Review Article

Autoantibodies to platelets: Roles in thrombocytopenia

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Circulating platelets are targeted by autoantibodies in various pathologic conditions, such as immune thrombocytopenic purpura (ITP). Anti-platelet antibodies cause thrombocytopenia through enhanced platelet clearance via Fcγ receptor-mediated platelet destruction by the reticuloendothelial system and impaired platelet production. Moreover, functional blockade of platelet surface receptors by autoantibodies may further promote bleeding tendency. The major targets of these autoantibodies are platelet membrane glycoproteins, including GPIIb/IIIa and GPIb/IX, receptors for fibrinogen and other platelet-activating ligands, but some patients with ITP have antibodies to a receptor for thrombopoietin, which is a growth factor required for megakaryocytogenesis and platelet production. Several antigen-specific assays have been developed to measure anti-glycoprotein antibodies, whereas we have recently established an enzyme-linked immunospot assay for the detection of circulating B cells secreting IgG anti-GPIIb/IIIa antibodies, which is a sensitive, specific, and convenient method for evaluating the presence or absence of ITP. Production of pathogenic anti-platelet antibodies is maintained by a continuous loop, in which B cells produce anti-platelet antibodies, antibody-coated platelets are phagocytosed and GPIIb/IIIa-derived cryptic peptides presented by splenic macrophages, and GPIIb/IIIa-reactive $CD4^+$ T cells exert their helper activity. Important discoveries on cellular and molecular mechanisms for anti-platelet autoantibody production contribute to development of diagnostic assays and therapeutic strategies for ITP.

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Introduction

Platelets are a common target of autoantibody responses in a variety of pathologic conditions, including immune thrombocytopenic purpura (ITP), bacterial and viral infections, and drug-induced thrombocytopenia. ITP is an acquired hemorrhagic condition of accelerated platelet consumption caused by anti-platelet autoantibodies[1]. This condition is seen in patients with various diseases, such as systemic lupus erythematosus (SLE) and human immunodeficiency virus infection, and can also occur without an underlying disease, in which case it is known as idiopathic form of ITP. The classic studies of Harrington and coworkers provided the first evidence for the existence of a serum anti-platelet factor in ITP patients in 1951.These investigators infused plasma from ITP patients into healthy volunteers or patients with inoperable malignant neoplasms, resulting in a marked and transient thrombocytopenia in many recipients[2]. The factor responsible for platelet destruction was subsequently shown to be present within the immunoglobulin fraction, and this activity was cancelled by preincubation of plasma with normal platelets. These early studies demonstrated the role of anti-platelet autoantibodies in the thrombocytopenic state[3]. Until now, a variety of platelet membrane glycoproteins (GPs), including GPIIb/IIIa and GPIb/IX, have been shown to be targets recognized by anti-platelet autoantibodies[4]. In addition, we have recently found that some patients with ITP have autoantibody to thrombopoietin (TPO) receptor, which suppresses megakaryocyte differentiation and platelet production[5]. This review summarizes autoantibodies to a series of platelet autoantigens and their roles in pathophysiologies of the autoimmune thrombocytopenic state.

Pathogenic roles of anti-platelet autoantibodies

1) Enhanced platelet clearance

Anti-platelet antibodies bind to circulating platelets, resulting in Fcγ receptor-mediated platelet destruction by the reticuloendothelial system[1]. Antibody-coated platelets may be destroyed by complement-mediated lysis, but the clinical response of most ITP patients to a monoclonal antibody to the Fcγ receptor[6] suggests that the Fcγ receptor-mediated mechanism is more important. Both *in vitro* and clinical studies have shown that the spleen is the dominant organ for the clearance of antibody-coated platelets, while hepatic clearance predominates in a minority of patients[7].

2) Defective platelet function

Since platelet GPs targeted by autoantibodies are involved in platelet activation, anti-platelet antibodies may affect platelet function and, rarely, mimic thombasthenia or Bernard-Soulier syndrome. In fact, there was a case report of the patient with a normal platelet count who had clinically significant bleeding with defective platelet aggregation[8]. This patient had a high titer anti-GPIIb/IIIa antibody, which blocked the binding of fibrinogen to this complex. The predominant autoantibody subclass was IgG4 with low affinity to Fcγ receptors and complement. This functional impairment may promote bleeding tendency in patients with ITP.

3) Impaired platelet production

Recent studies suggest that some anti-platelet autoantibodies also affect platelet production. In the 1980s, it was shown that platelet turnover in the majority of ITP patients was either normal or reduced rather than increased as would be expected, suggesting either inhibition of megakaryocytopoiesis or destruction of megakaryocytes in bone marrow[9]. Circulating TPO levels, which is regulated by a 'sponge effect', meaning it is controlled solely by binding to its receptor mainly expressed on bone marrow megakaryocytes and their precursors[10], are normal or slightly elevated in ITP patients, suggesting a normal or decreased megakaryocyte mass[11]. In recent *in vitro* study, plasma antibody containing anti-GPIIb/IIIa antibodies from 12 of 18 adults with severe ITP inhibited maturation of hematopoietic stem cells into megakaryocytes[12]. An ultrastructural study of bone marrow from ITP patients showed that 78% were morphologically abnormal, manifesting mitochondrial swelling with cytoplasmic vacuolization, distention of the demarcation membranes, and chromatin condensation within the nucleus, all of which are features of para-apoptosis[13]. These findings together indicate that impaired platelet production induced by anti-platelet autoantibodies in some ITP patients is mediated through two distinct processes: suppression of megakaryocytopoiesis and megakaryocyte damage.

Platelet autoantigens

In 1975, Dixon and colleagues showed that platelets from ITP patients had an elevated level of platelet-associated IgG (PAIgG)[14]. Subsequent studies, however, showed that PAIgG was also increased to some extent in many patients with non-immune thrombocytopenia and was therefore too non-specific for its measurement to be clinically useful. This is because normal platelets contain two distinct pools of IgG, one located on the surface as a form complexed with Fcγ receptors and the other located in the intracellular α-granules[15]. Later, IgG eluted from ITP platelets was shown to bind to normal platelets, but not to platelets from patients with Granzmann thrombasthenia, who genetically lack GPIIb/IIIa on platelet surface[16]. This was the first evidence for autoantibodies to the platelet surface GP. Table 1 lists platelet surface autoantigens, most of which are platelet membrane GPs. Anti-GP antibodies induce thrombocytopenia, primarily by enhancing platelet clearance through opsonization of circulating platelets. Since platelet GPs are not only expressed on platelets, but also present on the surface of magakaryocytes and their precursors, anti-GP antibodies also suppress platelet production.

Table 1 Targets of anti-platelet autoantibodies

Platelet antigen	CD nomenclature	Molecular weight (kDa)	Ligand	Frequency in ITP patients (%)
GPIIb/IIIa	CD41/61	145/110	Fibrinogen, collagen (I), fibronectin, vitronectin	50-90
GPIb/IX	CD42abc	170/17	vWF	30-80
GPV	CD42d	82	vWF	~20
GPIa/IIa	CD49b/29	160/138	Collagen (I)	~20
GPIV	CD36	88	Collagen (I), thrombospondin	~10
TPO receptor	CD110	71	TPO	10-20

1)GPIIb/IIIa

GPIIb/IIIa, also designated CD41/CD61or αIIbβ3 integrin, is specific to the magakaryocyte lineage, including platelets. GPIIb and GPIIIa are major platelet membrane proteins and make up of ~17% of the total platelet membrane protein mass. These two subunits form a calcium-dependent, non-covalently bound complex. In resting platelets, GPIIb/IIIa exists in a low-affinity state and does not bind its ligands. During platelet activation, a conformational change results in the exposure of the binding site for a variety of ligands, most notably fibrinogen, which allows firm adhesion to the extracellular matrix and aggregation[17). GPIIb/IIIa is the most common target recognized by anti-platelet autoantibodies in ITP patients; the frequency ranged from 50 to 90%, while GPIIb/IIIa is also targeted by antibodies found in alloimmune thrombocytopenia and drug-induced thrombocytopenia. Anti-GPIIb/IIIa antibodies in ITP patients mainly recognize cation-dependent conformational epitopes located at extracellular structure of the complex. These epitopes are localized to the region close to the ligand-binding site in GPIIb and/or the structure that requires discontinuous amino acids from both GPIIb and GPIIIa[18]. On the other hand, plasma samples from some ITP patients have antibodies reactive with intracellular epitopes of GPIIIa, which are considered non-pathogenic and produced secondary in response to massive platelet destruction[19].

Several antigen-specific assays have been developed to measure autoantibodies that recognize one or more platelet surface GPs[4]. These monoclonal antibody-based assays include immunobead assay and monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay. Using these assays, platelet-associated anti-GPIIb/IIIa antibodies can be demonstrated in about 50-60% of ITP patients, but specificity was relatively high (78-93%) when patients suspected of having ITP are compared with healthy individuals or patients with non-immune thrombocytopenia. Thus, a positive antigen-specific assay provides confirmatory evidence for the diagnosis in patients suspected of having ITP while a negative test does not rule it out. Moreover, the presence or absence of platelet-associated anti-GPIIb/IIIa antibodies has prognostic significance[20]. However, inter-laboratory standardization of platelet antigen-specific assays has been difficult to achieve[21]. In these assays, it is necessary to use platelets, instead of serum or plasma, as the source of the antibodies, because the majority of pathogenic anti-platelet antibodies are present as platelet-associated antibodies. For this reason, these assays require complicated procedures, such as platelet solubilization, and a relatively large blood sample, especially from patients with a low platelet count. These limitations have prevented the assays to be routinely used in clinical laboratories. We have recently established an enzyme-linked immunospot (ELISPOT) assay for detection of circulating B cells secreting IgG anti-GPIIb/IIIa antibodies[22]. This assay is shown to be a sensitive, specific, and convenient method for evaluating the presence or absence of ITP. We have recently conducted a prospective study to identify initial laboratory findings that are useful for predicting a diagnosis of ITP, and identified increased anti-GPIIb/IIIa antibody-producing B cells and platelet-associated anti-GPIIb/IIIa antibody, elevated proportion of reticulated platelets, and normal or slightly increased circulating TPO[23]. Based on these findings, we have proposed diagnostic criteria for ITP that depend solely on non-invasive laboratory tests using peripheral blood samples[24].

2)GPIb/IX and GPV

GPIb/IX, a receptor for von Willebrand factor (vWF), is the second major GP complex on platelets, and unique to platelets and megakaryocytes. GPIb, which is composed of a heavy chain

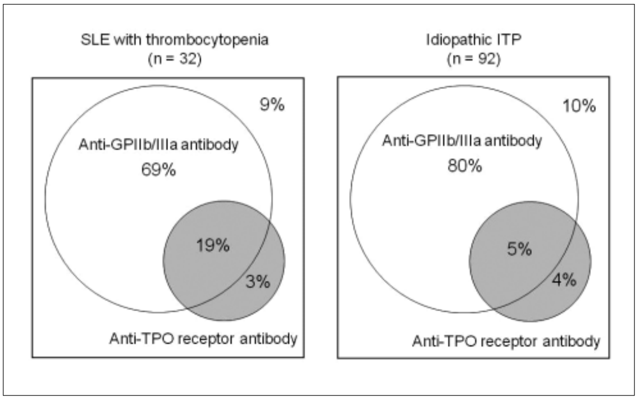

Fig.1 Distribution of anti-GPIIb/IIIa and anti-TPO receptor antibodies in patients with SLE and thrombocytopenia (left) and in those with idiopathic ITP (right).

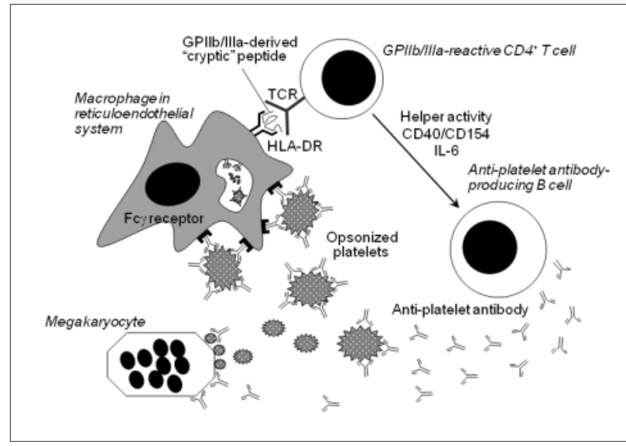

Fig.2 Schematic representation of pathophysiology of ITP and a continuous pathogenic loop carried out by macrophages in the reticuloendothelial system, GPIIb/IIIa-reactive CD4+ T cells, and anti-platelet antibody-producing B cells that maintains anti-platelet antibody production. TCR = T-cell receptor.

Ibα and a light chain Ibβ, is non-covalently associated with GPIX. The majority of GPIb/IX molecules on the platelet surface are present in association with GPV. All four subunits belong to the leucine-rich GP family. An extracellular domain of GPIbα, named glycocalicin, is cleaved off by a Ca^{2+}-dependent protease calpain, and present in circulation. Thrombus formation mediated by high hemodynamic shear stress is mediated primarily by the binding of GPIb/IX/V to immobilized vWF, resulting in a complex series of events that include platelet adhesion, activation, and aggregation. GPIb/IX is the second common target recognized by anti-platelet autoantibodies in ITP patients; the frequency ranged from 30 to 80%, while anti-GPV antibodies occur in 10% to 20% of patients with ITP[4]. Platelet-associated and plasma antibodies to GPIb/IX and GPV are detectable by monoclonal antibody-based assays. The majority of sera and platelet eluates positive for anti-GPIb/IX antibodies reacted with glycocalicin[25]. The epitopes on this extracellular region are thought to be conformational, but one of them has been mapped at a linear amino acid sequence of GPIbα. Platelet-associated antibodies reactive with GPV, but not with GPIb/IX, were frequently detected in rheumatoid arthritis patients with gold-induced thrombocytopenia[26].

3)Other platelet GPs

A small proportion of anti-platelet antibodies are shown to recognize GPIa/IIa or GPIV, which are highly expressed on platelets, but also in several other cell types. In a cohort of adult ITP patients, 93% of sera reacted with more than one GP, but GPIa/IIa and GPIV were never the sole targets[27].

4)TPO receptor

TPO receptor, also named as c-Mpl, is a type I transmembrane protein and expressed specifically on hematopietic stem cells and cells in the megakaryocyte lineage. We have demonstrated the presence of autoantibodies to TPO receptor in SLE patients with thrombocytopenia by enzyme-linked immunosorbent assay using recombinant TPO receptor as an antigen[5]. This antibody specificity was clinically associated with thrombocytopenia with megakaryocytic hypoplasia, and interfered with TPO function by blocking its ligation to the receptor *in vitro*. The involvement of the anti-TPO receptor antibody in impaired thrombopoiesis was further supported by the clinical course of a patient with amegakaryocytic thrombocytopenia, in whom the platelet count was negatively correlated with circulating anti-TPO receptor antibody titer and TPO concentration[28]. Anti-TPO receptor antibody was detected in 22% of patients with SLE and thrombocytopenia and in 10% of patients diagnosed as having idiopathic ITP[29]. More than 90% of patients with ITP had either anti-GPIIb/IIIa or anti-TPO receptor antibodies, independent of the idiopathic or secondary form (Fig.1). In addition, the majority of patients with anti-TPO receptor antibody had concomitant anti-GPIIb/IIIa antibody. In SLE patients with thrombocytopenia, patients with anti-TPO receptor antibody had significantly higher frequencies of megakaryocytic hypoplasia and poor therapeutic responses to corticosteroids and intravenous immunoglobulin than did the patients without this antibody, most of whom had the anti-GPIIb/IIIa antibody alone.

Mechanisms for anti-platelet autoantibody production

Earlier studies representing interleukin (IL)-2 production from peripheral blood T cells in response to autologous platelets indicated the presence of T cells autoreactive to platelets in patients with ITP[30]. We subsequently found that GPIIb/IIIa was one of the major target antigens recognized by platelet-reactive CD4+ T cells in ITP patients[31]. These T cells had the ability to stimulate IgG anti-platelet antibody production from autologous B cells in the presence of the GPIIb/IIIa antigen. This helper activity depended on two types of stimuli: T cell-derived IL-6 and CD40-CD154 engagement[32]. Interestingly, GPIIb/IIIa-reactive T cells recognize "cryptic" epitope peptides that were not generated from native GPIIb/IIIa molecule, but from structurally modified protein or bacterially expressed recombinant fragments of GPIIb/IIIa[33]. Therefore, it is likely that these autoreactive T cells exist in the normal T-cell repertoire, and are activated *in vivo* in ITP patients, but not in healthy individuals. In our recent study evaluating frequencies and activation status of GPIIb/IIIa-reactive T and B cells in peripheral blood and spleen obtained from ITP patients undergoing splenectomy, we found that the T-B-cell interaction through recognition of the cryptic peptides of GPIIb/IIIa occurred primarily in the spleen[34]. Further *in vitro* analyses using GPIIb/IIIa-reactive CD4+ T-cell lines and freshly isolated splenocytes from the same ITP patients demonstrated that splenic macrophages that phagocytosed opsonized platelets via Fcγ receptor had the ability to efficiently concentrate small quantities of platelet antigens that were previously cryptic[35]. Based on these findings together, we propose that a pathogenic loop maintains the ongoing anti-platelet antibody response in ITP patients (Fig.2). That is, macrophages in the reticuloendothelial system capture opsonized platelets via the Fcγ receptors, process them, and present GPIIb/IIIa-derived cryptic peptides to T cells. GPIIb/IIIa-reactive CD4+ T cells are then activated and exert helper activity when their T-cell receptor recognizes the antigenic peptide in the context of the HLA-DR molecule. Finally, B cells produce pathogenic IgG anti-platelet antibodies, and the platelets are opsonized and phagocytosed by macrophages. The mechanism that triggers this response in ITP patients remains unclear, but once this pathogenic loop is established, the anti-platelet autoantibody production would, theoretically, go on endlessly. Thus, therapeutic strategies that inhibit pathogenic anti-platelet antibody production should be aimed at interrupting this continuous autoimmune loop. In this regard, we recently demonstrated that the platelet recovery observed in a subset of *Helicobacter pylori*-infected ITP patients after *H. pylori* eradication is likely to be mediated through a change in the Fcγ receptor balance on macrophages toward the inhibitory phenotype[36].

Conclusion

In recent years, considerable information has been obtained concerning the characteristics of anti-platelet autoantibodies, their pathogenic roles in inducing thrombocytopenia, and cellular mechanisms controlling the production of these antibodies. Since platelet GP-specific antibody assays are not widely used in routine laboratories at this moment, it is necessary in clinical settings to establish convenient commercial kits. Further studies examining the mechanisms that trigger a pathogenic loop effected by macrophages, and GPIIb/IIIa-reactive CD4+ T cells and B cells in ITP patients should be useful in clarifying the etiology of ITP and in developing novel therapeutic strategies for refractory ITP.

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