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Molecular and cellular pathogenesis of heparin-induced thrombocytopenia (HIT)

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1. The clinical problem

Heparin-induced thrombocytopenia (HIT) is a potentially devastating thrombotic disorder caused by autoantibodies to complexes between a protein released by activated platelets, platelet factor 4 (PF4), and polyanions such as heparin [1,2]. Patients typically develop thrombocytopenia 5–14 days after initial exposure to heparin, consistent with antibody development, but clinical manifestations can occur within hours after drug exposure in those in whom antibodies persist. HIT develops in 2–3% of patients who receive unfractionated heparin (UFH) in therapeutic concentrations for at least 6 consecutive days. The risk is about 10-fold higher with exposure to UFH than with low molecular weight heparin (LMWH) and is somewhat greater in those undergoing cardiopulmonary bypass (coronary or other major arterial surgery, extracorporeal membrane oxygenation, heart/lung transplantation, ventricular assist devices) and orthopedic or other major surgical procedures or trauma than in medical patients. Approximately 50% of thrombocytopenic patients present with, or soon develop, venous, arterial or microvascular thrombotic complications. HIT is likely to remain an important iatrogenic medical problem until acceptable substitutes for UFH are found for cardiopulmonary bypass that provide rapid high intensity anticoagulation that can be easily monitored and readily reversed.

New or recurrent thromboembolic complications, sometimes multiple, develop in about 50% of patients if the disease is unrecognized or if management consists solely of preventing re-exposure to heparin [3,4]. The risk is greatest in the first 36–48 h (> 5%/day) [4,5], making rapid recognition and intervention critical. Maximally tolerated doses of direct thrombin inhibitors (argatroban, bivalirudin, rivaroxaban) and inhibitors of Factor Xa (fondaparinux, danaparoid) reduce the development of new thromboembolic complications to a varying extent, but mortality has remained at about 10%, amputations around 1–3%, and major

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bleeding, for which there is no antidote, occurs at a rate that approaches 1%/day in treated patients [6–9].

A commonly employed clinical algorithm to assess pre-test probability of HIT (the 4Ts score) has a high negative predictive value, but low specificity when applied to patients in complicated settings such as intensive care in which more than one cause of thrombocytopenia and thrombosis are possible. Rapid assays to detect anti-PF4 antibodies are being developed but additional high quality evidence is needed to validate their utility. Management of potentially affected patients is complicated by the fact that HIT-related antibodies develop in 20–50% of those exposed to UFH in settings of intense platelet activation, such as cardiopulmonary bypass [10]. This means that development of autoantibodies vastly exceeds the frequency of HIT, leading to over-diagnosis [6,11,12], which likely increases the risk of bleeding when irreversible anticoagulation is given to high-risk patients in whom thrombocytopenia has another etiology [13–15]. There is a correlation between antibody (IgG) anti-PF4 titer, presence of heparin-dependent platelet activating antibodies, and clinical disease [16–18], but standardization and interpretation of these assays has been difficult and availability within the acute time frame required for optimal diagnosis and management is often not feasible [19]. Thus, there is a continued need to develop diagnostics and rationale non-anticoagulant management strategies.

2. Objectives

The goal of this chapter is to provide insights into four questions related to the pathogenesis of HIT. First, how does an autoantigen develop when a normal host protein such as PF4 binds to an endogenously expressed polyanion, here in the form of heparin? Second, how do the resultant immune complexes induce such an acute and intense systemic prothrombotic disorder, not typical of most disorders associated with immune complexes and platelet activation per se? Third, why are only a subset of anti-PF4 antibodies associated with clinical disease? Fourth, can this information be used to develop novel diagnostics and rational therapeutics?

3. Formation of the HIT antigen

PF4 is synthesized as a 7.8 kDa monomer, which forms a stable dimer that is in equilibrium with PF4 tetramers. It is presumed that PF4 is packed within the alpha granules of platelets primarily in form of a tetramer bound to serglycin, a chondroitin sulfate containing proteoglycan. PF4, released when platelets are activated, binds to the more highly anionic heparin or more ubiquitous and heterogeneous glycosaminoglycans, forming HIT antigens on diverse vascular and intravascular cell surfaces. Formation of the HIT antigen is defined by the binding of IgG from patients with clinical HIT or binding of the monoclonal antibody KKO that competes with HIT-IgG for binding to PF4, and induces thrombocytopenia and thrombosis in an engineered animal model [20]. Binding of KKO and most HIT antibodies to PF4 is markedly enhanced in the presence of polyanions such as heparins, but also synthetic sugars, cell surface glycosaminoglycans, polyphosphates, RNA aptamers and DNA [21–26]. Antigen formation requires a minimal chain length of 8–11 residues in the case of heparin, consistent with the difference in the incidence of HIT in patients exposed to UFH

vs. LMWH, and is modulated by sulfate content and extent of branching [25,27]. These chemical features may change the secondary structure of PF4 and/or are required to maintain PF4 as a tetramer, which in turn is required for oligomerization, as discussed below [27–29].

Although high-affinity heparin-independent anti-PF4 antibodies have been described, formation of complexes between PF4 and polyanions causes a marked increase in the binding affinity of most HIT antibodies [30]. However, the HIT antigen is formed only within restricted molar ratios of PF4 to heparin or other polyanions, such as those encountered after intense platelet activation in the setting of therapeutic concentrations of UFH. This is true both in solution and on cell surfaces [31], which may help to explain in part why HIT occurs more commonly in patients after cardiopulmonary bypass when both reactants are at the relevant concentrations and ratios and why not all exposures to heparin lead to recurrent thrombocytopenia and thrombosis. PF4 and UFH undergo dramatic biophysical changes at these “pathologic” ratios, forming ultralarge complexes (ULCs) that exceed 600 kDa in size and can be imaged by electron microscopy (Fig. 1) and followed by dynamic light scattering (DLS) [32,33]. Subsequent binding of KKO or HIT IgG leads to formation of immune complexes (Fig. 1D) that can exceed a micron in size and that are exceedingly stable unless dissociated by high concentrations of heparin [32]. Formation of ULCs undoubtedly requires oligomerization of PF4 tetramers and incorporation of multiple polyanion molecules. As discussed below, the size and stability of these antigenic complexes and their subsequent effect on antibody binding, formation of ultralarge immune complexes, and intracellular signal transduction through IgG Fc receptors (FcR γ) on platelets and other cell surfaces may underlie the unusually intense prothrombotic features of HIT not shared by most other disorders mediated by immune complexes. These features also suggest that it is the propensity of PF4 to oligomerize in the presence of polyanions that in part underlies its involvement in the pathogenesis of HIT in contrast to other heparin-binding proteins.

Although seemingly diverse polyanions share the capacity to induce the HIT antigen, mutations within a restricted sequence of amino acids on the surface of the PF4 tetramer abolish binding of most HIT antibodies [34,35]. Insights from recent crystallography studies show that PF4 monomers assemble into an asymmetric tetramer with the “antigenic region” identified by mutation analysis lying on the surface of its open end where the C-terminal regions of monomers B and D converge, and a closed end where a polyanion such as fondaparinux, the pentamer in UFH that binds anti-thrombin III, resides [29] (Fig. 2A). Analysis of the co-crystal of Fab fragments of KKO with PF4 shows that the CDRs of antibody’s heavy and light chains interface with three of the four monomers [29] (Fig. 2B). Therefore, formation and stability of the PF4 tetramer is likely integral to its pathogenicity. Binding of polyanions may induce the autoantigen by stabilizing the conformation of the tetramer, thereby helping to maintain accessibility of the antigenic site in PF4 to pathogenic HIT antibodies, which bind with higher on-rates than non-pathogenic antibodies [30,36].

4. Generation of pathogenic HIT antibodies and immunocomplexes

Less is known about the mechanism by which heparin/PF4 complexes initiate antibody formation. Monocytes, and likely dendritic cells, internalize ULCs, which might initiate or propagate antibody formation [37]. However, the prevalence of antibody formation is

remarkable and there is as yet no evidence for MHC-restricted presentation of peptide fragments to T-helper cells. Recent data indicates that polyreactive IgM antibodies bind ULCs and activate the classic pathway of complement to generate C3c [38–40]. Complement containing immune complexes bind to B-cell CD21, which may amplify T-cell responses within germinal centers leading to the generation of isotype switched high affinity IgG antibodies [41], although T cell independent processes have been invoked as well.

As mentioned, the higher affinity of some HIT antibodies for PF4/polyanion complexes may underlie their capacity to form clinically relevant ultralarge immune complexes [36,42]. It has also been proposed that the propensity of HIT antibodies to generate intracellular procoagulant pathways may depend in part on epitope specificity. KKO induces oligomerization of PF4 directly, perhaps due to its positioning on the surface of tetramers [42,43]. A model based on the co-crystal structure of the Fab fragment of KKO with PF4 aligns and approximates the tetramers along a rigid linear polyanion template, which enhances the capacity of the antibody to bridge tetramers [29,30,43] (Fig. 3). This further stabilizes the structure of the immune complex and permits assembly of multiple antibodies leading to enhanced antibody avidity. Antibodies that bind to other epitopes on PF4 may be unaffected by the presence of polyanions and lack the capacity to propagate and stabilize the antigenic complex. This hypothesis is supported by the recent finding that an assay based on the capacity of HIT antibodies to inhibit binding of KKO to beads coated with PF4 shows high sensitivity and specificity for diagnosing clinical disease and suggests the possibility of developing an “antigen-restricted” PF4 diagnostic [44].

5. Induction of the procoagulant state

It is uncertain whether ultralarge immune complexes containing PF4/UFH bind directly to Fc γ receptors, to cell surface glycosaminoglycans that might then be incorporated and thereby enhance their size, stability and/or proximity to Fc γ receptors, or if they form primarily on cell surfaces directly [23,27]. Stable occupation or crosslinking of Fc γ IIA receptors on platelets initiates aggregation and release of presumably procoagulant microparticles in vitro, and thrombocytopenia and thrombosis in a murine model in which mouse PF4 has been replaced with human PF4 and platelets have been invested with Fc γ IIA receptors [20]. In turn, activated platelets release additional PF4 as well as polyphosphates that are capable of generating HIT-antigen independent of heparin and which thereby may recruit and activate newly available platelets [21,24].

However, the intensity of the prothrombotic state suggests that the ultralarge immune complexes in HIT generate procoagulant properties on additional cell surfaces as well. Activation of monocytes through Fc γ IIA receptors [22,45] leads to expression of tissue factor and generation of thrombin, which provides a second signal for platelet activation with formation of procoagulant (coated) platelets [46] and backactivation of monocytes, granulocytes and endothelial cells. Activation of granulocytes generates NETs that can form HIT antigen by binding of PF4 to DNA [47]. Thrombin and complement-mediated activation of endothelial cells generates tissue factor and initiates the elaboration of von Willebrand factor, which also has the capacity to bind PF4 [48–50]. HIT antigen formed on released NETs or von Willebrand factor may help perpetuate immune complex formation

and generation of a procoagulant response. Undoubtedly, additional procoagulant pathways will be uncovered.

The net effect of these events is the generation of multiple interactive procoagulant intravascular cell surfaces that propagate and perpetuate the risk of thrombosis even when heparin has been cleared from the circulation and metabolized. The diversity of procoagulant pathways likely to be operative helps explain the absence of data supporting the efficacy of anti-platelet agents and the incomplete benefit of inhibiting thrombin and Factor Xa even with maximally tolerated doses of anticoagulants.

6. Development of rationale intervention in HIT

The multiplicity of cells and pathways involved in the pathogenesis of HIT suggests the potential benefit of creating novel agents that act at more proximal steps in pathogenesis and that can be used in concert with existing therapies. Inhibitors of syk and intracellular signaling events downstream of Fc γ RIIA have shown efficacy in vitro but are not specific to HIT [51,52]. Small molecules that interfere with PF4 oligomerization and cell activation by HIT antibodies have been developed and are undergoing optimization [53]. Investigation of the cocrystal structure of PF4 with the Fab fragment of a “non-pathogenic” monoclonal anti-PF4 antibody, RTO, demonstrated its interaction with PF4 monomers at a site that clashed with monomer-monomer binding [29] (Fig. 4). In theory, inhibition of tetramer formation should reduce the affinity of pathogenic antibodies and prevent formation of ultralarge immune complexes. In line with this hypothesis, RTO inhibited platelet activation by KKO in vitro and thrombus formation following laser injury to the microvasculature in a mouse model of HIT [29] (Fig. 5).

7. Unmet needs/future directions

Our current understanding of the pathogenesis of HIT is summarized in Fig. 6. UFH will continue to be in widespread use until an effective substitute can be found that permits cardiovascular procedures requiring high intensity anticoagulation to be performed with comparable effectiveness and safety. Therefore, there will continue to be a need to identify patients at risk to develop HIT either prior to generation of IgG anti-PF4/heparin antibodies or soon thereafter based on their serologic or cell-activating properties. The current limitations in disease prevention or treatment of established disease identify a need for greater in-depth understanding of antigen and immune complex formation and their effect on cellular procoagulant pathways in order to optimize the development of rational and safe disease-specific interventions.

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Fig. 1.

Transmission electron microscopy of rotary shadowed PF4-heparin complexes. Panel A. In the absence of heparin very small, relatively homogeneous molecules are seen that likely represent PF4 tetramers (35 kDa). Panel B. Complexes formed at a heparin-PF4 ratio (HPR) of 1.2 were more uniform in size, quite large and appear to be composed of multiple PF4 tetramers. Panel C. At a HPR of 2.1, a heterogeneous mixture of molecules is seen, most smaller than those seen at an HPR of 1.2 but virtually all larger than seen in the absence of heparin. Panel D. Examples of PF4-heparin complexes with multiple KKO antibodies bound. Below each frame is a very high contrast image to highlight the shadowed molecules and eliminate the background. Arrows point to the antibodies. Magnification bar = 50 nm. Adobe Photoshop 7.0 (Adobe, San Jose, CA) was used to produce high-contrast images. (Modified from Blood 105 [1]:131–8. 2005. PMID: 15304392).

Fig. 2.

Crystal structure of PF4 tetramer. Panel A. Measurement of the E28 to K50 distances in the PF4/fondaparinux complex crystal shows that PF4 adopts an asymmetric 'open'-'closed' conformation. C terminal ends of the B and D chains are further apart than are the A and C chains. Heparin binds to the 'closed' end of the PF4 tetramer. This interaction stabilizes the conformation of the "antigenic region" on the 'open' end of the tetramer. Depicted are the residues, which were also found to be critical for HIT antibodies binding based on mutagenesis. Panel B. Molecular surface representations of the PF4/KKO-Fab complex structure. The heavy chain and light chain of KKO-Fab are colored in blue and light blue, respectively. Monomers A, B, C and D in one PF4 tetramer are colored in green, cyan, magenta and yellow, respectively. The KKO-Fab fragment binds to the PF4 tetramer by making contacts with three monomers (for example, chains A, B and D within one PF4 tetramer). (Modified from Nature Communications 6: Article No. 8277, doi:<https://doi.org/10.1038/ncomms9277> 2015. PMID: 26391892; PMCID: PMC4580983).

Fig. 3.

Model of the growth of a PF4/heparin/KKO-Fab ternary complex. Heparin first binds to the groove within the PF4 tetramer. Binding of the 1st tetramer imparts a local linearized structure on heparin. This enhances the binding and approximation of a second PF4 tetramer, which also fosters PF4-PF4 interactions. Progression of this process eventuates in the formation of an ultralarge antigenic complex (ULC). The model assumes the heparin molecule is composed of about 7 structures similar to fondaparinux, depicted in the figure as a non-continuous chain. Intact UFH may further enhance the stability of the holo-complex compared with the fondaparinux fragment, thereby rendering it more antigenic and more capable of binding multiple IgG antibodies. Not only is heparin shared by more than one tetramer, KKO can cross-link tetramers. Surface representations of KKO-Fab are colored in blue (heavy chain) and light blue (light chain) and PF4 tetramers forming ULCs are colored in various shades of green. (Modified from Nature Communications 6: Article No. 8277, doi:<https://doi.org/10.1038/ncomms9277> 2015. PMID: 26391892; PMCID: PMC4580983).

Fig. 4.

Crystal structure of the RTO-Fab/PF4 monomer complex. Panel A. Cartoon representations of the complex. The heavy chain and light chain of RTO-Fab are colored in blue and light blue, respectively, PF4 monomer in green. Panel B. Superposition of the PF4 monomer (green) in complex with RTO-Fab (blue and light blue) with the unbound PF4 tetramer (grey and red). The three arrows indicate the sites where binding of RTO-Fab to one PF4 monomer causes steric clashes with a second PF4 monomer, thereby preventing tetramer formation. (Modified from Nature Communications 6: Article No. 8277, doi:<https://doi.org/10.1038/ncomms9277> 2015. PMID: 26391892; PMCID: PMC4580983).

Fig. 5.

Inhibition of thrombosis in a mouse model of HIT. Anti-mouse CD41-F(ab')₂ fragments conjugated to Alexa 647 were infused to double transgenic HIT mice to label platelets. Arterioles were injured via a pulsed nitrogen dye laser. KKO (1 μg/g) was infused to induce thrombus growth after initial snapshot were taken. Panel A. Representative composite images of overlaid platelet fluorescence on brightfield snapshots of injuries in mice receiving either RTO or the IgG₂κb isotype control TRA are shown. Pre KKO images represent the thrombus 15 min after initial injury and post KKO images represent the same thrombus 15 min after KKO was injected intravenously. Arrows represent the direction of blood flow. Panel B. Each dot is a single injury in mice receiving either RTO or the IgG₂κb isotype control TRA corresponding to the percent size of platelet fluorescence after dosing the animal with KKO. Error bars are the standard deviation. *N*=19 injuries in 3 mice for RTO, *N*=19 injuries in 3 mice for TRA. *P* < .005. (Modified from Nature Communications 6: Article No. 8277, doi:<https://doi.org/10.1038/ncomms9277> 2015. PMID:26391892; PMCID: PMC4580983).

Fig. 6.

Overview of current understanding of the pathogenesis of HIT. Step 1 (Inception): The afferent arm of the immune response is shown in the upper panel. Polyreactive IgM antibodies (cross) react with PF4 complexed with heparin, activating the classic pathway of complement and forming C3c-containing immune complexes that bind to B-cell C21 and likely accelerate antigen presentation. The efferent arm shown in the lower panel can be conceptually subdivided into two sequential processes. In Step 2 (Activation), PF4-containing complexes can bind to cell surface glycosaminoglycans (GAGs) and/or FcγRIIA (left side) or they may incorporate within or, more likely, form on cell surfaces bound to GAGs. These immune complexes activate FcγRIIA-dependent intracellular signaling pathways on platelets, monocytes and neutrophils and complement-mediated activation of the endothelial cells leading to a prothrombotic state. Step 3 (Propagation/Amplification) involves: a) the expression of tissue factor and generation of thrombin back-activates target cells releasing additional PF4 and b) large polymeric products from the activated cells, including DNA-containing NETs by granulocytes and von Willebrand Factor by endothelial cells that bind PF4 and thereby provide additional sources of HIT antigen. Activated cells also release polyphosphates that bind PF4 and HIT antibody. Mixed polymeric complexes may form as shown at the extreme right. The net effect of Steps 2 and 3 is a feed-forward amplification loop that may help explain the intense and protracted prothrombotic clinical presentation of HIT, which persists even after heparin has been discontinued.