top Vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ) tubes with citrate anticoagulant prior to and after the completion of LDL apheresis treatment. These samples were labeled as pre-treatment, post-treatment, or thirty minute (30') post-treatment samples. After ~350-500 ml of plasma had been processed by the Liposorber[®] System (~10-15 minutes), 50 ml of plasma was collected proximal and distal to the dextran sulfate LDL-adsorption column. These samples were labeled as pre-column and post-column samples, respectively. Samples were identified by study ID numbers and kept at room temperature prior to processing to prevent platelet activation. All samples were processed within 1 hour of collection.

Platelet poor plasma (PPP) preparation

Whole blood samples were centrifuged twice at 2000 g for 10 minutes in a swinging bucket rotor. The supernatant was then transferred to a fresh 15ml conical tube. Small aliquots were made and all tubes were frozen at -80°C until analysis. Plasma samples collected in 50ml conical tubes were aliquoted and frozen at -80°C until analysis.

Platelet factor 4 (PF4) measurement

PF4 was quantitated using the ZYMUTEST PF4 ELISA kit (Hyphen BioMed, Neuville-sur-Oise, France) according to manufacturer's instructions with slight modifications. Briefly, 200 µl PF4 standard (supplied by the manufacturer) or diluted patient samples were introduced into each well in duplicate and incubated for 1 hour at room temperature (RT). Patient samples were diluted as follows to ensure that all measurements were in assay range: pre-treatment and post-treatment samples, 1:20; pre-column samples, 1:100; and post-column samples, 1:2. Each well was then washed five times with 300 µl wash solution. 200 µl conjugate (anti-PF4 polyclonal antibody coupled with peroxidase) was added into each well and incubated for 1 hour at RT. After washing the wells five times, substrate (TMB/H₂O₂) was added and incubated in each well for 2 minutes at RT. The reaction was stopped with 50 µl 0.45 M sulfuric acid and the absorbance at 450 nm was measured.

Anti-PF4-heparin antibody (HITT antibody) measurement

HITT antibodies were measured using the PF4 Enhanced kit (GTI Diagnostics, Waukesha, WI) according to manufacturer's instructions. Briefly, 300 μ l of working wash solution was added to each well and incubated for 10 minutes at RT. 50 μ l of diluted (1:50) positive control, negative control and patient samples were introduced into each well in duplicate and incubated for 45 minutes at 37°C. The wells were then washed 4 times with 300 μ l working wash solution. 50 μ l diluted conjugate (goat anti-human immunoglobulin (IgG/A/M) conjugated to alkaline phosphatase) was added to each well and incubated for 45 minutes at 37°C. The wells were washed 4 times and incubated with 100 μ l of substrate (p-nitrophenyl phosphate) in the dark for 30 minutes at room RT. The reaction was stopped by adding 100 μ l of 3M sodium hydroxide stopping solution and the absorbance at 405 nm was measured.

Serotonin release assay

The serotonin release assay was performed as previously described²³. Briefly, platelet rich plasma (PRP) from healthy donors was incubated with 0.5 μ l carbon-14 labeled 5-hydroxytryptamine creatinine sulfate (GE Life Sciences, Piscataway, NJ) per milliliter of PRP for 20 minutes at 37°C. Serotonin uptake was inhibited by adding 1 mmole/ml imipramine (Sigma-Aldrich, St. Louis, MO) to the PRP.

Sera from subjects with a positive HITT antibody ELISA were tested for heparin dependent serotonin release. Negative and positive controls contained sera from patients previously known to have negative or positive serotonin release, respectively. Five different concentrations of heparin (0.05 units/ml, 0.1 units/ml, 0.5 units/ml, 1 unit/ml and 2 units/ml) (Abbott Laboratories, Abbott Park, IL) were studied. In addition, a positive ADP control was also performed (in the absence of heparin). The percent release was calculated for all conditions as previously described²³. The normal range is 0-5%. A serotonin release >10% in the presence of 0.5-2 U/ml heparin was considered positive.

Flow cytometric measurement of platelet surface antigens

2 μ l of whole blood samples collected in citrate anticoagulant was incubated with PE labeled anti-P-selectin, PerCP-Cy5.5 labeled anti-CD41a and Alexa fluor 488 labeled anti-PF4 antibodies for 30 minutes at room temperature. The cells were then fixed with 1% paraformaldehyde solution (BD Cytotfix, Franklin Lakes, NJ) for 30 minutes at 4°C. Flow cytometric analysis of platelet surface antigens was performed on an LSRII flow cytometer (BD, Franklin Lakes, NJ) and the data were analyzed using FlowJo version 7.6.1. The geometric mean fluorescence intensity of PF4 and P-selectin were compared between samples.

Heparin anti-Xa chromogenic assay

Heparin concentration in plasma samples were measured using the heparin anti-Xa chromogenic assay (Diagnostica stago, Asnières-sur-Seine, France) and the Coag-A-Mate II (Biomerieux, Marcy l'Etoile, France) instrument according to the manufacturer's instructions. Briefly, plasma was diluted 1:9 in factor Xa buffer and warmed to 37°C. Heparin antithrombin III reagent is then added after which factor Xa is added to the reaction. The reaction was allowed to proceed for 60 seconds at 37°C. Heparin substrate was then added and the absorbance change was monitored for 30 seconds.

Statistical analysis

Data were plotted and statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA) and STATA (StataCorp LP, College station, TX). A 2-tailed Student's t test was used to compare mean values between 2 paired samples.

ANOVA was used to determine if differences among 3 repeated-measures variable samples were present and the Wald test was used to determine which pairs were significantly different. $P < 0.05$ was considered statistically significant.

Results

Twenty-five patients were enrolled in this study, with demographic information available from 24 patients (Table 1). There were more males than females (15 vs 9). The mean age for males was 51 years and the mean age for females was 60 years. Patients were diagnosed with familial hypercholesterolemia ($n=21$, 87.5%), high lipoprotein (a) ($n=2$, 8.3%) or primary biliary cirrhosis ($n=1$, 4.2%). None of the patients had any clinical symptoms of HIT.

Virtually all of the PF4 in the plasma being treated was removed by the LA-15 column (pre-column vs post-column plasma concentrations: 133.8 ± 88.26 IU/ml vs 0 IU/ml; $P < 0.0001$) (Figure 1A). PF4 levels in the plasma from peripheral blood drawn after completion of the treatment did not differ significantly from pre-treatment levels (pre-treatment vs post-treatment concentrations: 116.5 ± 79.14 IU/ml vs 110.8 ± 67.28 IU/ml; $P = \text{NS}$) (Figure 1B). However, the amount of PF4 on platelet surfaces was significantly decreased by LDL apheresis (pre-treatment vs post-treatment MFI: 46.32 ± 20.98 vs 31.37 ± 10.11 ; $P < 0.0001$). This difference was maintained for at least 30 minutes after completion of the treatment (30' post-treatment MFI 29.83 ± 10.35 ; $P < 0.0001$ compared to pre-treatment) (Figure 2).

A low level of heparin (0.05 IU/ml) was present in the pre-treatment plasma due to the use of heparin during peripheral intravenous line placement (Figure 3). As expected, the heparin concentration in the plasma increased significantly during treatment (pre-treatment vs post treatment, 0.05 ± 0.10 IU/ml vs 0.89 ± 0.09 IU/ml; $P < 0.0001$) Thirty minutes post treatment, although still significantly elevated, heparin levels began decreasing (0.79 ± 0.17 IU/ml; $P < 0.05$ compared to post-treatment).

Only 2 of 17 patients demonstrated HIT antibodies ($\text{OD} \geq 0.4$) (Figure 4). The serotonin release assay revealed that they were nonfunctional (serotonin release less than or equal to 5%). A trend toward a decrease in HIT antibodies was observed in the post-column plasma (pre-column vs post-column absorbance 0.19 ± 0.15 vs 0.15 ± 0.10 ; $P < 0.01$) as well as in the post-treatment peripheral blood plasma (pre-treatment vs post-treatment absorbance, 0.32 ± 0.40 vs 0.23 ± 0.24 ; $P = \text{NS}$) although the latter was not statistically significant.

Platelet surface P-selectin was measured as a marker of platelet activation. There were no significant changes in P-selectin on the surface of platelets before and after LDL apheresis treatment (pre-treatment vs post-treatment MFI, 49.07 ± 27.24 vs 45.14 ± 25.77 ; $P = \text{NS}$) (Figure 5). The level of another marker of platelet activation, sCD40L, was low in the plasma samples at baseline and remained low during treatment (data not shown). These data suggest that LDL apheresis via dextran sulfate adsorption does not significantly activate platelets.

Discussion

Patients undergoing LDL apheresis have a high rate of cardiovascular disease and are exposed to large amounts of heparin on a regular basis yet HIT has rarely been reported in this patient population. To date, only one case report has been published²² where HIT developed in a patient undergoing LDL apheresis using the HELP system. We have not observed HIT in our LDL apheresis patients. This is the first study to our knowledge that has systematically investigated a potential protective mechanism against HIT in patients undergoing LDL apheresis.

We previously showed that ultralarge (>670kDa) complexes (ULC) of PF4 and heparin that are formed over a narrow molar range with an optimal ratio of heparin to PF4 of approximately 1:1 are necessary for heparin dependent antibody binding and platelet activation that is central to the pathophysiology of HIT¹⁰. Increasing or decreasing the concentrations of heparin relative to PF4 or vice versa lead to a loss of antigenicity and lack of formation of HIT antibodies¹¹. Therefore, when patients are exposed to heparin, the risk of HIT is predicted to be greatest when the molar amount of heparin balances that of PF4. Importantly, PF4 also forms antigenic complexes with endogenous glycosaminoglycans (GAGs) on the surface of platelets similar to the ULC that are formed between unfractionated heparin and PF4 in plasma²⁴. Thus, decreasing PF4 bound to cell surfaces is expected to be beneficial for patients with HIT.

We show that plasma PF4 is efficiently removed by the LA-15 column. This removal can be explained by the charge characteristics of the column and PF4. The dextran sulfate that is immobilized on cellulose gel beads in the column is negatively charged so it may readily bind to the positively charged PF4. Because post-treatment plasma PF4 levels were similar to pre-treatment plasma PF4 levels, we hypothesized that PF4 from cell surfaces, which is the major reservoir of PF4 in vivo, had re-equilibrated with plasma PF4 to restore plasma PF4 levels. We show in figure 2 that platelet surface PF4 levels decreased significantly immediately after treatment and remained low 30 minutes after treatment ended. Based on the work of Rauova et al²⁴, we predict that this decrease in platelet surface PF4, at a time when heparin levels are elevated, significantly decreases antigenic complexes on the platelet surface and disfavors platelet clearance and/or activation. This decrease may also lessen the antigenic stimulus for antibody formation.

Patients undergoing LDL apheresis are exposed to 8000-12000 units of heparin per treatment. Not surprisingly, the heparin concentration in the plasma after treatment was significantly elevated compared to pre-treatment levels (figure 3). Heparin infusion is stopped approximately 45 minutes prior to the end of the procedure. Thirty minutes after the end of the procedure, heparin concentration in the plasma had begun to decrease, while platelet surface PF4 remained low (Figure 2).

In patients undergoing LDL apheresis, the molar ratio of heparin to PF4 may be shifted from the optimal 1:1 ratio in two ways. The first is by the administration of large amounts of heparin and the second is by decreasing PF4 on the surface of vascular cells, specifically platelets. Both ways result in a dramatic increase in the ratio of heparin to PF4 which should result in the formation of less ULC and a greater number of smaller complexes which are relatively benign¹⁰. This increased ratio persisted for at least 30 minutes after treatment, with heparin returning toward pretreatment levels in the presence of persistently lowered platelet surface PF4.

HIT antibodies were present in only two patients and these antibodies were not functional as determined by the serotonin release assay. No thrombocytopenia or thrombotic events have been reported by these two patients. This could be attributed to the reduction in platelet surface PF4 levels. Rauova et al demonstrated that cell surface PF4 complexes are antigenic only over a narrow concentration of PF4. If the optimal ratio of heparin/GAG to PF4 is exceeded, HIT antibody binding is impaired²⁴. Furthermore, PF4 on the platelet surface may also be important for HIT antibody formation. With a decrease in platelet surface PF4, the probability of formation of HIT antibodies is decreased due to an alteration of the heparin/GAG to PF4 ratio. However, further studies are required to address this possibility.

No significant change in platelet activation was detected in these patients after LDL apheresis as shown by a similarity in pre-treatment and post-treatment platelet surface P-

selectin levels (figure 5) as well as plasma sCD40L levels (data not shown), suggesting that the apheresis process should not aggravate or induce HIT by increasing PF4 release. Importantly, these platelets were activatable by a thrombin receptor activator peptide which caused a significant increase (14.5 fold, $P < 0.05$) in platelet surface P-selectin levels (data not shown).

The reported heparin dose administered during each dialysis session is approximately 6000 units, which is similar to the amount administered during LDL apheresis²⁹. Yamamoto et al²⁵ report that in patients newly enrolled for treatment with hemodialysis in Japan, the incidence of HIT was 3.9% (6/154). This is comparable to the overall reported incidence of HIT of 5%²⁶. The mean duration to the development of HIT after the initiation of hemodialysis was 18 days. However, patients undergoing chronic intermittent hemodialysis for 3 months or longer (mean period of dialysis, 70.3 ± 74 months; median period of dialysis, 41 months), the onset of HIT is rarely seen (0.6%)²⁷⁻²⁹. Thus, chronic hemodialysis patients represent another population in which HIT is relatively rare. One possible reason for this, similar to our patient population, is the repeated systemic exposure to large amounts of heparin which increases the heparin to PF4 ratio beyond the optimal ratio for antigenicity. It is not known if PF4 is also removed by hemodialysis. Other mechanisms may also be responsible for the low rates of HIT in patients undergoing chronic intermittent hemodialysis.

In conclusion, we provide a potential mechanism for the rare occurrence of HIT in hypercholesterolemic patients undergoing LDL apheresis. Removal of PF4 from the surface of platelets and plasma at a time when patients are exposed to heparin prevent the formation of pathogenic ULCs of heparin and PF4. Furthermore, the decreased surface levels of PF4 prevent HIT antibodies, when present, from binding thereby preventing platelet activation via the FcγRIIa. This study raises the possibility that the Liposorber® system (or the dextran sulfate adsorption column) may be a useful therapeutic option for patients with HIT through its ability to lower PF4 since PF4 is the pathologic target of HIT antibodies. To accomplish this, an alternate anticoagulant such as citrate or direct thrombin inhibitor would need to be used in the Liposorber® apheresis system in place of heparin. The data in this study provide a rational basis for testing the efficacy of such an intervention.

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Figure 1. Plasma PF4 is removed by LDL apheresis

PF4 levels in plasma samples were measured using the Zymutest PF4 ELISA kit. A. PF4 was completely removed by the LA-15 LDL apheresis column B. The amount of PF4 in the peripheral blood plasma did not change significantly after LDL apheresis. Data are the mean $\pm$ SD. *** $P < 0.0001$

Figure 2. Platelet surface PF4 decreases with LDL apheresis

Platelet surface PF4 levels were measured by flow cytometry as mean fluorescent intensity (MFI). PF4 levels on the surface of platelets decreased post-treatment. Data are the mean $\pm$ SD. *** $P < 0.0001$

Figure 3. Heparin concentration remains elevated 30 minutes after treatment

Heparin concentration in plasma samples was measured using the heparin anti-Xa chromogenic assay. A low level of heparin was present pre-treatment due to heparin use during line placement. Heparin concentration in the plasma increased significantly during treatment and remained high 30 minutes after treatment. Data are the mean $\pm$ SD. *** $P < 0.0001$ compared to pre-treatment. * $P < 0.05$ compared to post-treatment.

Figure 4. HITT antibodies are not developed during LDL apheresis

HITT antibody levels were measured using the PF4 Enhanced ELISA kit. HITT antibodies were not generated in patients undergoing LDL apheresis. Two patients had HITT antibodies before treatment started. Data are the mean $\pm$ SD.

Figure 5. Platelets are not activated by LDL apheresis

Platelet surface P-selectin levels were measured by flow cytometry. There was no change in surface levels of P-selectin after treatment. Data are the mean $\pm$ SD.

Table 1
Demographic and baseline characteristics of patients

Characteristics	Number ^a (%)
N	24
Gender	
Male	15 (62.5%)
Female	9 (37.5%)
Age (years)	
Male	50.9
Female	59.6
Diagnosis	
Familial Hypercholesterolemia	21 (87.5%)
High Lipoprotein(a)	2 (8.3%)
Primary Biliary Cirrhosis	1 (4.2%)

^a A total of 25 patients were enrolled in the study; however, demographic information could only be obtained from 24 patients.