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 $Fc\gamma$ 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 $Fc\gamma$ 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 $Fc\gamma$ 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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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.

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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*.

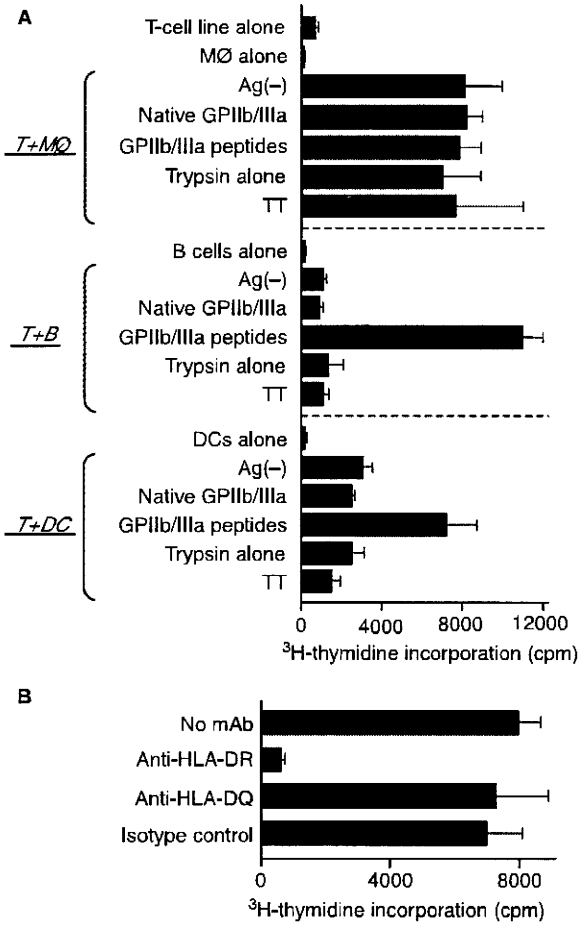

Fig. 1. Response of GPIIb/IIIa-reactive T-cell line to various splenic APCs in the presence or absence of GPIIb/IIIa. (A) GPIIb/IIIa-reactive T-cell line NE1 was cultured with autologous splenic macrophages (MØ), B cells or DCs in the presence or absence of native GPIIb/IIIa, its tryptic peptides, mock-treated 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

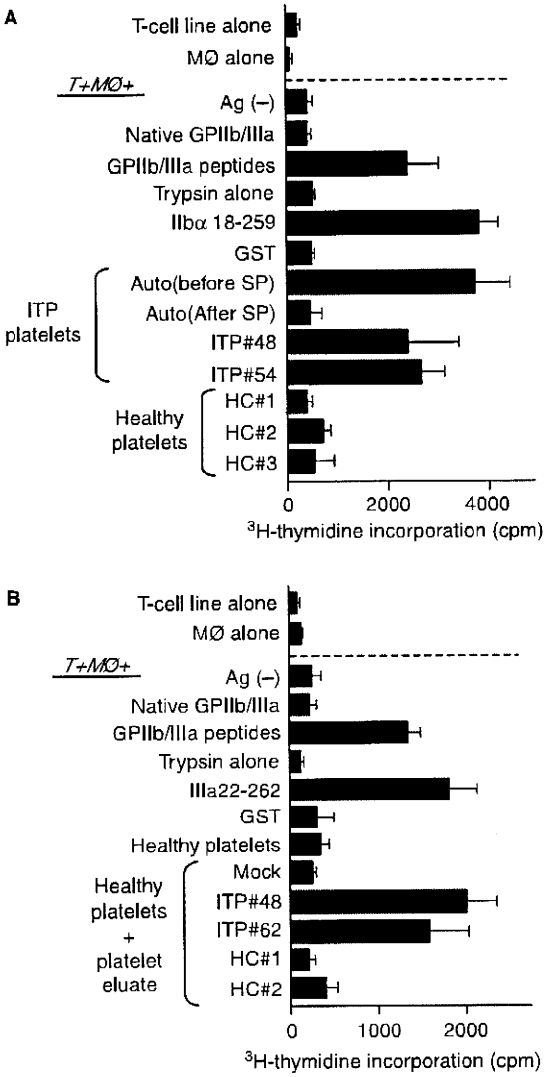

Fig. 2. Response of GPIIb/IIIa-reactive T-cell lines to cultured macrophages in the presence of ITP or healthy platelets. (A) GPIIb/IIIa-reactive T-cell line NE1 was cultured with autologous macrophages (MØ), which were prepared from circulating monocytes obtained when the patient was in remission after 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

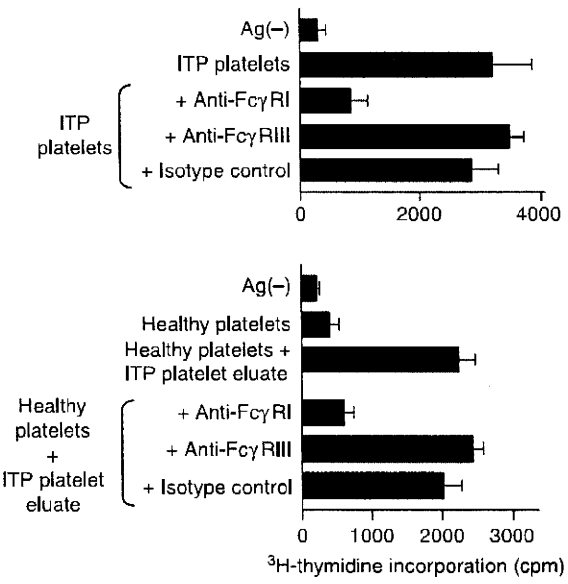

Fig. 3. Effects of anti-Fcγ receptor mAb on the proliferation of GPIIb/IIIa-reactive T-cell lines induced by cultured macrophages carrying opsonized platelets. GPIIb/IIIa-reactive T-cell lines MH3 (upper panel) and MH6 (lower panel) were cultured with autologous macrophages and ITP platelets (#48) or 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.

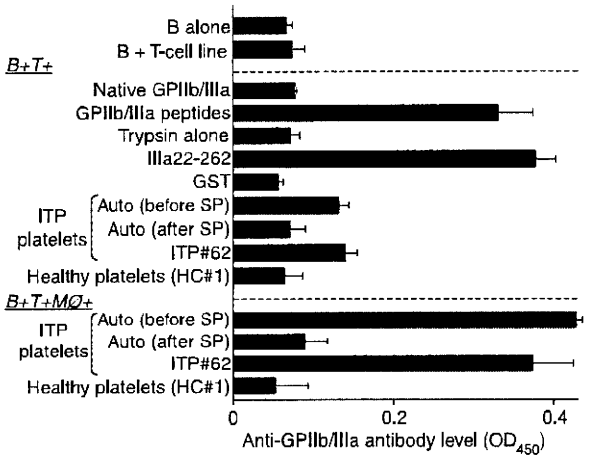

Fig. 4. Anti-GPIIb/IIIa antibody production in cultures of a GPIIb/IIIa-reactive T-cell line, autologous B cells, and macrophages carrying sensitized platelets. GPIIb/IIIa-reactive T-cell line SuM8 was cultured with autologous peripheral blood B cells in the presence or absence of various antigen sources, 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.

Acknowledgements

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Antagonism of Sphingosine 1-Phosphate Receptor-2 Enhances Migration of Neural Progenitor Cells Toward an Area of Brain Infarction

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Background and Purpose—We have previously shown that the sphingosine 1-phosphate (S1P)/S1P receptor-1 ($S1P_1R$) axis contributes to the migration of transplanted neural progenitor cells (NPCs) toward areas of spinal cord injury. In the current study, we examined a strategy to increase endogenous NPC migration toward the injured central nervous system to modify S1PR.

Methods—S1P concentration in the ischemic brain was measured in a mouse thrombosis model of the middle cerebral artery. NPC migration in vitro was assessed by a Boyden chamber assay. Endogenous NPC migration toward the insult was evaluated after ventricular administration of the $S1P_2R$ antagonist JTE-013.

Results—The concentration of S1P in the brain was increased after ischemia and was maximal 14 days after the insult. The increase in S1P in the infarcted brain was primarily caused by accumulation of microglia at the insult. Mouse NPCs mainly expressed $S1P_1R$ and $S1P_2R$ as S1PRs, and S1P significantly induced the migration of NPCs in vitro through activation of $S1P_1R$. However, an $S1P_1R$ agonist failed to have any synergistic effect on S1P-mediated NPC migration, whereas pharmacologic or genetic inhibition of $S1P_2R$ by JTE-013 or short hairpin RNA expression enhanced S1P-mediated NPC migration but did not affect proliferation and differentiation. Interestingly, administration of JTE-013 into a brain ventricle significantly enhanced endogenous NPC migration toward the area of ischemia.

Conclusions—Our findings suggest that S1P is a chemoattractant for NPCs released from an infarcted area and regulation of $S1P_2R$ function further enhances the migration of NPCs toward a brain infarction. (***Stroke*. 2008;39:3411-3417.)**

Key Words: migration ■ sphingosine 1-phosphate receptor-2 ■ sphingosine 1-phosphate ■ neural progenitor cells

Neural stem/progenitor cells (NPCs), self-renewing cells with the capacity to differentiate into neural cells, have been shown to exist mainly in 2 specific brain regions within the adult central nervous system (CNS): the subventricular zone (SVZ) and the hippocampal subgranular zone.[1] NPCs proliferate in the SVZ and migrate through it in a pattern reminiscent of the rostral migratory stream toward the olfactory bulb, where they differentiate into mature neurons.[2] NPCs also migrate to sites of brain injury. This may represent an adaptive response to limit or repair damage.[3,4] Newly generated NPCs are recruited from the SVZ to nearby areas of neural damage, and some show region-specific differentiation, known as neurogenesis.[3,4] A recent study showed that neurogenesis after brain injury is a meaningful response and that blockade of neurogenic cell division by irradiation worsens the outcome of cerebral ischemia.[5] Thus, a precise understanding of the mechanism underlying injury-mediated NPC migration may contribute to improving the effectiveness of stem cell–based therapies for CNS disorders.

Recently, we reported the importance of sphingosine 1-phosphate (S1P), a lysophospholipid mediator, for injury-mediated NPC migration.[6] S1P is currently attracting a great deal of attention as a bioactive sphingolipid that has various cellular functions and acts via the 7 transmembrane S1P receptors (S1PRs), $S1P_1R$ through $S1P_5R$.[7–9] We reported that the S1P concentration in the spinal cord increased after a contusion injury and contributed to the migration of transplanted NPCs in vivo via the $S1P_1R$.[6] In the current study, we show that S1P-mediated NPC migration toward an area of brain injury is enhanced by modulation of $S1P_2R$ function. We propose new therapeutic approaches to enhance the mobilization of endogenous NPCs toward areas of CNS injury.

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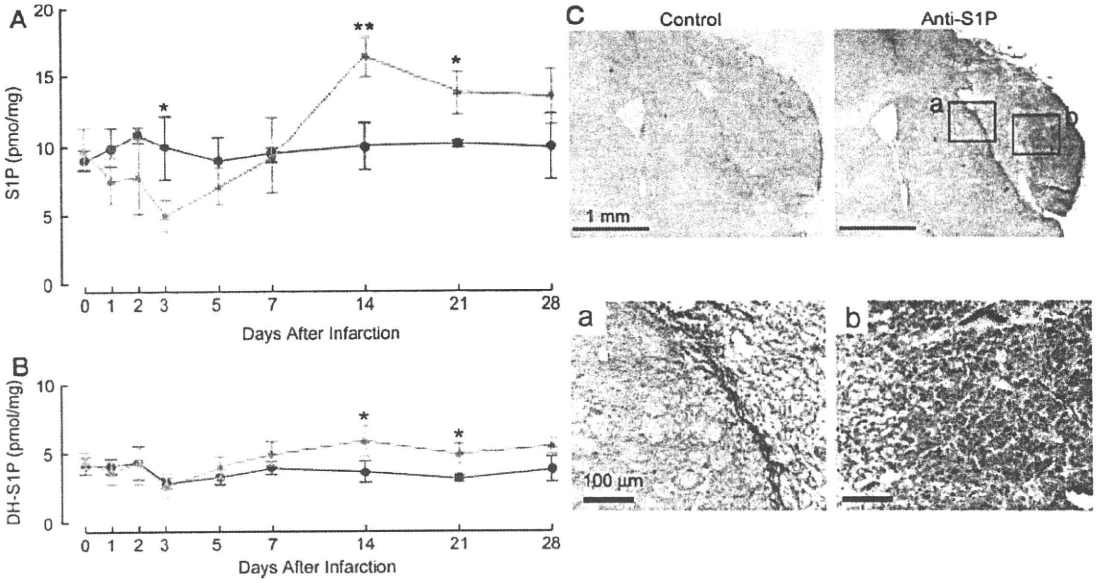

Figure 1. Alterations in S1P and dihydro-S1P concentrations after brain ischemia. The concentrations of S1P (A) and dihydro-S1P (B) in ischemic brain tissue (orange) or in contralateral control tissue (green) at various time points after brain ischemia were measured by high-performance liquid chromatography. Data represent mean±SD (n=3). *$P<0.05$, **$P<0.001$, 2-tailed Student's t test. C, Distribution of S1P in the brain 14 days after injury. Sections were immunostained with an antibody against S1P and visualized with Vector SG (black; anti-S1P). Counterstaining was carried out with Nuclear Fast Red (red). The same section stained with an isotype-matched control antibody is also shown (Control). Higher magnifications of the numbered boxed regions are shown in the lower panel.

Materials and Methods

Materials, measurement of S1P, immunohistochemistry, methods for migration, cell proliferation, NPC differentiation, construction of a lentiviral vector, and reverse transcription–polymerase chain reaction (RT-PCR) are described in detail in the supplemental materials, available online at http://stroke.ahajournals.org.

Photochemically Induced Brain Infarction

All animal procedures were approved by the institutional animal care and concern committee at Jichi Medical University. Animal care was performed in accordance with the committee's guidelines. C57BL/6 female mice (8 to 12 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Thrombosis of the middle cerebral artery (MCA) in mice was performed as described.[10] In brief, mice were anesthetized with 1.7% to 2.0% isoflurane and the temporal muscle was transected to expose the skull. The left distal MCA could be observed through the skull. Photoillumination was achieved with a xenon lamp (model L4887–03; Hamamatsu Photonics, Hamamatsu, Japan) via an optic fiber with a focus. The light was focused onto the MCA over the intact skull at a power of 2.3×10^6 lux. The photosensitizing dye Rose Bengal was simultaneously administered at a dose of 20 mg/kg IV within 5 minutes of laser irradiation.

Cell Culture

Mouse NPCs were isolated and cultured, as described previously.[6] In brief, the forebrains of E14.5 mouse embryos were isolated and mechanically dissociated into a suspension of single cells. The dissociated cells were cultured in Dulbecco's modified Eagle's medium/F12 supplemented with B27 supplement (Invitrogen, Carlsbad, Calif), 20 ng/mL basic fibroblast growth factor, and 20 ng/mL endothelial growth factor. The cells were used for experiments between passages 2 and 4.

Infusion of JTE-013 Into Brain Ventricles

For studies involving pharmacologic blockade of $S1P_2R$, 0.25 μL/h Alzet Minipumps (Durect Corp, Cupertino, Calif) were used for drug delivery. Empty pumps with flow moderators were weighed, filled with 1 mmol/L JTE-013 or phosphate-buffered saline (containing the same concentration of dimethyl sulfoxide as control), and then reweighed. Flow moderators were connected to a catheter (Alzet brain infusion kit 2; Durect Corp), which was connected to an infusion cannula. Pumps were stored overnight at 37°C in sterile saline to prime drug release. Two days after MCA thrombosis was induced, mice were anesthetized with isoflurane and placed in a small-animal stereotaxic frame. JTE-013 infusion during the acute phase of infarction may change the infarct size because inhibition of $S1P_2R$ is reported to affect vascular function.[11,12] Hence, we started the JTE-013 infusion 2 days after ischemia. The scalp was shaved, cleaned, and opened with a scalpel. A small burr hole was drilled 1 mm lateral and 0.2 mm caudal from the bregma. The catheter was lowered into the left ventricle (depth, 2.5 mm ventral) and affixed with dental cement, and the opening was sutured shut. Mice were euthanized by decapitation after 14 days. Cannula placement was verified by direct observation by cutting an insertion point. Once confirming the correct insertion, we proceeded to histologic analysis. The transverse sections (0.5 mm forward of the bregma) were analyzed to assess NPC migration in vivo. Endogenous NPC migration was detected by immunostaining of NPCs with an anti-doublecortin (DCX) polyclonal antibody (Santa Cruz Biotechnology). The distance to the ischemic area from the SVZ was separated into 3 parts (0 to 300 μm, 301 to 600 μm, and 601 to 900 μm). The DCX-positive area in each part was quantified with the use of Scion Image for Windows (Scion Corp). For 3-dimensional counting of migrated cells, 3 separate sections (0.5 mm, 0.7 mm, and 0.9 mm forward of the bregma) were prepared for analyses. To count the number of DCX-positive cells, the number of nuclei stained with DAPI in the DCX-positive area was manually counted by a blinded observer and expressed as the total number of DCX-positive cells in each area.

Results

Changes of S1P Concentration After Brain Infarction

To investigate the physiologic role of S1P in ischemic stroke, brain S1P concentrations were measured in a mouse model of brain ischemia. After brain infarction by photochemical induction of thrombosis in the distal MCA, most glial cells and neurons in the affected brain would be dead during the acute

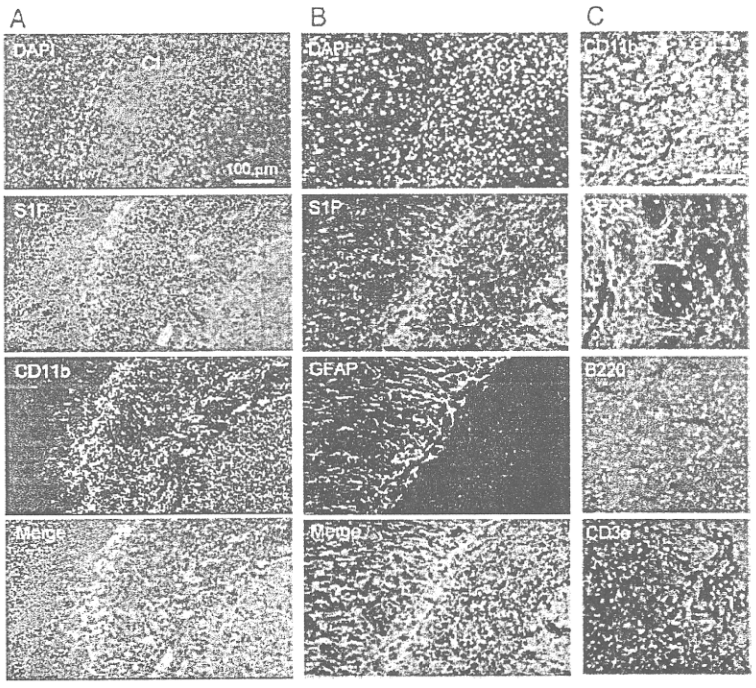

Figure 2. Localization of S1P after brain ischemia. Sections obtained from the brain 14 days after the insult were double-immunostained for CD11b and S1P (A) or glial fibrillary acid protein and S1P (B). Nuclear localization was simultaneously examined by DAPI staining. The merged images show colocalization of CD11b and S1P or of glial fibrillary acid protein and S1P. Areas of infarct are separated by a dotted line. CI indicates cerebral infarction). C, Representative images obtained from S1P (green) and merged with CD11b, glial fibrillary acid protein, B220, and CD3e (red) at higher magnification are shown.

phase of infarction. Reflecting this cell deterioration, S1P content was significantly decreased 3 days after infarction (Figure 1A). Interestingly, S1P content in the ischemic brain was significantly increased thereafter and reached a maximum 14 days after ischemia (Figure 1A). On the other hand, changes in dihydro-S1P concentration after ischemia were marginal (Figure 1B). Immunohistologic analysis with an anti-S1P antibody confirmed that the site of infarction contained a large amount of S1P (Figure 1C). S1P was highly expressed at the boundary zone and in the central core of the infarct (Figure 1C).

Next, we examined the cellular location of the elevated S1P content after brain infarction by immunohistochemistry. Destruction of the normal structure of the CNS and accumulation of microglia and immunoreactive cells of nonneural lineages expressing CD11b were observed after the insult (Figure 2A). S1P was highly expressed in the region of microglia accumulation in the infarct area and boundary zone (Figure 2A). On the other hand, astrocytes expressing glial fibrillary acid protein (GFAP) accumulated primarily at the boundary zone, whereas S1P immunoreactivity was partly colocalized, but S1P in the infarcted area was not (Figure 2B). As expected, few microtubule-associated protein 2 (MAP-2)–positive neurons were observed in the insult areas (data not shown). As well, most S1P immunoreactivity did not merge into B lymphocytes (B220) and T lymphocytes (CD3e; Figure 2C) in the infarcted area. These data suggest that microglia that accumulate at the site of injury are the main sources responsible for S1P elevation, whereas astrocytes might partly contribute to the increase in S1P in the boundary zone.

Expression of S1PR in Mouse NPCs

Many if not all of the biologic responses induced by S1P are mediated by its cell surface receptors, ie, S1PRs (S1P$_1$R through S1P$_5$R).[8,9] Next, we examined the expression of S1PRs in several adult mouse tissues and NPCs. Although S1P$_1$R seemed to be ubiquitously expressed, the patterns of S1PR expression were quite different among the tissues examined (Figure 3A). In this study, the NPCs expressed all known S1PRs (Figure 3A); real-time, quantitative RT-PCR analysis of NPCs revealed that S1P$_1$R and S1P$_2$R were the most highly expressed (Figure 3B).

Migration of NPCs Toward S1P

We next evaluated the effects of S1P on NPC migration. As shown in Figure 4A, S1P-induced migration resulted in a

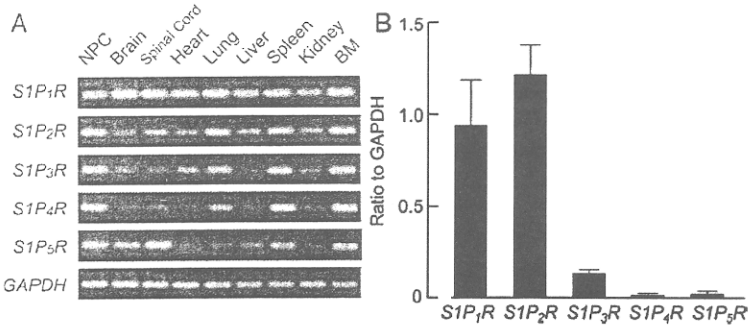

Figure 3. Expression of S1PRs in murine NPCs. A, RT-PCR analyses of transcripts derived from the genes for *S1P$_1$R* to *S1P$_5$R* in NPCs and in various mouse tissues. As a control, RT-PCR analysis for the mouse glyceraldehyde 3-phosphate dehydrogenase transcript was performed simultaneously. BM indicates bone marrow. B, mRNAs for the *S1P1R* to *S1P5R* genes in NPCs were quantified by real-time quantitative RT-PCR. Data represent mean±SD (n=3 per group).

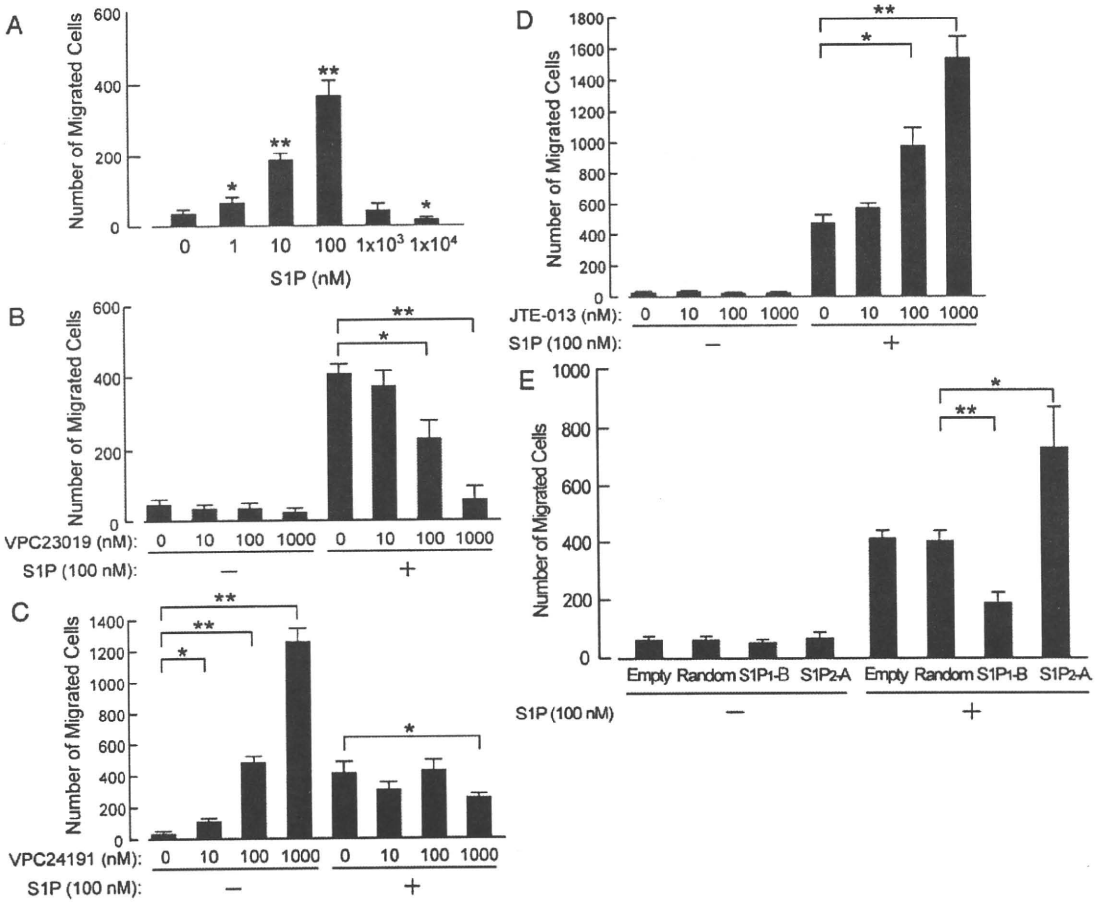

Figure 4. Modulation of NPC migration by agonists and antagonists for S1PR and by shRNA expression in vitro. A, NPC migration was assessed with use of a modified Boyden chamber assay. The indicated concentrations of S1P were placed in each lower chamber, and NPCs were allowed to migrate for 12 hours (n=3). B–D, NPC migration toward VPC23019 (B), VPC24191 (C), or JTE-013 (D), alone or in combination with 100 nmol/L S1P, was examined (n=3). E, NPCs were transduced with a lentiviral vector expressing no shRNA sequence (Empty), a random sequence (Random), an $S1P_1R$ sequence (S1P1B), or an $S1P_2R$ sequence (S1P2A). Transduced NPC migration with or without 100 nmol/L S1P was examined (n=3). Data represent mean±SD. *$P<0.05$, **$P<0.01$, 2-tailed Student's t test.

bell-shaped concentration-response curve, and the maximal response was observed at 100 nmol/L. To explore which S1PRs were involved in S1P-mediated NPC migration, we used 2 S1P-related synthetic compounds, VPC23019 and VPC24191. VPC23019 acts as a competitive inhibitor of $S1P_1R$ and $S1P_3R$ but partly stimulates $S1P_4R$ and $S1P_5R$.[13] On the other hand, VPC24191 is an agonist against $S1P_1R$ and $S1P_3R$. VPC23019 failed to induce NPC migration and abolished migration toward S1P (Figure 4B). VPC24191 by itself dramatically enhanced NPC migration in a concentration-dependent manner (Figure 4C). Interestingly, the addition of S1P inhibited the NPC migration elicited by a higher concentration of VPC24191 (1 to 10 μmol/L; Figure 4C). These data suggest that $S1P_1R$ is the primary receptor involved in S1P-mediated NPC migration, in support of our previous study,[6] but also indicated that other S1PRs can regulate the $S1P_1R$-mediated response.

We next focused on $S1P_2R$, a receptor that is responsible for inhibition of migratory responses.[14,15] Although JTE-013, a specific antagonist of $S1P_2R$, had no effect on NPC migration, S1P-mediated migration modulated by JTE-013 reached that induced by VPC24191 (Figure 4D). Similar results were observed after expression of short hairpin (sh) RNA: short interfering RNA against $S1P_1R$ ($S1P_1B$) inhibited S1P-mediated NPC migration, whereas $S1P_2R$ knockdown ($S1P_2A$) significantly enhanced the migration of NPCs elicited by S1P (Figure 4E). Considering that the concentration of S1P increased in the ischemic area, modulation of $S1P_2R$ instead of $S1P_1R$, could be a more practical approach to increase the mobilization of NPCs.

To examine whether inhibition of $S1P_2R$ affects other stem cell properties, we examined the effects of JTE-013 on the proliferation and differentiation of NPCs. S1P reportedly induced cell differentiation and proliferation[16]; however, S1P had only a marginal effect on NPC proliferation and differentiation in this study (Figure 5), and JTE-013 was without effect (Figure 5). These data suggest that modulation of $S1P_2R$ enhances S1P-mediated migration of NPCs without the loss of cell viability and the potential to differentiate.

Involvement of $S1P_2R$ in Endogenous NPC Migration

Finally, we attempted to increase the mobilization of endogenous NPCs by $S1P_2R$ antagonism. After infarction, we analyzed

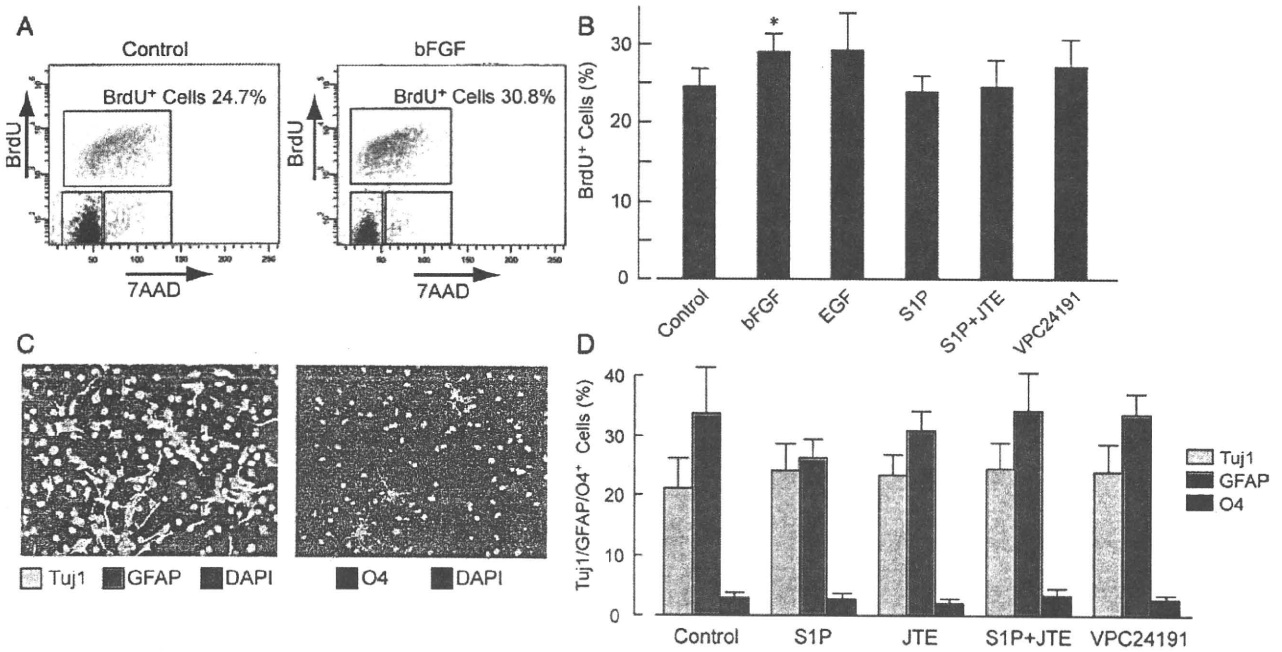

Figure 5. The S1P$_2$R antagonist JTE-013 had no affect on the proliferation and differentiation of NPCs. A, Bromodeoxyuridine incorporation was analyzed by flow cytometry after stimulation without (Control) or with 20 ng/mL basic fibroblast growth factor for 1 hour. Representative flow cytometry data are shown. B, Bromodeoxyuridine incorporation stimulated without (Control) or with 20 ng/mL basic fibroblast growth factor, 20 ng/mL endothelial growth factor, 1 μmol/L S1P, 1 μmol/L S1P and 1 μmol/L JTE-013, or 1 μmol/L VPC23419 was quantified. Data represent mean±SD (n=4). C, NPCs were cultured in the presence of 1% serum for 5 days, and lineage-specific differentiation was observed by immunocytochemistry. Representative data of double staining against Tuj1 and glial fibrillary acid protein (left) and single staining with O4 (right) is shown. D, The ratio of lineage-specific differentiation with addition of the indicated agent was quantified. Data represent mean±SD (n=4). *P<0.05, 2-tailed Student's t test.

NPC migration from the lateral ventricle wall by immunofluorescence against DCX, a microtubule-associated protein that is specifically expressed in NPCs and immature neurons. We started continuous administration of JTE-013 into the ventricle 2 days after focal brain ischemia to examine whether targeting S1P$_2$R would enhance endogenous NPC migration toward the area of the insult (Figure 6A). NPCs expressing DCX migrated from the lateral ventricle wall and accumulated around the

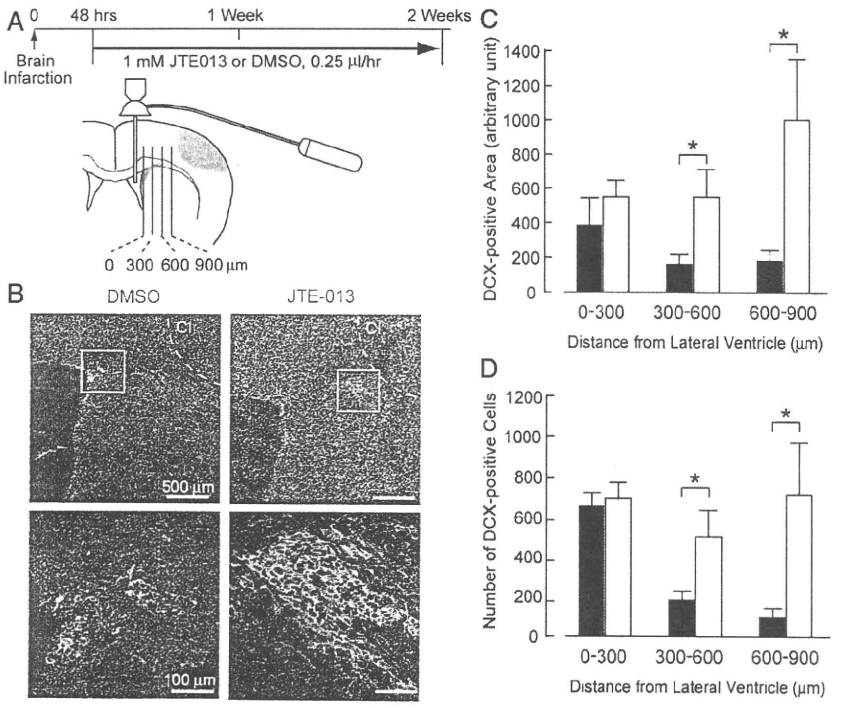

Figure 6. Enhancement of endogenous NPC migration toward areas of brain ischemia by ventricular administration of the S1P$_2$R antagonist JTE-013. A, Schematic presentation of the procedure and quantification of migrated DCX-positive cells. Two days after brain infarction, vehicle (dimethyl sulfoxide) or JTE-013 was continuously infused into the cerebral lateral ventricle. Histologic analysis of DCX was done at day 14. The distance to the ischemic area from the SVZ was separated into 3 parts (0 to 300 μm, 301 to 600 μm, and 601 to 900 μm). B, Representative data of histologic analyses of DCX are shown (red). Higher magnifications of the boxed regions are shown in the lower panel. Areas of infarction are separated by a dotted line (CI). C, Each area of DCX-positive cells was separately quantified in the section at 0.5 mm forward of the bregma. Vehicle is indicated by black bars; JTE-013, by white bars. Data represent mean±SE (n=6 per each group). D, Migrated DCX-positive cells were stereologically assessed by counting the total number of cells in 3 separate cross sections (0.5 mm, 0.7 mm, and 0.9 mm forward of the bregma). Vehicle is indicated by black bars; JTE-013, by white bars. Data represent mean±SE (n=3 per each group). *P<0.05, 2-tailed Student's t test.

ischemic area (Figure 6B). Interestingly, NPC migration toward the ischemic area was dramatically enhanced by ventricular infusion of JTE-013 (Figures 6B through 6D). Administration of JTE-013 into ventricles could diffuse into many areas in the brain and so might affect many neurologic functions; many kind of neural cells reportedly express S1PRs, including $S1P_2R$.[17] However, enhanced NPC migration after administration of JTE-013 was not observed in the contralateral side of the infarct (data not shown). These data suggest that pharmacologic inhibition of $S1P_2R$ in NPCs by JTE-013 promotes endogenous NPC migration toward the areas of brain ischemia where S1P has increased.

Discussion

Migration of NPCs is important not only for development of the embryonic nervous system but also for repair of the nervous system after injury.[3,4] Identifying candidate molecules that could play a role in NPC migration is crucial for understanding proper tissue formation by newly formed neural cells, as well as for developing novel therapies to promote neural repair after CNS injury.[18,19] We previously showed a role for S1P in NPC migration toward a pathologic area of the CNS and proposed that elevation of S1P at the site of injury was a guiding factor for NPC migration.[6] Here, we reveal that $S1P_2R$ is a potential target for strategies aiming to increase NPC migration after brain ischemia.

We have already shown that $S1P_1R$ contributes to NPC migration toward areas of high S1P expression in the injured CNS.[6] This occurs in a variety of cell types through Gi-mediated Rac activation,[20,21] whereas $S1P_2R$ abolishes migration after the coupling of $S1P_2R$ to the $G_{12/13}$/Rho pathway.[11,22] The $S1P_2R$ abolished migration not only through $S1P_1R$ but also other types of receptors for growth factor.[14,15] Overactivation of the Rho/Rho-kinase pathway is thought to be 1 of the important mechanisms that strongly inhibit cell migration.[11] These data present 2 strategies for the induction of NPC migration to a site of injury: elevation of $S1P_1R$ signaling or blockade of $S1P_2R$ signaling. However, the $S1P_1R$-specific agonist failed to enhance NPC migration in the presence of S1P, suggesting that activation of $S1P_1R$ itself could not overcome the inhibitory effect of $S1P_2R$ in NPCs. Considering that the concentration of S1P increases at the site of an insult, modulation of $S1P_2R$ function could be a more practical approach for the mobilization of NPCs when compared with modulation of $S1P_1R$ function. We confirmed the involvement of $S1P_2R$ in in vitro NPC migration by RNA interference experiments; however, the effects of JTE-013 unrelated to $S1P_2R$ antagonism could not be completely eliminated because JTE-013 is reported to inhibit vasoconstriction through unknown mechanism(s) independent of the $S1P_2R$.[23] Further studies with gene-deficient mice would be required to confirm the full effects of $S1P_2R$ inhibition.

Migration of NPCs is an important process in neurogenesis.[4] The neural stem cells of the SVZ are the principal source of NPCs, which form chainlike structures and migrate laterally toward the injured striatal regions before differentiating into mature neurons.[3,4,19] Our data suggest that local elevation of S1P concentration after brain ischemia acts as an activation signal for NPCs in the SVZ and that modulation of $S1P_2R$ function could enhance endogenous NPC migration. Recent studies have suggested that endogenous NPC migration and angiogenesis are mechanistically linked in the nervous system; neuroblast cells migrate toward blood vessels in areas undergoing early vascular remodeling.[24,25] Vascular endothelial growth factor, a major angiogenic factor that acts as a guiding factor for endothelial progenitors, is an attractive guidance cue for the migration of undifferentiated NPCs.[26,27] Our data show that S1P, another important angiogenic factor,[20] also guides the migration of NPCs, suggesting a mechanistic link between NPC migration and angiogenesis via the S1P/S1PR axis in the nervous system.

The change in S1P concentration after brain ischemia is quite different from that of known inflammatory cytokines, chemokines, and growth factors. Intercellular adhesion molecules, including intercellular adhesion molecule-1 and selectins, are rapidly induced 3 to 6 hours after ischemia and peak at 6 to 12 hours.[28,29] The expression of adhesion molecules on microvessels promotes neutrophil recruitment and trafficking into the brain.[28,29] Adhesion molecules and inflammatory mediators play a role in focal ischemic brain injury. In this study, the concentration of S1P was gradually enhanced at the site of ischemia, and S1P was highly expressed at the site of microglia accumulation. The gradual increase in S1P led us to postulate that it plays an important role in regeneration after CNS injury. Microglia reportedly release an unidentified chemoattractant(s) for NPCs after CNS injury and thus play an important role in directing the replacement of damaged or lost cells in the CNS.[30] Recently, selective ablation of microglial cells was shown to exacerbate ischemic injury in the brain, suggesting that microglial cells serve as an endogenous pool of neurotrophic molecules.[31] Because S1P enhances the migration of NPCs, we postulate that S1P is a physiologic, neuroprotective substance released from microglia.

An issue that remains to be addressed is what stimulates S1P biosynthesis after injury. A common product of sphingolipid breakdown is ceramide, which is generated primarily by hydrolysis of membrane sphingomyelin.[32] Ceramide can be further catabolized by ceramidases to generate sphingosine, which can be phosphorylated through the action of sphingosine kinase, generating S1P.[33] Sphingosine kinase activity in the brain was recently reported to increase 24 hours after ischemia in a mouse model of MCA occlusion[34]; however, those increases were not necessarily the same as the S1P concentration in our study. As well, if elevation of sphingosine kinase activity accounts for the increase in S1P concentration after insult, the concentration of dihydro-S1P would increase because dihydrosphingosine is phosphorylated by sphingosine kinase isozymes to an extent similar to that of naturally occurring sphingosine.[35,36] One possible mechanism is sphingomyelin breakdown after phagocytosis of neural cells by activated microglia. Microglial activation and phagocytic potential are reported to occur gradually between 1 and 12 weeks after an ischemic insult.[37] Activated microglial cells phagocytose dead neural cells and may induce sphingomyelin breakdown during the neuroregeneration phase. Investigations into the precise mechanisms of injury-mediated S1P elevation and sphingosine metabolism at injury sites are now under way in our laboratory.

Summary

In summary, increased S1P at the site of brain infarction acts as a chemoattractant for NPCs. Although activation of S1P$_1$R failed to enhance NPC migration in the presence of S1P, S1P$_2$R, a specific antagonist of the migration-inhibitory receptor, upregulates migration responses induced by S1P and augments endogenous NPC migration toward the ischemic insult. These data suggest that S1P$_2$R blockade is a promising candidate to enhance NPC migration toward sites of brain infarction. Further studies in gene-deficient mice will be needed, as will behavioral and functional analyses after the administration of S1P$_2$R antagonists for treatment of ischemic stroke.

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Disclosures

None.

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Phenotypic Correction of Hemophilia A by Ectopic Expression of Activated Factor VII in Platelets

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Platelets are receiving much attention as novel target cells to secrete a coagulation factor for hemophilia gene therapy. In order to extend the application of platelet-directed gene therapy, we examined whether ectopic expression of activated factor VII (FVIIa) in platelets would result in an efficient bypass therapy to induce sufficient thrombin generation on platelet surfaces in mice with hemophilia A. Transduction of bone marrow cells with a simian immunodeficiency virus (SIV)-based lentiviral vector harboring the platelet-specific *GPIbα* promoter resulted in efficient transgene expression in platelets. FVIIa antigen was expressed in platelets by this SIV system; FVII transgene products were found to localize in the cytoplasm and translocate toward the sub-membrane zone and cell surface after activation. Although FVII antigen levels in platelets did not reach the therapeutic levels seen with FVIIa infusion therapy, whole-blood coagulation, as assessed by thromboelastography, was significantly improved in mice with hemophilia A. Further, we observed correction of the bleeding phenotype in mice with hemophilia A after transplantation, even in the presence of FVIII-neutralizing antibodies. Our results demonstrate that FVIIa-expressing platelets can strengthen hemostatic function and may be useful in treating hemophilia and other inherited bleeding disorders. These findings are comparable to the proven therapeutic effects of FVIIa infusion.

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INTRODUCTION

Platelets are differentiated anucleate cells whose functions are essential for hemostasis. Because platelets can circulate throughout the body, release a number of mediators on demand, and provide a scaffold for the coagulation cascade, the targeting of platelets as a circulating delivery system would seem a reasonable approach to genetic modification of hemostasis. The feasibility of such a platelet-directed approach was originally demonstrated by Poncz *et al.* in transgenic mice.[1] Platelet expression of urokinase-type plasminogen activator, using a platelet-specific *platelet factor-4* promoter, enabled urokinase-type plasminogen activator to be stored in platelets and then released within developing thrombi when the platelets became activated.[1] Further, platelet-specific expression of factor VIII (FVIII) can be achieved in a transgenic setting, with the resultant FVIII predominantly or exclusively stored in platelet granules rather than being released into the plasma.[2] In addition, Shi *et al.* have demonstrated that ectopically expressed FVIII in platelets can be used in the treatment of hemophilia with or without FVIII-neutralizing antibodies, and that targeted FVIII expression in platelets continues to support hemostasis even in the presence of high titers of FVIII-neutralizing antibodies.[3] We and others have applied this approach to gene therapy, and have demonstrated that transplantation of hematopoietic stem cells (HSCs) transduced with a lentiviral vector containing *human FVIII* driven by a platelet-specific promoter improves the hemostatic function of mice with FVIII-deficient hemophilia A, despite the levels of FVIII in their plasma being scant or undetectable.[4,5]

In order to further extend the application of platelet-directed gene therapy, we focused our attention on the extrinsic pathway initiated by tissue factor (TF). Assembly of TF and activated Factor VII (FVIIa) complexes on anionic phospholipids expressed on activated cell membranes is the most important initiation mechanism for blood coagulation.[6] Recently, recombinant human FVIIa (rhFVIIa: NovoSeven) has proven to be a highly successful alternative treatment for hemophilia patients.[7] Patients with a variety of other coagulation deficiencies that are characterized by impaired thrombin generation have been successfully treated with rhFVIIa.[8] In addition, liver-directed gene therapy with an adeno-associated virus vector equipped with hFVIIa achieved therapeutic plasma hFVIIa levels in a mouse model of hemophilia B, and phenotypic correction was observed when a murine FVIIa (mFVIIa) homolog was used.[9] In addition, it is possible that platelets that stably express FVIIa can efficiently induce hemostasis at the site of vascular injury in a variety of hemorrhagic disorders. In this study, we used gene therapy to examine whether platelet-specific FVIIa expression would result in an efficient bypass therapy for

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factor X activation, thereby generating sufficient thrombin on platelet surfaces in FVIII-deficient mice.

RESULTS

Enhanced green fluorescent protein expression in platelets after transplantation of HSCs transduced with a simian immunodeficiency virus lentiviral vector harboring the platelet *GPIbα* promoter

We have previously shown that transplantation of c-kit$^+$, sca-1$^+$, and lineage$^-$ (KSL) murine HSCs that are transduced with an simian immunodeficiency virus (SIV)-based lentiviral vector carrying enhanced green fluorescent protein (*eGFP*), driven by a platelet-specific *GPIbα* promoter, enables efficient expression of eGFP in platelets.[4] Because transplantation of KSL cells requires nontransduced bone marrow cells, engraftment by the transduced cells is no >40–55% after transplantation.[4] In order to obviate the need for competitor cells, we validated the transplantation procedure using unfractionated bone marrow cells. As shown in **Figure 1**, transplantation using unfractionated bone marrow cells resulted in more efficient gene targeting to platelets. eGFP expression in platelets was sustained for at least 3 months after transplantation (**Figure 1b**), and we found that 0.60–2.78 vector copies/genome had integrated into the cells of the mice that had received the transplants (**Figure 1c**).

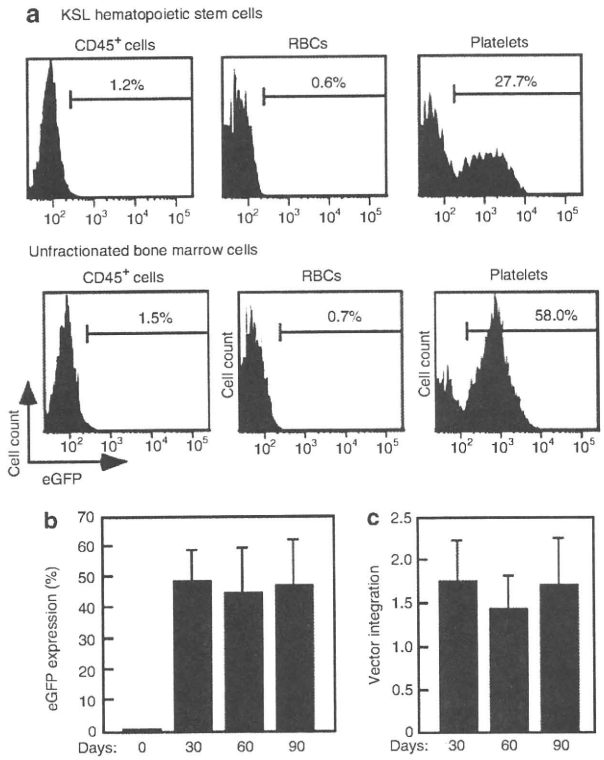

Figure 1 Effects of different stem cell sources on enhanced green fluorescent protein (eGFP) expression in platelets *in vivo*. KSL cells or whole bone marrow cells obtained from Ly5.1 mice were transduced with SIV-GPIbα-eGFP at a multiplicity of infection of 30. Irradiated Ly5.2 mice received either transduced KSL cells (1×10^4) together with competitor cells (2×10^5), or transduced unfractionated bone marrow cells (2×10^6). (**a**) Representative flow cytometry analysis of eGFP-positive cells among CD45$^+$ lymphocytes and granulocytes, red blood cells (RBCs), and platelets in peripheral blood 30 days after transplantation. (**b**) Percentages of eGFP-positive platelets at 30, 60, and 90 days after transplantation. Columns and error bars represent the mean ± SD ($n = 7$). (**c**) Proviral integration into the genomic DNA of bone marrow cells was quantified at 30, 60, and 90 days after transplantation by real-time quantitative PCR. Columns and error bars represent the mean ± SD ($n = 7$). SIV, simian immunodeficiency virus.

hFVII-2RKR expression in platelets induced by platelet-directed gene transduction

The rhFVIIa product currently in clinical use is produced in a single-chain form and activated to the two-chain form during protein purification.[7] Approximately 1% of circulating hFVII in healthy individuals is in the activated form, and the amount of hFVIIa required for bypassing is much larger than the physiological concentration.[7] In order to secrete the activated form of hFVII from transduced cells, we inserted into the factor X activation–cleavage site two arginine/lysine/arginine (RKR) sequences recognized by an intracellular paired basic amino-acid cleaving enzyme/furin type protease, resulting in the secretion of the two-chain molecule with a structure similar to hFVIIa (hFVII-2RKR; **Figure 2a**).[9]

We first examined whether functional FVIIa was produced in megakaryocytes. After transduction with SIV vector containing *hFVII-2RKR* driven by cytomegalovirus promoter, hFVII antigen in the supernatant from the megakaryoblastic cell line UT-7/TPO was detected and found to have activity similar to that from HEP-G2 cells (**Figure 3a** and **c**). The FVII activity of FVII-2RKR was much higher than that of plasma-derived hFVII (**Figure 3c**), thereby suggesting that FVII-2RKR could be cleaved into two chains. In addition, mRNA expression of γ-glutamyl carboxylase, which post-translationally modifies glutamyl residues into γ-carboxyglutamyl residues of vitamin K–dependent coagulation

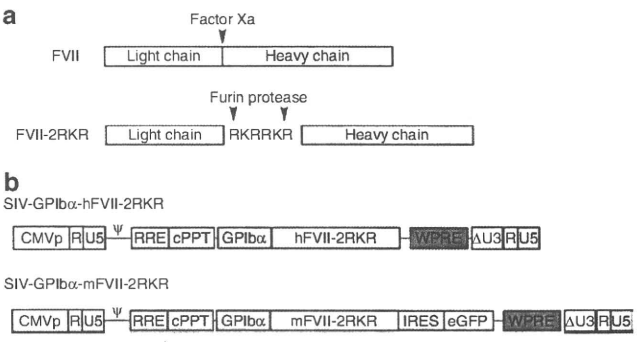

Figure 2 Schematic representation of factor VII (FVII) and simian immunodeficiency virus (SIV) lentiviral vector. (**a**) FVII and engineered activated FVII (FVII-2RKR) construct showing the light and heavy chains. Arrows indicate the recognition sites of physiological factor X activation (FXa) and the intracellular paired basic amino-acid cleaving enzyme/furin type protease. (**b**) The SIV lentiviral vector for platelet-specific gene expression consisted of a cytomegalovirus (CMV)/long-terminal repeat (LTR) chimeric promoter followed by a packaging signal (ψ), a rev-binding element (RRE) for cytoplasmic export of the RNA, the transgene expression region consisting of an internal promoter (*GPIbα*) and the transgene (*hFVII-2RKR* or *mFVII-2RKR-IRES-eGFP*), woodchuck hepatitis virus post regulatory element (WPRE), and a 3′-self-inactivating LTR. cPPT, central polypurine tract; eGFP, enhanced green fluorescent protein; hFVII, human FVII; IRES, internal ribosomal entry site; mFVII, murine FVII.