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IN FOCUS

Splenic macrophages maintain the anti-platelet autoimmune response via uptake of opsonized platelets in patients with immune thrombocytopenic purpura

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Summary. *Background:* Immune thrombocytopenic purpura (ITP) is an autoimmune disease primarily caused by IgG anti-platelet autoantibodies. Activation of autoreactive $CD4^+$ T cells upon recognition of cryptic GPIIb/IIIa peptides presented by antigen-presenting cells (APCs) is a critical step for triggering and maintaining the pathogenic anti-platelet autoantibody response. *Objectives:* We investigated which APCs carry the cryptic peptides of GPIIb/IIIa that activate autoreactive $CD4^+$ T cells in ITP patients. *Methods:* GPIIb/IIIa-reactive T-cell lines generated from ITP patients were cultured with autologous freshly isolated splenic macrophages, B cells or dendritic cells. To further investigate how the macrophages presented the antigenic GPIIb/IIIa peptides, we prepared macrophages from the peripheral blood monocytes of the same patients during remission. *Results:* Macrophages induced the proliferation of GPIIb/IIIa-reactive T-cell lines without an exogenous antigen, but B cells and dendritic cells required GPIIb/IIIa peptides to stimulate the T cells. Macrophages derived from peripheral blood during remission required an exogenous antigen to induce the GPIIb/IIIa-reactive T-cell line response, but could elicit a response without added antigen if they were preincubated with platelets from ITP patients with platelet-associated anti-GPIIb/IIIa antibodies or healthy platelets pretreated with ITP platelet eluates. The T-cell response was inhibited by anti-FcγRI antibody. Finally, cultured macrophages that captured opsonized platelets promoted anti-GPIIb/IIIa antibody production in mixed cultures of autologous GPIIb/IIIa-reactive T-cell lines and B cells. *Conclusions:* Splenic macrophages that take up opsonized platelets via FcγRI are major APCs for cryptic GPIIb/IIIa peptides, and are central to the maintenance of anti-platelet autoantibody production in ITP patients.

Keywords: antigen-presenting cell, anti-platelet antibody, immune thrombocytopenic purpura, macrophage, T cell.

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Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by increased platelet clearance, which is primarily caused by IgG autoantibodies to platelet membrane glycoproteins (GPs), such as GPIIb/IIIa [1]. The presence of T cells autoreactive to platelets was first described by Semple and colleagues in 1991 [2]. In that report, they demonstrated that peripheral blood T cells from ITP patients secreted interleukin (IL)-2 upon stimulation with autologous platelets. The IL-2 production from circulating T cells in response to platelets was further confirmed by other studies [3,4]. We recently found that these platelet-reactive T cells recognize GPIIb/IIIa in ITP patients [5]. These $CD4^+$ T cells are considered to be pathogenic because they have a helper activity that promotes the production of IgG anti-GPIIb/IIIa antibodies that can bind normal platelets *in vitro* [5]. The GPIIb/IIIa-reactive T cells respond to tryptic peptides of GPIIb/IIIa or recombinant GPIIb/IIIa fragments produced in bacteria, but not to native GPIIb/IIIa [5,6], indicating that the epitopes they recognize are 'cryptic' determinants, generated at a subthreshold level by the processing of native GPIIb/IIIa under normal circumstances [7]. These autoreactive T cells are present in the normal T-cell repertoire, but they do not normally encounter these antigenic peptides, and thus these peptides are ignored by the immune system. In ITP patients, these T cells are activated upon recognition of cryptic GPIIb/IIIa peptides presented by antigen-presenting cells (APCs) in the periphery [8]. Later, cryptic epitopes on GPIIIa that are recognized by $CD4^+$ T cells from British patients with ITP were identified [9]. Therefore, exposure of the cryptic peptides of GPIIb/IIIa to the immune

system is a critical step for triggering and maintaining the anti-platelet autoantibody response.

In patients with ITP, the spleen is considered to be the primary site of platelet destruction and anti-platelet antibody production [10]. We recently found that the activation of GPIIb/IIIa-reactive T cells and the subsequent anti-platelet antibody production occur primarily in the spleen [11], suggesting that APCs in the spleen are a potential source for T-cell exposure to the cryptic determinants of GPIIb/IIIa. However, it remains unclear which splenic APCs are responsible for presenting the peptides, although splenic macrophages are thought to be a major APC. We previously established GPIIb/IIIa-reactive $CD4^+$ T-cell lines from the peripheral blood of ITP patients [6], and stored them after detailed characterization. We recently had a chance to obtain the spleen from some of these patients, because they received a splenectomy later in the course of the disease. In this study, freshly isolated splenocytes were used to identify the APCs that present the cryptic peptides of GPIIb/IIIa by co-culturing splenic macrophages, B cells or dendritic cells with the autologous pre-established GPIIb/IIIa-reactive $CD4^+$ T-cell lines. In addition, we investigated the mechanisms for the processing and presentation of the GPIIb/IIIa cryptic peptides by the APCs.

Materials and methods

Study subjects

Samples from three patients with ITP (ITP#24, #25 and #47) were analyzed in this study. ITP was defined as thrombocytopenia (platelet count $< 150 \times 10^9$/L) persisting longer than 6 months, no morphological evidence of dysplasia in bone marrow cells, and no secondary immune or non-immune diseases that could account for the thrombocytopenic state [1]. All the patients were female; their ages at diagnosis and splenectomy were 19 and 21 (ITP#24), 45 and 51 (ITP#25), and 22 and 24 (ITP#47) years. At diagnosis, all the patients were positive for anti-GPIIb/IIIa antibodies response, which was evaluated by detection of B cells producing IgG anti-GPIIb/IIIa antibodies using the enzyme-linked immunospot assay [12], and platelet-associated IgG anti-GPIIb/IIIa antibodies in platelet eluates measured by enzyme-linked immunosorbent assay using purified human GPIIb/IIIa as the antigen [13]. The platelet count before splenectomy was $< 20 \times 10^9$/L, and all the patients responded to treatment by maintaining a platelet count $> 100 \times 10^9$/L for at least 1 year after the splenectomy.

At the time that we took blood samples for establishing the T-cell lines, all the patients were on low-dose prednisolone (< 10 mg day^{-1}). One week before the splenectomy, intravenous immunoglobulin (IVIG; 0.4 g m^{-2} day^{-1} for 5 days) was given to two patients (ITP#25 and #47), but one of them (ITP#25) did not respond adequately and required a platelet transfusion during the operation. The remaining patient (ITP#24) also received a platelet transfusion instead of IVIG, because of a history of aseptic meningitis related to IVIG. Additional peripheral blood samples were obtained on several occasions when the patients were in remission and negative for platelet-associated anti-GPIIb/IIIa antibodies or anti-GPIIb/IIIa antibody-producing B cells. Circulating platelets were prepared from the three ITP patients whose samples were used for the T-cell lines, from three additional ITP patients (ITP#48, #54, and #62), in whom the platelet count was $< 50 \times 10^9$/L and whose samples showed increased platelet-associated anti-GPIIb/IIIa antibodies, and from three healthy individuals (HC#1, #2, and #3). All samples were obtained after the patients and control subjects gave their written informed consent in accordance with the Declaration of Helsinki. The study protocol was approved by the Keio University International Review Board.

Antigen preparation

Human GPIIb/IIIa was purified from outdated platelet concentrates using affinity chromatography and chemically modified by treatment with porcine trypsin (0.1 μg mL^{-1}), as described previously [5]. Phosphate-buffered saline containing porcine trypsin in the absence of GPIIb/IIIa was also prepared for use as a mock-treated control antigen. Seven recombinant fragments encompassing amino acid residues 18–259, 244–575 and 566–841 of GPIIbα (IIbα18-259, IIbα244–575 and IIbα566–841, respectively) and amino acid residues 22–262, 254–462, 455–723 and 708–762 of GPIIIa (IIIa22–262, IIIa254–462, IIIa455–723 and IIIa708–762, respectively) were prepared as glutathione S-transferase (GST) fusion proteins [6].

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) density-gradient centrifugation. Circulating B cells were isolated from PBMCs using anti-CD19 monoclonal antibody (mAb)-coupled magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) followed by MACS column separation according to the manufacturer's protocol. Flow cytometric analysis showed that the purity of the B cell fraction was >98%. Monocyte-derived cultured macrophages were isolated as plastic-adherent PBMCs in the presence of macrophage-colony stimulating factor (R&D Systems, Minneapolis, MN, USA) [14]. Epstein-Barr virus-transformed lymphoblastoid B-cell line cells (LBLs) were generated from the samples of all the patients.

Sterile spleen tissue was washed twice to remove peripheral blood and crushed with a syringe plunger. After all of the cells were dissociated, the cell suspension was filtered through nylon mesh and subjected to Lymphoprep density gradient centrifugation. The recovered cells were used as splenocytes [11]. Splenic macrophages and B cells were isolated from splenocytes using anti-CD14 or anti-CD19 mAb-coupled magnetic beads (Miltenyi Biotech). Dendritic cells (DCs) were isolated from splenocytes by negative selection of lineage-positive cells followed by positive selection of $CD11c^+$ cells, using the MACS system, according to the manufacturer's protocol. Flow

cytometric analysis showed that the B-cell and macrophage fractions contained >98% $CD19^+$ cells and $CD14^+CD80^+$ cells, respectively, and the DC fraction contained >90% $CD11c^+HLA\text{-}DR^+$ cells.

Platelets were separated from platelet-rich plasma using a modified gel filtration method [15] to minimize their activation during the isolation procedure. In some instances, platelets were aliquoted and stored at −80 °C. Platelet eluates were prepared from platelet aliquots using 0.1 M hydrochloric acid, followed by prompt neutralization with 0.2 M sodium hydroxide [5]. The platelets were sensitized by incubating them (10^6) with platelet eluates derived from 5×10^6 platelets for 30 min at room temperature, and they were washed once before being cultured.

GPIIb/IIIa-reactive T-cell lines

GPIIb/IIIa-reactive T-cell lines were established by repeated stimulation of peripheral blood T cells with tryptic peptides of GPIIb/IIIa, followed by a limiting dilution [6]. A portion of each T-cell line was stored in liquid nitrogen, and the remaining cells were maintained by stimulation with an antigenic recombinant GPIIb/IIIa fragment, IL-2 (100 U mL^{-1}), and irradiated (100 Gy) autologous LBLs at 7–10 day intervals. Cytokine profiles were determined by evaluating the production of IFN-γ, IL-4, IL-6 and IL-10 in response to treatment with phytohemagglutinin (1 μg mL^{-1}) and anti-CD3 mAb (30 ng mL^{-1}) for 48 h [6].

Antigen-induced T-cell proliferation

GPIIb/IIIa-reactive T-cell lines (5×10^4 T cells) were cultured with autologous irradiated LBLs, autologous macrophages, B cells, or DCs freshly prepared from the spleen, or with autologous cultured macrophages derived from the peripheral blood (10^5), in the presence or absence of antigen for 3 days [6]. After a final 16-h incubation with 0.5 mCi/well of ^{3}H-thymidine, the cells were harvested and ^{3}H-thymidine incorporation was determined in a TopCount microplate scintillation counter (Packard, Meriden, CT, USA). The antigens used included native GPIIb/IIIa, tryptic peptides of GPIIb/IIIa, mock-treated control (trypsin alone), individual recombinant GPIIb/IIIa fragments, GST, and tetanus toxoid (List Biological Laboratories, Campbell, CA, USA) at a final concentration of 5 μg mL^{-1}. Untreated or sensitized platelets (10^6) were also used as an antigen source. All experiments were carried out in duplicate or triplicate, and the values are the mean cpm ± standard deviation of multiple determinations. To examine the roles of HLA class II and Fcγ receptors in GPIIb/IIIa-specific T-cell proliferation, mAbs were added when the cultures were started. Anti-HLA-DR, anti-HLA-DQ (Leinco Technologies, Ballwin, MO, USA), anti-FcγRI (reactive with FcγRIA; R&D system), anti-FcγRIII (reactive with FcγRIIIA and FcγRIIIB; BD PharMingen, San Jose, CA, USA), and isotype-matched control mAb were used at a final concentration of 2.5 μg mL^{-1}.

In vitro assay for anti-GPIIb/IIIa antibody production

An *in vitro* assay to analyze the antigen-induced anti-GPIIb/IIIa antibody synthesis in cultures of GPIIb/IIIa-reactive T-cell line cells and autologous peripheral blood B cells was carried out as described [6]. Briefly, GPIIb/IIIa-reactive T cells (2×10^5) plus autologous peripheral blood B cells (10^5) were cultured with or without native GPIIb/IIIa, tryptic peptides of GPIIb/IIIa (5 μg mL^{-1}) or mock-treated control in the presence of pokeweed mitogen (1 mg mL^{-1}) for 10 days. Untreated or sensitized platelets (10^6) were also used as an antigen source. These cultures were carried out in the presence or absence of autologous cultured macrophages (10^5). The level of IgG anti-GPIIb/IIIa antibodies in culture supernatants was measured in duplicate by an enzyme-linked immunosorbent assay using affinity-purified GPIIb/IIIa as the antigen [13]. All cultures were prepared in duplicate, and the anti-GPIIb/IIIa antibody results shown represent the mean OD_{450} ± standard deviation of four values.

Results

GPIIb/IIIa-reactive T-cell lines

A total of six GPIIb/IIIa-reactive T-cell lines established from three ITP patients were used in this study. All the T-cell lines failed to proliferate in response to native GPIIb/IIIa, but responded to tryptic GPIIb/IIIa peptides as well as one of the recombinant GPIIb/IIIa fragments in the presence of autologous LBLs, consistent with previous studies [5,6]. Table 1 presents the characteristics of the GPIIb/IIIa-reactive T-cell lines used in this study. The antigenic fragments recognized were heterogeneous: two lines recognized IIbα18–259, one recognized IIbα566–841, and three recognized IIIa22–262. All the lines were HLA-DR-restricted T cells that produced high levels of IFN-γ with minimal or no IL-4 expression upon

Table 1 Characteristics of GPIIb/IIIa-reactive $CD4^+$ T-cell lines derived from ITP patients

T-cell line #	ITP donor	Antigenic fragment	HLA class II restriction	Cytokine profiles	*In vitro* helper activity
NE1	ITP#24	IIbα18–259	HLA-DR	IFN-γ, IL-6, IL-10	+
NE4	ITP#24	IIIa22–262	HLA-DR	IFN-γ, IL-6, IL-10	+
SuM7	ITP#25	IIbα566–841	HLA-DR	IFN-γ, IL-4, IL-10	−
SuM8	ITP#25	IIIa22–262	HLA-DR	IFN-γ, IL-4, IL-6, IL-10	+
MH3	ITP#47	IIbα18–259	HLA-DR	IFN-γ, IL-6	+
MH6	ITP#47	IIIa22–262	HLA-DR	IFN-γ, IL-4, IL-6, IL-10	+

mitogenic stimulation, consistent with previous studies showing Th0/Th1 cytokine response in childhood ITP [4,16]. All except one line (SuM7) were able to induce the production of anti-GPIIb/IIIa antibodies from autologous B cells *in vitro*.

Macrophages as APCs for antigenic GPIIb/IIIa peptides in vivo

To evaluate which splenic APCs presented the antigenic GPIIb/IIIa peptides *in vivo* in ITP patients, GPIIb/IIIa-reactive T-cell line cells were co-cultured with freshly isolated autologous macrophages, B cells or DCs in the presence or absence of GPIIb/IIIa antigens (Fig. 1A). B cells and DCs required the addition of tryptic GPIIb/IIIa peptides to induce the proliferation in the GPIIb/IIIa-reactive T-cell lines. In contrast, the splenic macrophages stimulated the cells regardless of the presence of exogenous antigens, suggesting that these splenic macrophages had already phagocytosed opsonized platelets *in vivo* before splenectomy and had processed GPIIb/IIIa for presentation to T cells. The T-cell response induced by splenic macrophages in the absence of exogenous antigen was completely blocked by an anti-HLA-DR mAb, but not by an anti-HLA-DQ mAb, indicating a requirement for HLA-DR/T-cell receptor engagement (Fig. 1B). The remaining five T-cell lines also showed an HLA-DR-restricted response in cultures with macrophages but without exogenous antigen. These findings together suggest that splenic macrophages are the dominant APCs for antigenic GPIIb/IIIa peptides *in vivo*.

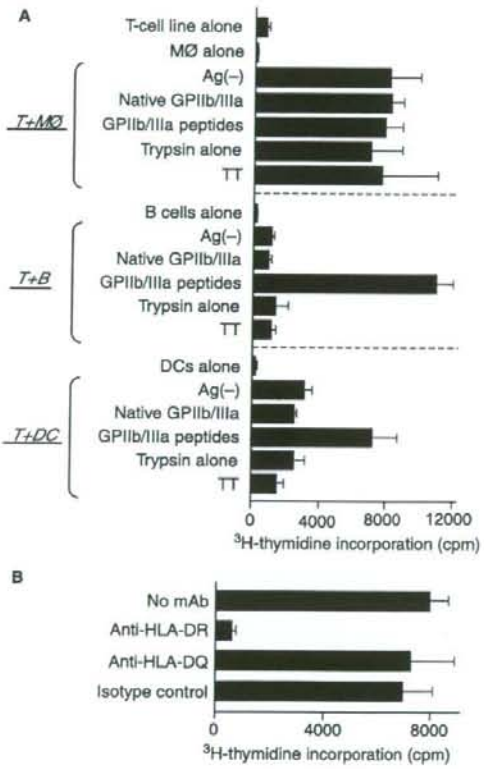

Fig. 1. Response of GPIIb/IIIa-reactive T-cell line to various splenic APCs in the presence or absence of GPIIb/IIIa. (A) GPIIb/IIIa-reactive T-cell line NE1 was cultured with autologous splenic macrophages (MØ), B cells or DCs in the presence or absence of native GPIIb/IIIa, its tryptic peptides, mock-treated control (trypsin alone), or tetanus toxoid (TT). The antigen-induced T-cell response was measured by ^{3}H-thymidine incorporation. (B) GPIIb/IIIa-reactive T-cell line NE1 was cultured with autologous splenic macrophages without exogenous antigen in the presence or absence of anti-HLA-DR, anti-HLA-DQ or isotype-matched control mAbs. The antigen-induced T-cell response was measured by ^{3}H-thymidine incorporation.

Platelet opsonization is required for macrophages to present antigenic GPIIb/IIIa peptides

To investigate the mechanisms for macrophages' processing and presentation of the antigenic GPIIb/IIIa peptides, we used autologous macrophages prepared from circulating monocytes in samples collected when the patients were in remission after splenectomy. In contrast to freshly isolated splenic macrophages, these cultured macrophages failed to induce the proliferation of GPIIb/IIIa-reactive T-cell lines in the absence of the exogenous antigen, but induced a response in the presence of tryptic GPIIb/IIIa peptides or the antigenic recombinant GPIIb/IIIa fragment (Fig. 2), suggesting that they did not carry antigenic GPIIb/IIIa peptides. Among various possible reasons for this difference in requiring exogenous antigen to induce the T-cell response, we focused on the macrophages' differing phagocytic states. Splelenic macrophages normally take up a large quantity of opsonized platelets *in vivo*, whereas it is unlikely that the cultured macrophages derived from patients in remission could have phagocytosed many platelets. Therefore, we evaluated whether the cultured macrophages could stimulate the GPIIb/IIIa-reactive T-cell lines in the presence of platelets derived from ITP patients who were thrombocytopenic and positive for platelet-associated anti-GPIIb/IIIa antibodies (Fig. 2A). As expected, macrophages preincubated with autologous platelets or allogenic platelets from ITP patients, but not those preincubated with healthy platelets, did induce the T-cell response. Interestingly, autologous platelets that were obtained before splenectomy also induced the T-cell response, even though those obtained during remission after splenectomy did not. Consistent results were obtained from the five additional T-cell lines. These findings indicate that presentation of the antigenic GPIIb/IIIa peptides by macrophages results from the uptake of platelets obtained under conditions of positive platelet-associated anti-GP antibodies.

The platelets derived from ITP patients at the time of splenectomy, from patients in remission and from healthy individuals differed in whether they were positive or negative for platelet-associated anti-GPIIb/IIIa antibodies. To evaluate if opsonization of the platelets was a critical factor for the

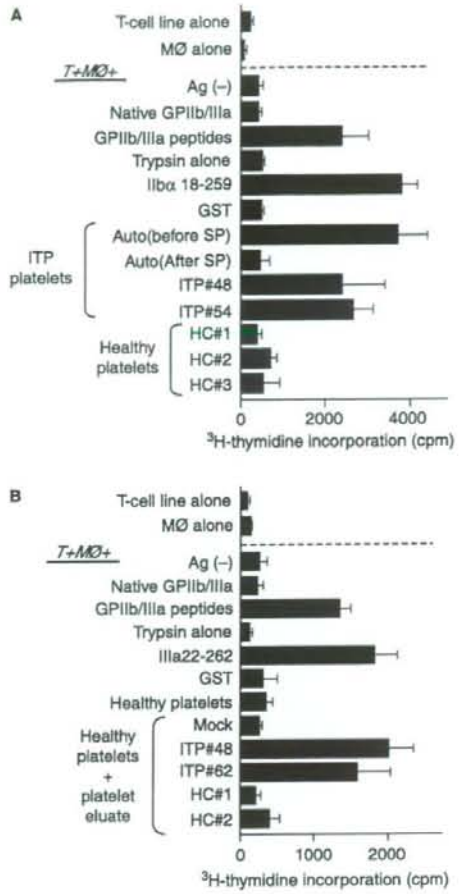

Fig. 2. Response of GPIIb/IIIa-reactive T-cell lines to cultured macrophages in the presence of ITP or healthy platelets. (A) GPIIb/IIIa-reactive T-cell line NE1 was cultured with autologous macrophages (MØ), which were prepared from circulating monocytes obtained when the patient was in remission after splenectomy, in the presence or absence of various antigen sources, including native GPIIb/IIIa, tryptic GPIIb/IIIa peptides, mock-treated control (trypsin alone), IIbα18-259, and GST. Autologous platelets (Auto) derived before and after splenectomy (SP), allogenic platelets from two ITP patients (ITP48 and #54), and platelets derived from three healthy individuals (HC#1-#3) were also added to some cultures as antigen sources. The antigen-induced T-cell response was measured by ^{3}H-thymidine incorporation. A representative result from two independent experiments is shown. (B) GPIIb/IIIa-reactive T-cell line MH6 was cultured with autologous cultured macrophages in the presence or absence of native GPIIb/IIIa, tryptic GPIIb/IIIa peptides, trypsin alone, IIIa22-262, or GST. Platelets derived from a healthy individual (HC#2) were preincubated with mock-treated eluates or platelet eluates from two ITP patients (ITP#48 and #62) or two healthy donors (HC#1 and #2), and added to the cultures. The antigen-induced T-cell response was measured by ^{3}H-thymidine incorporation. A representative result from three independent experiments is shown.

subsequent presentation of the antigenic GPIIb/IIIa peptides by macrophages, a T-cell line was incubated with cultured macrophages in the presence of healthy platelets pretreated with eluates from ITP platelets or healthy platelets (Fig. 2B). As expected, the T cells responded to macrophages carrying healthy platelets that had been presensitized with ITP platelet eluates, but not to those carrying healthy platelets pretreated with healthy platelet eluates. Consistent results were obtained from three additional T-cell lines, NE1, NE4 and SuM7.

The role of Fcγ receptors in the presentation of antigenic GPIIb/IIIa peptides by macrophages

We further evaluated the potential involvement of Fcγ receptors in the recognition of opsonized platelets by macrophages. Proliferation of the GPIIb/IIIa-reactive T cells induced by macrophages carrying opsonized platelets was inhibited by anti-FcγRI mAb, but not by anti-FcγRIII mAb (Fig. 3). This result was reproduced in seven independent experiments using three T-cell lines, NE1, SuM7 and SuM8.

Promotion of anti-GPIIb/IIIa antibody production by macrophages capturing sensitized platelets

Anti-GPIIb/IIIa antibody production was observed in cultures of GPIIb/IIIa-reactive T-cell lines and autologous B cells in the

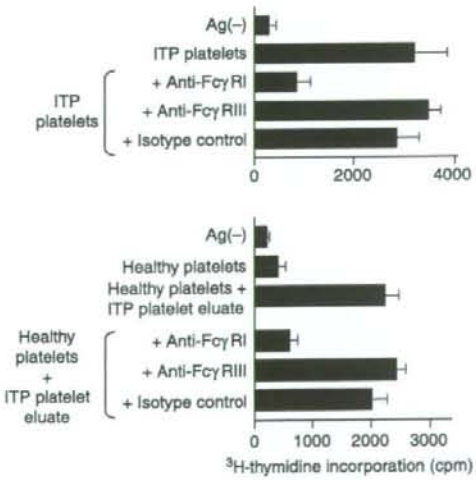

Fig. 3. Effects of anti-Fcγ receptor mAb on the proliferation of GPIIb/IIIa-reactive T-cell lines induced by cultured macrophages carrying opsonized platelets. GPIIb/IIIa-reactive T-cell lines MH3 (upper panel) and MH6 (lower panel) were cultured with autologous macrophages and ITP platelets (#48) or healthy platelets (HC#3) that were preincubated with ITP platelet eluates (#48). Anti-FcγRI, anti-FcγRIII or isotype control mAb was added when the cultures were started. The antigen-induced T-cell response was measured by ^{3}H-thymidine incorporation. A representative result from at least two independent experiments is shown.

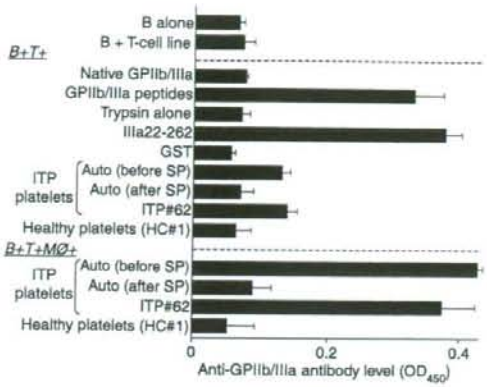

Fig. 4. Anti-GPIIb/IIIa antibody production in cultures of a GPIIb/IIIa-reactive T-cell line, autologous B cells, and macrophages carrying sensitized platelets. GPIIb/IIIa-reactive T-cell line SuM8 was cultured with autologous peripheral blood B cells in the presence or absence of various antigen sources, including native GPIIb/IIa, tryptic GPIIb/IIIa peptides, mock-treated control (trypsin alone), IIIa22-262, and GST. Autologous platelets (Auto) obtained before and after splenectomy (SP), allogenic ITP platelets (ITP62) and platelets derived from a healthy individual (HC#1) were also added to the cultures. These experiments were carried out in the presence or absence of autologous macrophages (MØ). The levels of IgG anti-GPIIb/IIIa antibodies in culture supernatants were measured by enzyme-linked immunosorbent assay. A representative result from two independent experiments is shown.

presence of tryptic GPIIb/IIIa peptides or an antigenic recombinant GPIIb/IIIa fragment (Fig. 4), consistent with our previous study [3]. In this system, the B cells capture the GPIIb/IIIa antigens, process them, and present the cryptic peptides. When we used platelets from ITP patients instead of the GPIIb/IIIa antigen in this culture system, no enhancement of anti-GPIIb/IIIa antibody production was observed. This might have been due to the inability of the B cells to capture and process platelets. Therefore, we improved this system by culturing T cells, B cells and platelets on autologous cultured macrophages. Under these conditions, the anti-GPIIb/IIIa antibody production was augmented when autologous or allogenic ITP platelets were added to the culture. Interestingly, autologous platelets obtained before the splenectomy promoted anti-GPIIb/IIIa antibody secretion by B cells, but those obtained in remission after the splenectomy did not. This response was blocked by the addition of an anti-HLA-DR or anti-FcγRI mAb (data not shown), suggesting the involvement of HLA-DR/T-cell receptor engagement and FcγRI-mediated platelet uptake in this process. Consistent results were obtained in six independent experiments involving two additional T-cell lines, NE1 and NH6.

Discussion

This study evaluated potential cellular mechanisms for inducing the sustained presentation of antigenic GPIIb/IIIa peptides that activate autoreactive T cells to subsequently produce pathogenic anti-GPIIb/IIIa antibodies from B cells in ITP patients. By using GPIIb/IIIa-reactive $CD4^+$ T-cell lines and freshly isolated splenocytes from the same ITP patients, who underwent splenectomy, we were able to identify splenic macrophages as the major APCs for presenting antigenic GPIIb/IIIa peptides *in vivo* in ITP patients. Further *in vitro* analyses revealed that presentation of the antigenic GPIIb/IIIa peptides by macrophages depended on their phagocytosis of opsonized platelets via the Fcγ receptor.

To elucidate the pathogenesis of ITP, it is necessary to clarify how cryptic determinants of GPIIb/IIIa become visible to the immune system and elicit a sustained pathogenic response in ITP patients. Because T cells that are responsive to cryptic self-determinants would not encounter antigenic peptides under normal circumstances, it is likely that a pathogenic autoreactive T-cell response is induced by the *de novo* presentation of a previously cryptic determinant under special conditions [17]. In this study, we have clearly shown that splenic macrophages present cryptic GPIIb/IIIa peptides and are capable of activating autoreactive $CD4^+$ T cells in ITP patients. In this regard, splenic macrophages in ITP patients are unique in terms of their state of phagocytosis, in that they carry a large quantity of opsonized platelets. Because increased antigen delivery to the processing compartment is one of the potential mechanisms for revealing cryptic self-determinants in APCs [17], it is likely that splenic macrophages that take up a large number of opsonized platelets via FcγRI efficiently concentrate the small quantities of platelet antigens that were previously cryptic. This process would be enhanced by the activation of the antigen-processing pathway and up-regulation of adhesion and costimulatory molecules through Fcγ receptor signaling [18]. In addition, the opsonization of platelets by anti-GPIIb/IIIa antibodies may amplify the generation of some minor epitopes, by protecting the antibody-binding site from protease attack during antigen processing [19]. These mechanisms could together promote the generation of previously cryptic peptides of GPIIb/IIIa and the resultant activation of GPIIb/IIIa-reactive T and B cells.

Based on the results from our *in vitro* assay for anti-GPIIb/IIIa antibody production, we propose that a pathogenic loop maintains the ongoing anti-platelet antibody response in ITP patients. That is, macrophages in the reticuloendothelial system capture opsonized platelets via the Fcγ receptor, process them, and present GPIIb/IIIa-derived cryptic peptides to T cells. GPIIb/IIIa-reactive $CD4^+$ T cells are activated when their T-cell receptor recognizes the protein complex consisting of the antigenic peptide and HLA-DR molecule, and they exert helper activity by secreting IL-6 [6] and up-regulating the expression of CD154 [20]. B cells produce pathogenic IgG anti-GPIIb/IIIa antibodies capable of binding to circulating platelets, 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. In this model, macrophages in the reticuloendothelial system play a central role in the chronic

phase of the ITP pathogenesis, including platelet destruction and the maintenance of the continuous production of pathogenic anti-platelet antibodies.

Our model suggests that therapeutic strategies that inhibit pathogenic anti-GPIIb/IIIa antibody production should be aimed at interrupting the continuous autoimmune loop effected by the macrophages and the GPIIb/IIIa-reactive $CD4^+$ T cells and B cells. T and B cells are already targets of many therapies for ITP, such as immunosuppressants [21], chimeric anti-CD20 mAb [22], and humanized anti-CD154 mAb [23], but the macrophages of the reticuloendothelial system could be another reasonable target. Clinical benefit has been already reported for Fcγ receptor blockade treatment using IVIG [24], anti-Rho(D) immunoglobulin [25], and anti-Fcγ receptor mAb [26]. In addition, 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 [27]. Further studies should be aimed at developing novel therapeutic strategies targeting macrophages, such as small molecules that inhibit the downstream signal of the activating Fcγ receptor [28].

One of the limitations of this study is the use of splenic APCs from patients who received IVIG treatment. This treatment might have affected the functional properties of the splenic APCs, but there was no substantial difference in the results of T-cell stimulation by APCs from patients who received IVIG and from the patient who did not. Another limitation is the use of T-cell lines, which may not represent the entire repertoire of pathogenic anti-GPIIb/IIIa-reactive T cells. Finally, antigenic GPIIb/IIIa peptides recognized by $CD4^+$ T cells in association with HLA class II alleles should be different among ethnic groups. Because our results were obtained from Japanese patients who have HLA class II alleles different from Caucasian and African populations, additional studies using samples from non-Japanese patients are necessary to confirm our findings.

In summary, splenic macrophages that phagocytose opsonized platelets via FcγRI play a central role in maintaining the continuous production of pathogenic anti-platelet antibodies in ITP patients. This information is useful in clarifying the pathogenesis of ITP and in developing novel therapeutic strategies for refractory ITP.

Addendum

M. Kuwana: contribution to concept and design, analysis, interpretation of data, and manuscript writing. Y. Okazaki: analysis, and interpretation of data. Y. Ikeda: patient recruitment and interpretation of data.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Positive and Negative Regulation of Integrin Function

Yoshiaki Tomiyama, Masamichi Shiraga, and
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Summary. Platelet integrin $\alpha_{IIb}\beta_3$, a prototypic non-I domain integrin, plays an essential role in platelet aggregation. The structure and function of $\alpha_{IIb}\beta_3$ is dramatically changed during platelet plug formation and pathological thrombus formation. The function of this integrin is regulated by the balance of actions of positive and negative regulatory factors. Several novel regulators have emerged from recent studies. As a positive regulator, the $P2Y_{12}$ plays a critical role in thrombus stability; and continuous interaction between ADP and $P2Y_{12}$ is essential for sustained $\alpha_{IIb}\beta_3$ activation. Semaphorin 3A and SHPS-1 have been identified as negative regulators. These molecules are secreted from or expressed on endothelial cells and inhibit the function of platelets as well as $\alpha_{IIb}\beta_3$. Investigation on these positive and negative regulatory factors should provide a new insight into the treatment of pathological thrombosis.

Key words. Inside-out signaling · Outside-in signaling · $P2Y_{12}$ · Semaphorin 3A · SHPS-1

Introduction

Platelets play a crucial role not only in hemostatic plug formation but also in a pathological thrombus formation, particularly in atherosclerotic arteries subjected to high shear stress [1, 2]. Moreover, recent studies have revealed that the platelet is a major player in the initiation of vascular remodeling as well as atherosclerotic lesion formation [3, 4]. As an initial step in thrombogenesis, platelets adhere to altered vascular surfaces or exposed subendothelial matrices and then become activated and aggregate with each other. As summarized in Fig. 1, it has been well documented that these processes are primarily mediated by platelet surface glycoproteins: GPIb-IX-V, integrin $\alpha_2\beta_1$ (also known as GPIa-IIa), GPVI, and integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) [5, 6].

Integrins comprise a family of heterodimeric adhesion receptors that mediate cellular attachment to the extracellular matrix and cell cohesion [7–9]. Platelets express at least five integrins on their surface: $\alpha_2\beta_1$(GPIa-IIa); $\alpha_5\beta_1$(GPIc-IIa); $\alpha_6\beta_1$(GPIc′-IIa); $\alpha_{IIb}\beta_3$(GPIIb-IIIa); $\alpha_v\beta_3$. Platelet integrin $\alpha_{IIb}\beta_3$ is a prototypic non-I domain integrin and plays an essential role in platelet aggregation as a physiological receptor for fibrinogen and von Willebrand factor. The importance of this integrin has been well documented by the clinical features of a congenital bleeding disorder, Glanzmann

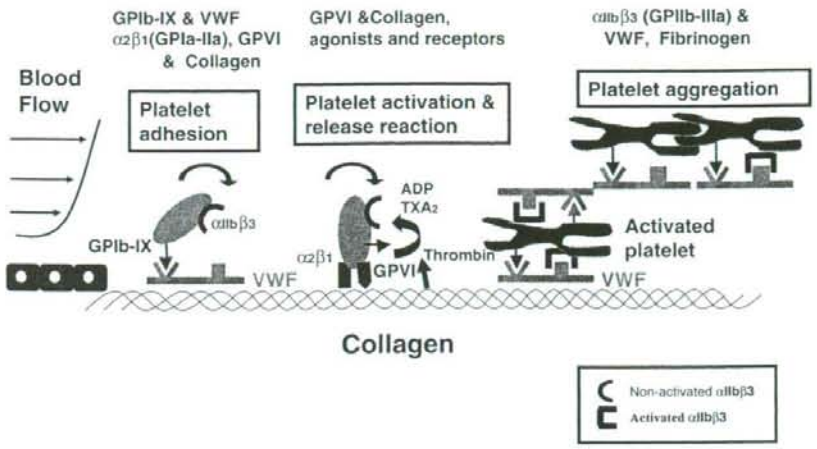

FIG. 1. Mechanisms of platelet plug formation and pathological thrombus formation. These processes depend primarily on platelet adhesive interactions with both platelet surface glycoproteins including integrins and extracellular matrix proteins. Platelet adhesion (or tethering) is mainly mediated by glycoprotein (*GP*)Ib-IX and von Willebrand factor (*VWF*) especially under high shear rates; and platelet aggregation is mediated by $\alpha_{IIb}\beta_3$ and VWF and fibrinogen. Platelet activation and released factors such as adenosine diphosphate (ADP) play a critical role in thrombus stability. *TXA_2*, thromboxane A_2

thrombasthenia (GT) [10, 11]. The crystal structure of $\alpha_{IIb}\beta_3$ revealed that the ligand-binding head is formed by a seven-bladed β-propeller domain from α_{IIb} and a β I-domain from β_3 [12, 13]. Despite the presence of integrin $\alpha_{IIb}\beta_3$ and its ligands, single platelets circulate freely within the vascular tree that is lined by an intact monolayer of endothelial cells. Thus, the function of integrin $\alpha_{IIb}\beta_3$ is regulated by the balance of actions of positive and negative regulatory factors. During thrombogenesis, the affinity of $\alpha_{IIb}\beta_3$ for macromolecular ligands is dynamically changed [8, 9]. In resting platelets, $\alpha_{IIb}\beta_3$ is in a low-affinity state and does not bind soluble macromolecular ligands. However, after exposure to subendothelial matrix and several mediators such as adenosine 5′-diphosphate (ADP), thromboxane A_2, and thrombin, platelets become activated, and activation signals (inside-out signaling) that induce a high-affinity state of $\alpha_{IIb}\beta_3$ for soluble ligands ($\alpha_{IIb}\beta_3$ activation) are generated. After ligand binding to $\alpha_{IIb}\beta_3$, postligand-binding signals (outside-in signaling) that induce tyrosine phosphorylation and cytoskeletal reorganization are further generated, leading to full expression of $\alpha_{IIb}\beta_3$ function. Molecular characterization of GT due to a dysfunctional $\alpha_{IIb}\beta_3$ (referred as variant GT) provides strong evidence that the cytoplasmic domain of β_3 is involved in inside-out signaling [14, 15]. Indeed, specific binding of the cytoskeletal protein talin to integrin β subunit cytoplasmic tails leads to $\alpha_{IIb}\beta_3$ activation as a final common step in integrin activation [16]. Major advances have been made regarding the structural basis of $\alpha_{IIb}\beta_3$ activation, resulting in the proposal of the "switchblade" model [17]. However, much remains to be elucidated about factors (or molecules) surrounding platelets that positively or negatively regulate $\alpha_{IIb}\beta_3$ function. In this review, we focus on recently identified factors and/or mechanisms that regulate $\alpha_{IIb}\beta_3$ function.

Positive Regulators for $\alpha_{IIb}\beta_3$ Function

In vivo fluorescence microscopy reveals that a few platelets are tethered to the intact vascular wall even under physiological conditions [18]. However, ~100% of these platelets were displaced from the vascular wall without firm arrest. Thus, a threshold for further platelet activation and the initiation of thrombus formation seems to exist, and $\alpha_{IIb}\beta_3$ function should be dynamically controlled by the balance of positive and negative regulators. A number of factors have been identified as a positive regulator for $\alpha_{IIb}\beta_3$ function (Table 1). These factors contribute to stabilize the platelet thrombus as well as initiate thrombus formation. ADP, collagen, and thrombin are classic, well-known factors that initiate thrombus formation by inducing $\alpha_{IIb}\beta_3$ activation. In contrast, serotonin acts as a potentiator, rather than an initiator, for $\alpha_{IIb}\beta_3$ activation. Recently, several factors that contribute to stabilize platelet thrombus have been identified: CD40L and $\alpha_{IIb}\beta_3$, Eph kinases and ephrins, Gas6 and its receptors, and ADP and $P2Y_{12}$ receptor (for review see ref. 19). CD40L, a member of the tumor necrosis factor (TNF) family, is expressed on the platelet surface after platelet activation, and a soluble form of CD40L (sCD40L) is generated by the activation as well. Although CD40 is known to be a receptor for CD40L, the effect of CD40L (and sCD40L) on platelets is mediated by $\alpha_{IIb}\beta_3$ but not by CD40. The interaction of CD40L and $\alpha_{IIb}\beta_3$ contributes to thrombus stability, probably via augmentation of $\alpha_{IIb}\beta_3$-mediated outside-in signaling [20, 21]. Eph kinases and ephrins also augment $\alpha_{IIb}\beta_3$ outside-in signaling [22, 23]. Platelets express the Eph receptor kinase (EphA4 and EphB1) and the Eph kinase ligand, ephrinB1; and blockade of the Eph/Ephrin interactions causes platelet disaggregation induced by low concentrations of ADP and decreased platelet thrombus volume on a collagen-coated surface at high shear rates. Gas6 is a secreted protein localized in α-granules; and its receptors Axl, Sky, and Mer are also expressed

TABLE 1. Regulators for $\alpha_{IIb}\beta_3$ function

Positive regulators
ADP
Collagen
Thrombin
Epinephrine
PAF
Serotonin
CD40L
Eph kinases/ephrins
Gas6
Leptin
Negative regulators
Prostacyclin
Nitric oxide
CD39 (NTPDase1)
PECAM-1
Semaphorin 3A
SHPS-1 (SIRPα1)

on platelets. It has been demonstrated that secreted Gas6 binds to its receptors, leading to the promotion and stabilization of platelet plug formation via $\alpha_{IIb}\beta_3$ outside-in signaling [24]. Thus, these newly identified factors may play a role in the stability of platelet aggregation in vivo. However, recent studies have revealed that the interaction between ADP and its receptor $P2Y_{12}$ play a critical role in the stability of platelet thrombus.

Role of the Interaction Between ADP and $P2Y_{12}$ in the Maintenance of $\alpha_{IIb}\beta_3$ Activation

ADP is stored within platelet dense granules and actively secreted upon platelet activation; approximately 2.5 μmol ADP exists in 10^{11} platelets [25]. Platelets have at least two major G protein-coupled ADP receptors: $P2Y_1$ is a G_q-coupled receptor responsible for mediating platelet shape change and reversible platelet aggregation through intracellular calcium mobilization, whereas $P2Y_{12}$ is a G_i-coupled receptor responsible for mediating the inhibition of adenylyl cyclase and sustained platelet aggregation [26]. $P2Y_{12}$ consists of 342 amino acid residues with seven transmembrane domains. The importance of $P2Y_{12}$ is well documented by the clinical feature of congenital bleeding disorder due to $P2Y_{12}$ deficiency [27–29]. We have identified a Japanese patient with $P2Y_{12}$ deficiency, OSP-1, caused by a point mutation in the translation initiation codon (ATG to AGG) [30]. $P2Y_{12}$-mediated signaling evoked by endogenous ADP plays a major role in platelet aggregation induced by low concentrations of collagen, U46619, and PAR1 TRAP in vitro. We and others have demonstrated impaired thrombus stability under flow conditions [29, 30]. Employing whole blood obtained from OSP-1, real-time analysis of thrombogenesis on a type I collagen-coated surface under a high shear rate ($2000\,s^{-1}$) revealed that $P2Y_{12}$ deficiency led to loosely packed thrombus and impaired thrombus growth with enhancing adhesion to collagen. The increase in platelet adhesion to collagen was probably due to the impaired platelet consumption by the growing thrombi. Moreover, our real-time observation indicated that the loosely packed aggregates were unable to resist against high shear stress, and most of the aggregates at the apex of the thrombi came off the thrombi [30]. In a mesenteric artery injury model $P2Y_{12}$-knockout mice also demonstrated the instability of thrombus formation [31]. Thus, the ADP-$P2Y_{12}$ interaction plays a major role in the stability of thrombus.

We assessed the $\alpha_{IIb}\beta_3$ activation on OPS-1 platelets in vitro by the binding of ligand-mimetic monoclonal antibody, PAC-1. Interestingly, $\alpha_{IIb}\beta_3$ activation is markedly impaired by stimulation with PAR1-TRAP, PAR4-TRAP, or U46619 in the absence of $P2Y_{12}$ [30]. On the other hand, PAR1-TRAP and U46619 are able to induce transient aggregation of OSP-1 platelets, indicating that $\alpha_{IIb}\beta_3$ could be transiently activated with these agonists. Based on these findings, we assume that $\alpha_{IIb}\beta_3$ activation may be too short and unstable to be detected by the PAC1 binding assay on OSP-1 platelets and that released ADP and $P2Y_{12}$-mediated signaling may play a critical role in the maintenance of $\alpha_{IIb}\beta_3$ activation. Employing modified ligand-binding assays, we have analyzed the mechanism of sustained $\alpha_{IIb}\beta_3$ activation induced by thrombin. After completion of $\alpha_{IIb}\beta_3$ activation and induction of α-granule secretion, a $P2Y_{12}$ antagonist (AR-C69931MX) was added to the activated platelets [32]. Under these conditions, the stimulated platelets showed long-lasting $\alpha_{IIb}\beta_3$ activation. However, the addition

of 1 μM AR-C69931MX at any time tested after thrombin stimulation disrupted the sustained $\alpha_{IIb}\beta_3$ activation without inhibiting CD62P expression (Fig. 2). Neither yohimbine (an adrenergic receptor antagonist), MIC-9042 (a 5-HT_2 receptor antagonist), nor SQ-29548 (a thromboxane A_2 receptor antagonist) inhibited sustained $\alpha_{IIb}\beta_3$ activation. Dilution of platelet concentrations from 50 000 platelets/μl to 500 platelets/μl also abolished sustained $\alpha_{IIb}\beta_3$ activation, and disruption of $\alpha_{IIb}\beta_3$ activation by the dilution was abrogated by the addition of small amounts of "exogenous" ADP. Thus, the continuous interaction between secreted ADP with $P2Y_{12}$ is necessary for sustained $\alpha_{IIb}\beta_3$ activation induced by thrombin; and substantial amounts of ADP (= substantial platelets) are needed to maintain $\alpha_{IIb}\beta_3$ activation. The critical role of the interaction between ADP and $P2Y_{12}$ is also evident in the sustained $\alpha_{IIb}\beta_3$ activation induced by U46619 (TXA_2 analogue) [32]. Even in the absence of $P2Y_{12}$, platelets can transiently aggregate with each other. However, platelets lacking G_q and G_{13} are completely unresponsive to thrombin, and the activation of G_i-mediated signaling alone is not sufficient to induce platelet aggregation [33]. Thus, it is likely that once $\alpha_{IIb}\beta_3$ is activated by G_q and/or G_{13}-mediated signaling the ADP-$P2Y_{12}$ may prevent the shift from the activated $\alpha_{IIb}\beta_3$ to the resting $\alpha_{IIb}\beta_3$ (Fig. 2).

Recent in vivo observations demonstrated that during platelet thrombus formation circulating platelets were tethered to the luminal surface of growing thrombi by VWF–GPIb interaction. However, more than 95% of tethered platelets were subsequently translocated and/or detached [18]. Activated $\alpha_{IIb}\beta_3$ on the detached platelets should become inactivated because the released ADP is immediately diluted by the

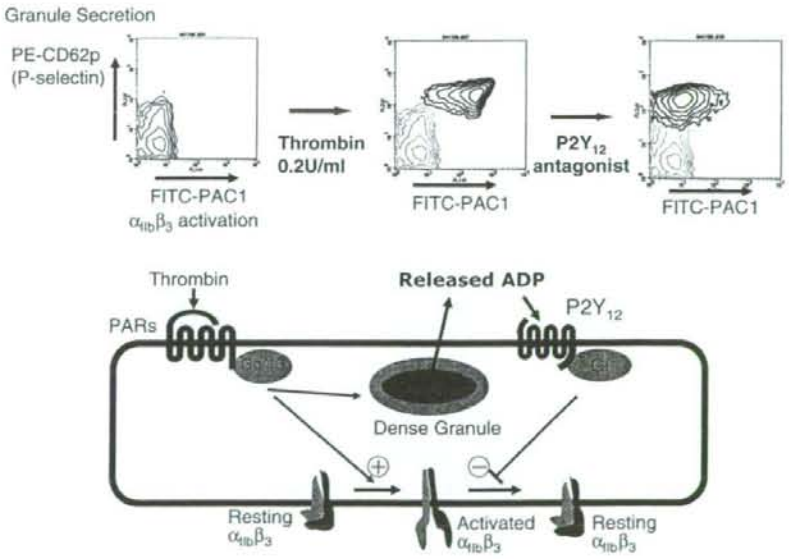

FIG. 2. Critical role of ADP–$P2Y_{12}$ interaction in the maintenance of $\alpha_{IIb}\beta_3$ activation. Blockade of ADP–$P2Y_{12}$ interaction at any time after thrombin stimulation disrupts $\alpha_{IIb}\beta_3$ activation. Once $\alpha_{IIb}\beta_3$ is activated by G_q- and/or G_{13}-mediated signaling, the ADP–$P2Y_{12}$ may prevent the shift from activated $\alpha_{IIb}\beta_3$ to resting $\alpha_{IIb}\beta_3$. *FITC-PAC1*, fluorescein isothiocyanate-conjugated PAC1

blood flow. At the luminal surface, activated $\alpha_{IIb}\beta_3$ on the tethered platelets would be maintained only when the platelets are continuously exposed to ADP released from adjacent activated platelets. At the inside of growing thrombi, it appears that platelets are constantly exposed to such high concentrations of released ADP that $\alpha_{IIb}\beta_3$ can be maintained in its high-affinity state in concert with the effects of thrombin and TXA_2. It is possible that ADP concentrations surrounding platelets may largely influence whether platelets participate in thrombus formation. Thus, $P2Y_{12}$ may serve as a sensor for thrombogenic status surrounding individual platelets, and the interaction between ADP and $P2Y_{12}$ likely determines thrombus size.

Negative Regulators for $\alpha_{IIb}\beta_3$ Function

Prostacyclin and nitric oxide produced by endothelial cells are well-known negative regulators for the platelet function [34]. In addition to these molecules several negative regulators have been emerged in recent studies (Table 1). We have identified that semaphorin 3A and SHPS-1 act as negative regulators for $\alpha_{IIb}\beta_3$ function [35, 36].

Semaphorin 3A as a Negative Regulator for Platelet Function

The semaphorin family comprises soluble and membrane-bound proteins that are defined by the presence of a conserved 500-amino-acid semaphorin domain at their amino termini. Class 3 semaphorins are secreted disulfide-bound homodimeric molecules; and Sema3A, a prototypic class 3 semaphorin, causes growth cone collapse and provides chemorepulsive guidance for migrating axons. Cell surface receptor for Sema3A consists of a complex of two distinct transmembrane receptors, neuropilin-1 and plexin A (A1-A3). It has been demonstrated that Sema3A is produced by endothelial cells and inhibits integrin function on endothelial cells in an autocrine manner [37]. Employing two distinct Sema3A chimera proteins, we have demonstrated that Sema3A has extensive inhibitory effects on platelet function [35]. Sema3A inhibited agonist-induced $\alpha_{IIb}\beta_3$ activation dose-dependently. Moreover, Sema3A inhibited granular secretion as well as platelet spreading on immobilized fibrinogen. However, Sema3A did not show any effects on the levels of cAMP or cGMP or thrombin-induced increase in intracellular Ca^{2+} concentrations. It is likely that Sema3A inhibits cytoskeletal reorganization in activated platelets as Sema3A inhibits platelet spreading and granule secretion.

Indeed, Sema3A inhibited agonist-induced elevation of filamentous actin (F-actin) contents and Rac1 activation. Rac1 activation is necessary for platelet actin assembly and lamellipodia formation after agonist stimulation. Therefore, marked impairment of Rac1 activation is likely to account for the Sema3A-induced impairment of actin rearrangement and spreading in platelets. There were two major downstream effectors of Rac1 identified: PAK and WAVEs [Wiskott-Aldrich syndrome protein (WASP) family verprolin-homologous proteins]. Several PAK substrates or binding partners have been implicated in the effects of PAK, including filamin, LIM kinase, myosin, and paxillin. Among them, LIM kinase phosphorylates and inactivates cofilin, a protein that promotes severing and depolymerization of F actin. Consistent with the inhibition of Rac1 activation, Sema3A inhibited phosphorylation of cofilin in both resting

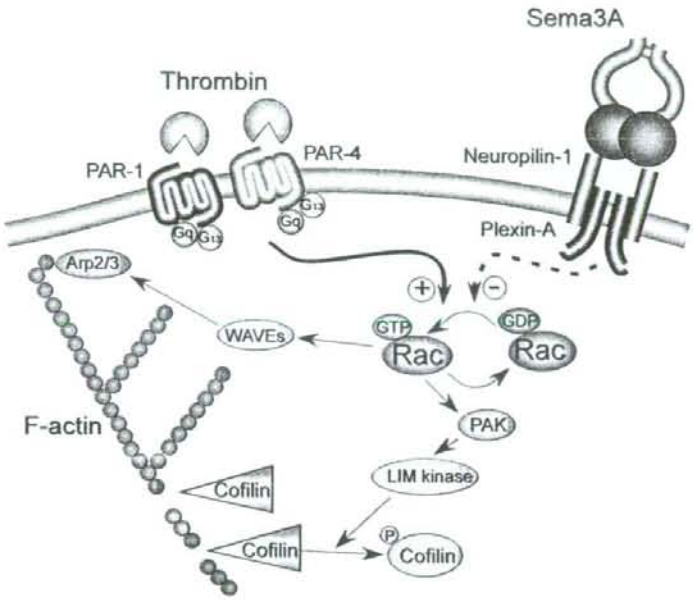

FIG. 3. Inhibitory mechanisms of platelet function by Sema 3A. Sema 3A inhibits platelet spreading and granular secretion as well as $\alpha_{IIb}\beta_3$ activation. The inhibitory effects are mediated in part by the inhibition of agonist-induced Rac1 activation and phosphorylation of cofilin. This inhibition leads to the inhibition of F-actin elevation and cytoskeleton rearrangement

and activated platelets, suggesting that Sema3A increases severing and depolymerization of F-actin by keeping cofilin in the activated state (Fig. 3). In addition to Rac1 inactivation, our recent data showed that Sema3A inhibited the PI3 kinase pathway, including Rap1B, which may account for the inhibition of $\alpha_{IIb}\beta_3$ activation (unpublished data).

SHPS-1 as a Negative Regulator for Platelet Function

SHPS-1 (Src homology 2 domain-containing protein tyrosine phosphatase substrate-1), also known as signal regulatory protein α1 (SIRP α1), is a membrane glycoprotein with three extracellular immunoglobulin (Ig)-like domains, a single transmembrane domain, and an intracellular domain containing two immunoreceptor tyrosine-based inhibitory motifs (ITIM) and expressed on endothelial cells and leukocytes. CD47 (integrin-associated protein, or IAP) is a ubiquitously expressed 50-kDa membrane glycoprotein with an extracellular Ig domain, five membrane-spanning domains, and a short cytoplasmic tail. CD47 physically associates with $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, and $\alpha_2\beta_1$ and modulates a variety of cell functions [38]. Two ligands are known to bind to CD47: thrombospondin-1 (TSP-1) and SHPS-1. The TSP-1–CD47 interaction has been believed to augment integrin-mediated platelet function. On the other hand, SHPS-1–Ig, a fusion protein consisting of the extracellular domain of SHPS-1 and human Ig Fc domain, impaired secondary platelet aggregation induced by a low concentration

of ADP (2.5μM). Moreover, SHPS-1–Ig markedly impaired $\alpha_{IIb}\beta_3$-mediated platelet spreading onto immobilized fibrinogen. The inhibition of platelet spreading is CD47-specific because it was not observed in CD47-deficient ($CD47^{-/-}$) murine platelets. Of particular interest is that SHPS-1 inhibits $\alpha_{IIb}\beta_3$-mediated platelet spreading without disturbing Syk and FAK tyrosine phosphorylation. SHPS-1 did inhibit tyrosin phosphorylation of α-actinin, a downstream effector of FAK. Thus, SHPS-1 negatively regulates platelet function through CD47, especially $\alpha_{IIb}\beta_3$-mediated outside-in signaling, by interfering with the downstream pathway of FAK.

Conclusion

Thrombogenesis is a complex process regulated by the balance of positive and negative regulatory proteins (or molecules). Further investigations of these regulatory molecules would provide a new insight into the more effective prevention of pathological thrombosis.

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特発性血小板減少性紫斑病

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Key words : Autoantibodies, Thrombopoietin, Reticulated platelets, GPIIb-IIIa

はじめに

特発性血小板減少性紫斑病（Idiopathic Thrombocytopenic Purpura, ITP）は，他の基礎疾患や薬剤などの原因が明らかではないにもかかわらず，血小板の破壊が亢進し血小板減少を来たす後天性の疾患である。欧米では特発性（idiopathic）というよりは，免疫性（immune）あるいは自己免疫性（autoimmune）という表現が用いられることが多い[1,2]。点状出血や紫斑など皮膚粘膜出血が主たる症状である。ITPの成因の詳細はいまだ不明であるが，主体となる血小板減少機序は，血小板に対する自己抗体を介した免疫反応による。特に慢性型（後述）では，血小板に対する自己抗体により早期に網内系で破壊され血小板減少をきたす自己免疫疾患と考えられている。

ITPはその発症様式と経過より，急性型と慢性型に分類され，6か月以内に自然寛解する病型は急性型，それ以後も血小板減少が持続する病型は慢性型と分類される。急性型は小児に多くみられ，ウイルス感染を主とする先行感染を伴うことが多い。一方，慢性型は成人女性に多く，内科においてITPといえば一般的に慢性型をさしている。しかしながら，発症時に急性型か慢性型かを区別することは極めて困難であり，実際には発症後6か月経過した時点において，6か月以内に寛解したものを急性型，そうでないものを慢性型として分類することになる（Table 1）。

疫　学

本邦における年間発症率は人口10万人あたり1.5～3.3人と推計される。慢性ITPは従来20～40歳台の若年女性に発症することが多いとされていたが，2005年の「血液凝固異常症に関する調査研究」班の調査では従来のピークに加え，60～80歳での発症ピークが認められるようになってきている。高齢者の発症には男女比に差はない。急性ITPは5歳以下の発症が圧倒的である（Fig. 1）。

病態生理

1951年，Harringtonらは彼自身も含め健常人に対しITP患者血漿の輸注試験を行いITPの原因が血漿中の血小板減少因子であることを初めて示した[3]。その後血小板減少因子がIgG分画に存在することが示され血小板減少は血小板に対する抗体に起因することが示唆された[4]。血小板自己抗体の主要な標的抗原に関しては，1982年von Leeuwenらは42例のITP患者血小板より自己抗体を解離し検討したところ，35例において血小板自己抗体は正常血小板と結合するが，血小板無力症血小板とは結合しないことを示した[5]。血小板無力症では血小板膜糖蛋白（GP）IIb-IIIaが欠損していることから[6]，GPIIb-IIIaが血小板抗体の主要な抗原であることを強く示唆する成績である。その後，血小板糖蛋白特異的抗体の検出法の開発などにより血小板抗体の多くが血小板膜糖蛋白GPIIb-IIIaあるいはGPIb-IXを標的としていることが明らかにされている[7~9]。ITPにおいてGPIIb-IIIaやGPIb-IX以外の血小板糖蛋白に対する抗体も存在するが，これらの抗体は比較的まれである[10]。特殊な例としては抗GPVI自己抗体に起因するITPが報告されている。GPVIは血小板におけるコラーゲン受容体であるが，抗GPVI自己抗体により軽度に血小板数が減少すると共にGPVIがinternalizeされ膜表面から欠損するため，血小板機能異常（コラーゲン惹起血小板凝集の欠如）をきたし出血傾向を示すという極めてユニークな病態を呈する[11~13]。

Harringtonらの成績は患者血清（血漿）抗体が重要であることを示唆しているものの，ITPにおいては血小板自己抗体の殆んどが既に患者血小板に結合しており，血

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