Thrombosis Research 131 (2013) 244–248

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Investigation of a potential protective mechanism against heparin-induced thrombocytopenia in patients on chronic intermittent hemodialysis

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ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form 16 November 2012
Accepted 15 December 2012
Available online 8 January 2013

Keywords:
Hemodialysis
HIT
PF4
Heparin
Platelet
Mechanism

ABSTRACT

Background: Heparin-induced thrombocytopenia (HIT) develops as a result of platelet (PLT) activation by anti-platelet factor 4 (PF4)/heparin complex antibodies. Despite repeated exposure to heparin, patients undergoing chronic intermittent hemodialysis (HD) rarely develop HIT. We investigated the possibility that HD decreases/removes PF4 from PLT surfaces and/or plasma, thereby disfavoring immune complex formation as a mechanism of protection against HIT.

Materials and methods: We enrolled 20 patients undergoing chronic HD at the Penn Presbyterian Medical Center. Blood samples were drawn before, during and after treatment in the presence and absence of heparin. PF4, anti-PF4/heparin antibody, heparin, and P-selectin levels were measured.

Results: No patients demonstrated clinical symptoms of HIT. PLT surface PF4 levels decreased and plasma PF4 levels increased concurrently with the increase in plasma heparin concentration. In the absence of heparin, PLT surface and plasma PF4 levels were unchanged. Anti-PF4/heparin antibodies, which were non-functional by the serotonin release assay, were detectable in 8 patients. PLT surface P-selectin levels did not change during treatment.

Conclusions: Removal of PLT surface and/or plasma PF4 as a mechanism of protection against HIT in patients undergoing HD is not supported by the results of our study, although the transient decrease in PLT surface PF4 in the presence of large amounts of heparin remains a candidate mechanism. The small sample size, single type of dialyzer membrane, and early sampling time points may have led to the inability to detect changes in PF4 levels. Future studies should explore other potential protective mechanisms.

Introduction

Heparin-induced thrombocytopenia (HIT) is a transient, prothrombotic, autoimmune disorder mediated by antibodies that recognize ultralarge complexes (ULCs) of platelet factor 4 (PF4) and heparin or other glycosaminoglycans (GAGs). Our laboratory has shown that unfractionated heparin and tetrameric PF4 form ULCs (>670 kDa) only over a narrow molar ratio of heparin to PF4 of approximately 1:1 [1]. These ULCs are thought to be central to HIT pathogenesis. Changes in the molar ratio of heparin to PF4 by as little as 40% reduce ULC formation and increase/favor formation of smaller, less pathogenic complexes [1,2]. 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 GAG itself [3].

PF4 (CXCL4) is a small molecule (70 amino acids) positively charged CXC chemokine that is released in high concentrations from PLT α-granules upon PLT activation [4]. Basal PF4 concentration in the plasma is very low (<1 nM) while the normal serum content is more than a thousand-fold higher (1–2.5 μM) [5,6]. PF4 monomers polymerize to form non-covalently linked tetramers with a molecular weight of approximately 32,000 Da at physiologic pH and ionic strength.

Hemodialysis (HD) removes waste products such as creatinine and urea as well as free water by diffusion of solutes across a semipermeable membrane and by a countercurrent flow mechanism whereby the dialysate flows in the opposite direction to blood flow in the extracorporeal circuit. Solute removal can be characterized as high-efficiency or low efficiency based on the ability to remove small solutes such as urea or high-flux or low-flux based on the ability to remove large solutes, such as β_2-microglobulin (~12,000 Da, negatively charged) [7,8]. Due

Abbreviations: HIT, Heparin-induced thrombocytopenia; HD, Hemodialysis; PLT, platelet; PF4, platelet factor 4; GAGs, glycosaminoglycans; ULC, ultralarge complexes; LDL, low-density lipoprotein; AVF, arterio-venous fistula; AVG, arterio-venous graft; CVC, central venous catheter; ELISA, enzyme-linked immunosorbent assay; PRP, platelet rich plasma; MFI, mean fluorescent intensity; OD, optical density; DSA, dextran sulfate adsorption; SD, standard deviation.

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http://dx.doi.org/10.1016/j.thromres.2012.12.010

to the larger pore size of high-flux dialyzers, it is possible that PF4 monomers (8,000 Da) and dimers (16,000 Da) may be removed during dialysis. Removal of PF4 during HD could shift the equilibrium to favor dissociation of ULCs.

HD commonly utilizes heparin as an anticoagulant. As much as 6000 U of heparin may be administered during each HD session [9]. Yamamoto and coworkers [10] reported that in patients newly initiated on HD in Japan, the incidence of HIT was 3.9% (6/154). The mean duration to the development of HIT after the initiation of HD was 18 days. However, in patients undergoing chronic intermittent HD for 3 months or longer (mean period of dialysis, 70.3 ± 74 months; median period of dialysis, 41 months), HIT is rare (0.6%) despite repeated heparin exposure [9,11,12].

Several mechanisms may be responsible for the low rates of HIT in patients undergoing chronic intermittent HD. Two possible mechanisms are the repeated systemic exposure to large amounts of heparin or removal of PF4 during HD, either of which may alter the heparin to PF4 ratio in a manner that disfavors the formation of ULCs [1]. PF4 is removed during low-density lipoprotein (LDL) apheresis by dextran sulfate removal [13] and represents a possible protective mechanism from HIT in that patient population. It is currently unknown if PF4 is removed by HD. We therefore investigated the ability of HD to decrease PF4 in the plasma as a potential protective mechanism against HIT.

Materials and methods

Study population and design

The study population included 20 consecutive patients receiving HD treatment in the DaVita outpatient dialysis clinic at Penn Presbyterian Medical Center. All patients were greater than 18 years old with end-stage renal disease due to hypertension, diabetes mellitus, or lupus nephritis. This study was approved by the University of Pennsylvania Institutional Review Board and written informed consent was obtained from the study participants. Inclusion criteria included age greater than or equal to 18 years; receiving outpatient chronic (≥3 months) thrice weekly HD for end-stage renal disease of any etiology; HD access via arteriovenous fistula, graft or central venous catheter; HD using the Polyflux Revaclear capillary dialyzer (Gambro, Lakewood, CO); and HD with heparin. Exclusion criteria included the inability to provide informed consent, a history of HIT, and current pregnancy.

This study occurred over two separate patient visits. In the first visit, HD was carried out according to standard procedures. A commercially available dialysis system (Phoenix, Gambro, Lakewood, CO) using the Polyflux Revaclear capillary dialyzer was used. Heparin dosing varied for each patient according to actual body weight. Patients less than 60 kg received a loading dose of 1000 U, patients weighing 60 to 90 kg received a loading dose of 1500 U, and patients weighing greater than 90 kg received a loading dose of 2000 U. All patients received a maintenance heparin dose of 500 U/hour. Heparin was discontinued 60 min prior to the end of the HD treatment if the patient had an arterio-venous fistula (AVF) or arterio-venous graft (AVG) as HD access. If the patient had a central venous catheter (CVC) as HD access, heparin was discontinued 30 min prior to the end of the HD treatment. Approximately 10 mL of whole blood was drawn into two citrated tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ) immediately before (pretreatment) and after the completion of HD (posttreatment) from the arterial port. Twenty minutes after starting HD, 10 mL of whole blood was also drawn proximal to the dialyzer from the arterial port (20 min predialyzer) and distal to the dialyzer from the venous port (20 min postdialyzer). At the second study visit, the standard HD procedure was modified by withholding heparin for the first 20 minutes. Whole blood (10 mL) was drawn into citrated tubes from the arterial port (No heparin) after 20 minutes. Heparin was then administered as regularly prescribed. After 20 minutes of HD with heparin, another whole blood sample was drawn from the arterial port (Heparin). All samples were kept at room temperature (RT) before processing to prevent PLT activation and were processed within 15 minutes of collection.

PLT-poor plasma 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 15 mL conical tube. Small aliquots were made and all tubes were frozen at −80 °C until analysis.

PF4 measurement

PF4 was quantified using a PF4 enzyme-linked immunosorbent assay (ELISA) kit (Zymutest, Hyphen BioMed, Neuville-sur-Oise, France) according to the manufacturer's instructions with slight modifications. Briefly, 200 μL of PF4 standard (supplied by the manufacturer) or diluted patient samples were introduced into each well in duplicate and incubated for one hour at RT. Patient samples were diluted as follows to ensure that all measurements were in assay range: pretreatment and posttreatment samples, 1:10 to 1:20; and precolumn and postcolumn samples, 1:5 to 1:20. Each well was then washed five times with 300 μL wash solution. Two- hundred microliters of conjugate (anti-PF4 polyclonal antibody coupled with peroxidase) was added into each well and incubated for one hour at RT. After the wells were washed five times, substrate (TMB/H_2O_2) was added and incubated in each well for two minutes at RT. The reaction was stopped with 50 μL of 0.45 mol/L sulfuric acid and the absorbance at 450 nm was measured.

Anti-PF4/heparin antibody measurement

Anti-PF4/heparin antibodies were measured using the polyspecific PF4 enhanced kit (GTI Diagnostics, Waukesha, WI) according to manufacturer's instructions. Briefly, 300 μL of working wash solution was added to each well and incubated for 10 minutes at RT. Fifty microliters of diluted (1:50) positive control, negative control and patient sample were introduced into each well in duplicate and incubated for 45 minutes at 37 °C. The wells were then washed four times with 300 μL working wash solution. Fifty microliters of 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 four times and incubated with 100 μL of substrate (p-nitrophenyl phosphate) in the dark for 30 minutes at room RT. The reaction was stopped by adding 100 μL of 3 mol/L sodium hydroxide stopping solution, and the absorbance (optical density, OD) at 405 nm was measured.

Serotonin release assay

The serotonin release assay was performed as previously described [14]. Briefly, platelet rich plasma (PRP) from healthy donors was incubated with 0.5 μl 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 PF4/heparin 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 performed (in the absence of heparin). The percent release was calculated for all conditions as previously described [14]. The normal range is 0–5%. A serotonin release >10% in the presence of 0.5–2 U/ml heparin and ≤5% in the absence of heparin was considered positive.