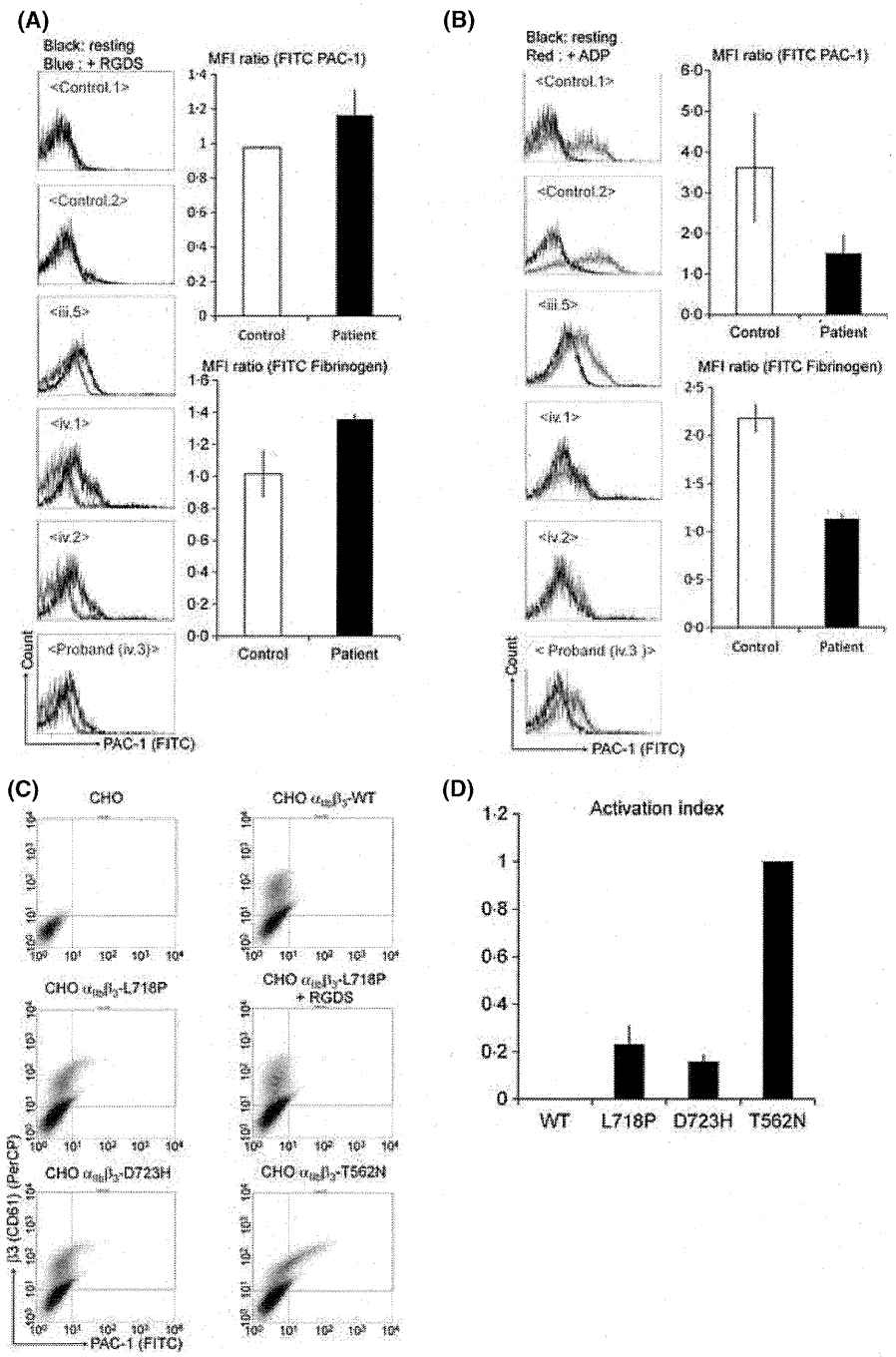

Fig 3. Functional analysis of integrin β3-L718P mutation. (A) Spontaneous binding of PAC-1 antibody to platelets obtained from affected individuals of the pedigree. Non-activated platelets (within 10 min after blood collection), incubated with or without 1 mM RGDS, were stained with FITC-conjugated PAC-1 antibody. After fixation, binding of PAC-1 to platelets was analysed by flow cytometry. Activation status of αIIbβ3 complex on resting platelets bound to FITC-PAC-1 (top) and FITC-fibrinogen (bottom). Mean fluorescence intensity (MFI) ratio was estimated by dividing the MFI of resting platelets by that of resting platelets incubated with RGDS. (B) Reduced activation of αIIbβ3 from affected individuals. The resting and ADP-stimulated platelets, stained with FITC-conjugated PAC-1 antibody were analysed by flow cytometry. Activation status of αIIbβ3 on stimulated platelets bound to FITC-PAC-1 (top) and FITC-fibrinogen (bottom). Values were estimated by dividing the MFI of platelets stimulated with ADP by those of resting platelets. (C) Partial activation of αIIbβ3-L718P and -D723H on CHO cells. CHO cells transfected with αIIbβ3 expression vectors (β3-WT, -L718P, -D723H and -T562N) were seeded on 100 μg/ml fibrinogen-coated coverslips in 6-well dishes. The cells, treated with or without RGDS, were stained with FITC-conjugated PAC-1 antibody and PerCP-conjugated anti-CD61 antibody and analysed by flow cytometry. (D) Activation index of αIIbβ3 mutants. Activation status of CHO cells expressing αIIbβ3-L718P and -D723H was compared with that of αIIbβ3-T562N as described in the "*Materials and methods*".

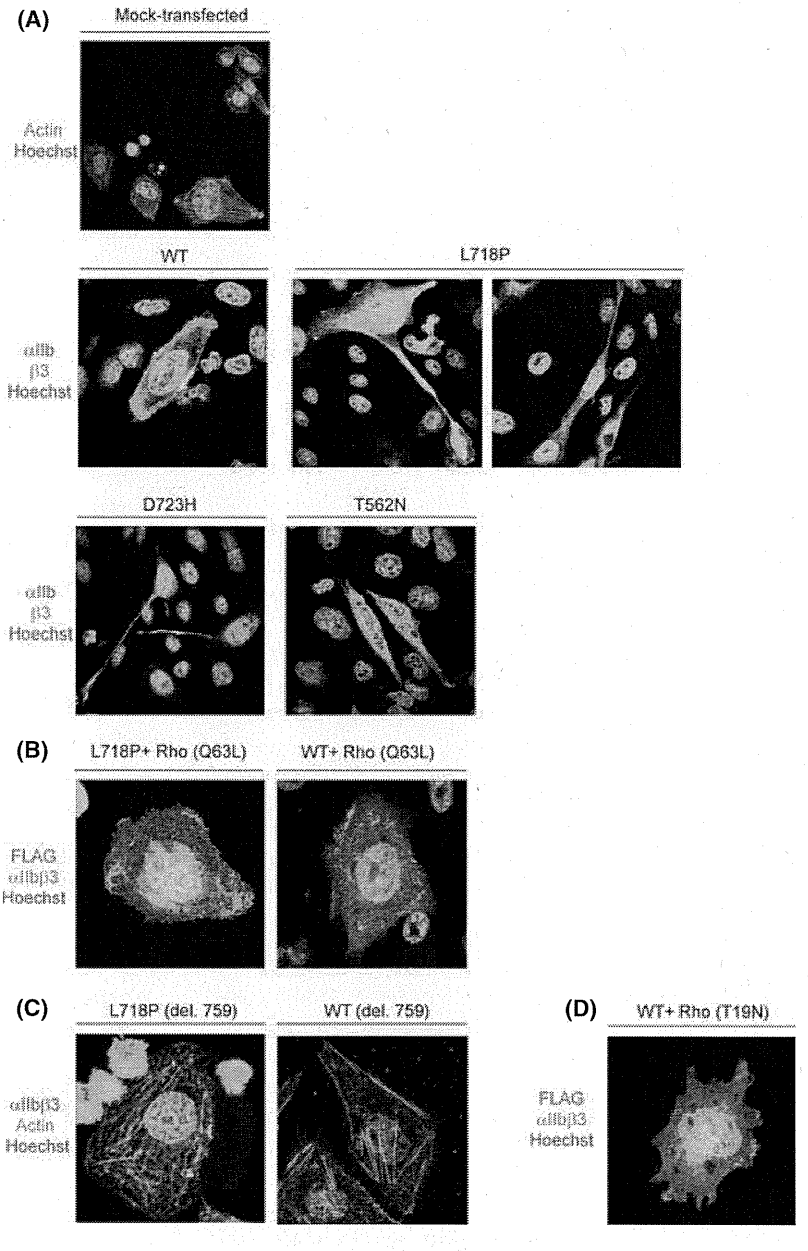

Fig 4. Overexpression of RhoA mutants or integrin β3-L718P (del. 759) modulates the formation of proplatelet-like cell protrusions in CHO cells. (A) Changes in CHO cell morphology by αIIbβ3 mutants. CHO cells transfected with αIIbβ3-L718P, -T562N and -D723H were seeded on fibrinogen-coated coverslips. After an 8-h incubation, the cells were fixed and stained with anti-CD41 and -CD61 antibodies followed by staining with Cy3- and Alexa 488-conjugated secondary antibodies. Mock-transfected cells were stained with Alexa 488-conjugated phalloidin and Hoechst 33342. (B) Inhibition of proplatelet-like protrusion formation by constitutively-active RhoA. An expression vector that encodes FLAG-tagged RhoA (Q63L) was transfected together with αIIbβ3-L718P or -WT expressing vectors into CHO cells. The cells grown on fibrinogen-coated coverslips were fixed and stained with anti-CD41 and anti-DDDDK-tag antibodies followed by staining with Alexa 488- and Cy3-conjugated secondary antibodies. (C) C-terminal deletion of β3-L718P inhibits the formation of proplatelet-like protrusions. C-terminal deleted integrin β3-L718P or -WT (del. 759) was expressed together with αIIb in CHO cells. The cells were fixed and stained with anti-CD41 antibody followed by staining with Cy3-conjugated secondary antibody and Alexa-488-labeled phalloidin. (D) A dominant-negative (T19N) form of RhoA was overexpressed in CHO cells. Images were taken as in (B).

Involvement of RhoA signalling in proplatelet-like protrusion formation

As previously reported by others (Ghevaert *et al*, 2008; Jayo *et al*, 2010), CHO cells expressing αIIbβ3-L718P, as well as αIIbβ D723H, formed long proplatelet-like protrusions on fibrinogen-coated dishes that were not observed in cells expressing wild-type αIIbβ3 (Fig 4A). In contrast, although cells expressing αIIbβ3-T562N, which yields a fully activated conformation (Kashiwagi *et al*, 1999), changed from their original round shape surrounded by a broad protrusion (Fig 4A, mock-transfected) to rhomboid-like cell morphology, proplatelet-like protrusions were rarely seen (Fig 4A). This suggests that mutants partially activating the integrin complex induce long proplatelet-like protrusions.

Recently, it was reported that the formation of proplatelet-like protrusions in CHO cells is mediated by the downregulation of RhoA activity (Chang *et al*, 2007; Schaffner-Reckinger *et al*, 2009), which is initiated by the binding of c-Src to the C-terminal tail (amino acid 760–762, Arg-Gln-Thr; RGT) of integrin β3 (Flevaris *et al*, 2007). We found that the formation of long cell protrusions was inhibited when a constitutively-active form of RhoA (Q63L) was introduced into αIIbβ3-L718P-expressing cells (Fig 4B). In addition, CHO cells expressing αIIbβ3-L718P (del. 759) mutant, which lacks the C-terminal c-Src binding site of in-

tegrin β3 (RGT), did not form any proplatelet-like protrusions (Fig 4C). Given that enforced activation of RhoA caused by introducing RhoA (Q63L), as well as de-repression of RhoA through C-terminal deletion of β3 in cells expressing αIIbβ3-WT, did not induce morphological changes in CHO cells (Figs 4B, C), it is proposed that constitutive inhibition but not activation through the c-terminal of β3 is responsible for the formation of abnormal cell protrusions in L718 mutants. However, as the enforced expression of a dominant negative form of RhoA (T19N) in αIIbβ3-WT expressing cells did not result in typical proplatelet-like protrusions (Fig 4D), this suggests that downregulation of RhoA was required but not sufficient for the formation of proplatelet-like protrusions induced by integrin β3-L718P.

Discussion

We report a pedigree with individuals suffering from a lifelong haemorrhagic syndrome, all of whom were carrying the integrin β3-L718P mutation. This had previously been reported only in a sporadic patient (Jayo *et al*, 2010). Next-generation sequencing, together with the clinical data of the patients, established that this integrin β3-L718P mutation causes thrombocytopenia resembling the disease caused by a different integrin mutation, β3-D723H, although the size of the platelets seems to differ somewhat between these mutations (Ghevaert *et al*, 2008; Schaffner-Reckinger *et al*, 2009).

Considering the dominant inheritance pattern of the haemorrhagic tendency caused by integrin β3-L718P as well as β3-D723H, these would be gain of function mutations, unlike those causing Glanzmann thrombasthenia. Indeed, expression of integrin β3-D723H partially activates the αIIbβ3 complex, resulting in downregulation of RhoA activity and induction of microtubule-dependent proplatelet-like cell protrusions considered relevant for production of macrothrombocytes (Ghevaert *et al*, 2008; Schaffner-Reckinger *et al*, 2009). Integrin β3-L718P appears to act in a similar fashion (Fig 4A and B). Interestingly, we demonstrate that the three C-terminal amino acid residues (RGT) of integrin β3 are required for L718P to form proplatelet-like cell protrusions (Fig 4C). RGT provides a binding site for c-Src tyrosine kinase, which was shown to inactivate RhoA (Flevaris *et al*, 2007), further supporting the hypothesis that integrin β3-L718P plays a role in causing megakaryocytes to produce abnormal platelets through the inhibition of RhoA.

In platelets derived from megakaryocytes that carry the integrin β3-L718P mutation, full activation of αIIbβ3 complex in response to inside-out stimuli is inhibited, as shown by reduced binding of PAC-1 and fibrinogen on stimulation with ADP (Fig 3B). A simple scenario is that, in platelets, integrin β3-L718P acts as a loss of function mutation. However, given that the carriers of Glanzmann's thrombasthenia who have both normal and mutant allele and express reduced amounts of the αIIbβ3 complex, in general show normal platelet aggregation, it is possible that the integrin β3-L718P mutation gains a function that ultimately results in the reduction of inside-out signals.

In summary, identification of a pedigree showing autosomal dominant inheritance leads to a model whereby the integrin β3-L718P mutation contributes to thrombocytopenia accompanied by anisocytosis most likely through gain-of-function mechanisms. Further investigations are necessary to fully elucidate these mechanisms by which this mutation exerts its abnormal effect on thrombocytosis and platelet aggregation.

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Author contributions

H.M., T.I. and M.K. designed the work. Y.K., H.M., A.K., S.O. and M.T. performed experiments and analysed data. S.K. contributed essential materials and interpreted data. M.M and K.N. contributed clinical materials and data. H.M, Y.K. and T.I. wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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CASE REPORT

A novel Wiskott–Aldrich syndrome protein mutation in an infant with thrombotic thrombocytopenic purpura

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Abstract

Thrombotic thrombocytopenic purpura (TTP) has not yet been reported to be associated with mutations in the Wiskott–Aldrich syndrome (WAS) gene. WAS is an X-linked recessive disorder characterized by thrombocytopenia, small platelet size, eczema, recurrent infections, and increased risk of autoimmune disorders and malignancies. A broad spectrum of mutations in the WAS protein (WASP) gene have been identified as causing the disease. In this study, we report on a 2-month-old Japanese boy who presented with cytomegalovirus (CMV) infection and TTP. The activity of von Willebrand factor cleaving metalloproteinase, ADAMTS13 was low and the antibody against ADAMTS13 was positive (3.6 Bethesda U/mL). Although TTP was improved by plasma exchange and steroid pulse therapy, thrombocytopenia persisted and regular transfusions of irradiated platelets were needed. Tiny platelets were found on a peripheral blood smear. CMV genome was positive in peripheral blood by polymerase chain reaction and the CMV viremia continued to persist despite intravenous gancyclovir therapy. Through direct sequencing of genomic DNA of the WASP gene in the patient, we identified a novel mutation of WASP gene: the seventh nucleotide in exon 11 (G) had been deleted (1345delG). This mutation causes a frameshift and a stop codon at amino acid 470. Western blotting demonstrated a truncated WAS protein. To our knowledge, this is the first report describing TTP in WAS patients with novel mutation in the WASP gene.

Key words Wiskott-Aldrich syndrome; thrombotic thrombocytopenic purpura; autoimmunity

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Wiskott–Aldrich syndrome (WAS) is a rare X-linked disorder with variable clinical phenotypes that correlate with the type of mutations in the WAS protein (WASP) gene (1). The WASP gene is composed of 12 exons containing 1823 base pairs and encodes a 502-amino acid protein that appears to be of central importance for the function of hematopoietic stem cells (2). Mutations of WASP gene are located throughout the gene, although some hot spots have been identified (3). The type of mutation strongly influences the clinical severity of WAS (3). Mutations that abolish WASP expression are mainly associated with a severe clinical phenotype (full blown WAS) and a life expectancy below 20 yr of age (4). Mutations, on the other hand, result in residual expression of a full-length point-mutated WASP, are often associated with X-linked thrombocytopenia (XLT) (5), corresponding to a longer life expectancy (6). A scoring system based on clinical symptoms has been developed to differentiate these distinct clinical phenotypes caused by WASP gene mutations (2, 3, 7). Autoimmune complications are frequently observed in WAS and patients who develop autoimmune diseases are assigned to a high-risk group with poor prognosis (1). The incidence of autoimmunity in WAS is high in the US and European populations (40–72%), whereas a lower incidence was reported in Japan (22%)

(1, 6). The most common autoimmune manifestation in WAS is hemolytic anemia (36%), followed by vasculitis (including cerebral vasculitis; 29%), arthritis (29%), neutropenia (25%), inflammatory bowel disease (9%), and IgA nephropathy (3%) (8). Henoch–Schönlein purpura, dermatomyositis, recurrent angioedema, and uveitis have also been reported in some patients (6, 9). Moreover, in some cases, multiple autoimmune manifestations are observed.

Autoimmune hematological diseases are characterized by the production of antibodies against blood proteins and cells, and comprise immune thrombocytopenia, autoimmune hemolytic anemia, acquired hemophilia, and thrombotic thrombocytopenic purpura (TTP). TTP is a rare but severe disease characterized by mechanical hemolytic anemia and consumptive thrombocytopenia leading to disseminated microvascular thrombosis that causes signs and symptoms of organ ischemia and functional damage. von Willebrand factor (vWF) is synthesised in endothelial cells and assembled in larger multimers that are present in normal plasma. The larger multimers, called unusually large vWF (ULvWF), are rapidly degraded in the circulation into the normal size range vWF multimers by a specific vWF-cleaving protease, ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13) (10). ADAMTS13 deficiency leads sequentially to the accumulation of ULvWF multimers, platelet aggregation and platelet clumping, which is characteristic of the disease. ULvWF multimer accumulation in TTP is associated with absent or markedly diminished ADAMTS13 activity due to an inherited or acquired deficiency (11). An inhibitory autoantibody to the ADAMTS13 metalloproteinase has been found in patients with acquired TTP (11).

Here, we report a male infant who presented with cytomegalovirus (CMV) infection and acquired TTP which led to the diagnosis of WAS. A novel mutation, one nucleotide deletion at position 1345 (1345delG) in exon 11 was identified. To our knowledge, this is the first report regarding WAS with TTP as an autoimmune disease.

Materials and methods

Flow cytometric analysis of WASP expression

Intracellular staining with anti-WASP mAb was performed as described by Kawai *et al.* (12) In brief, peripheral blood mononuclear cells (PBMCs) from both a healthy control and the patient were first fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and stained with phycoerythrin (PE)-labeled CD3 (PharMingen, San Diego, CA, USA), CD19 (Beckman Coulter, Fullerton, CA, USA), or CD56 (PharMingen) mAb. Then cells were permeabilized in 0.1% Triton X-100 in Tris-buffered saline (pH 7.4) with 1% fetal calf serum (FCS) and 0.1% NaN_3 for 5 min. Subsequently, these cells were reacted with 10 mg/mL of anti-WASP (5A5) (12) or isotype-matched control mouse IgG2a mAb (PharMingen) for 20 min on ice, washed, and then incubated with 10 mg/mL of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG2a antibody (Southern Biotechnology Associates, Birmingham, AL, USA). The stained cells were immediately analyzed on an EPICS XL (Beckman Coulter).

Anti-WASP antisera and Western blot analysis

B-Lymphoblatoid cell lines (B-LCLs) were established by inoculating PBMCs from healthy controls and the patient with Epstein–Barr virus (EBV) – containing supernatant (6). B-LCLs from healthy control and the patient were suspended at 1.0×10^7/mL in lysis buffer containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5% aprotinin, and 10 μg/mL leupeptin at pH 7.5 and were kept on ice for 30 min. From each sample, 40 μg total protein was loaded onto a sodium dodecyl sulfate polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were incubated with rabbit anti-WASP antibody (Ab 503) against a synthetic peptide (aa's 209–226 of WASP) (6) at 1 : 5000 dilutions. The membranes were incubated with alkaline phosphatase-conjugated goat antirabbit immunoglobulin (Promega, Madison, WI, USA). Results were visualized by incubation with AP buffer (100 mM Tris–HCl, pH 9.5; 100 mM NaCl; and 5 mM $MgCl_2$).

DNA purification and sequencing of genomic DNA

Genomic DNA was extracted from the patient's PBMCs using Sepa-Gene (Seikagaku kogyo, Tokyo, Japan). Purified genomic DNA samples were amplified with primer pairs designed to span each exon and exon/intron junction, and the specific causative mutation was identified by direct sequencing as described previously (6). For gene sequencing, informed consent by the patient's family and approval by institutional review boards was obtained.

Patient and results

The patient was the first son of healthy and non-consanguineous Japanese parents, born at term following an uncomplicated pregnancy, and his body weight at birth was 2888 g. His past medical history was unremarkable. At the age of 2 months, he presented with fever, intermittent tachypnea, and general petechiae. On examination, he looked pale and icteric. He had hepatosplenomegaly, but did not have lymphoadenopathy or eczema. Peripheral blood analysis disclosed severe anemia and thrombocytopenia with hemoglobin (Hb) of 3.9 g/dL (normocytic), reticulocytes of 37.8% and platelet count of 11×10^9/L. The mean platelet volume was 5.8–8.1 fL (normal range, 9.0–10.7 fL) and morphology

showed small platelets. White blood cell count (WBC) was 12.3 × 10^9/L. Laboratory investigations revealed the following: serum total bilirubin (T-bil) 3.5 mg/dL (indirect 2.4 mg/dL), lactate dehydrogenase (LDH) 3264 IU/L, aspartate aminotransferase (AST) 210 IU/L, alanine aminotransferase (ALT) 73 IU/L, gamma-glutamyltranspeptidase (γGTP) 257 IU/L, blood urea nitrogen (BUN) 12 mg/dL and creatinine (Cre) 0.22 mg/dL. His prothrombin time, activated partial thromboplastin time and fibrinogen were normal. D-dimer was 7.8 μg/mL (normal range, 0–0.5 μg/mL) and haptoglobin was 8.9 mg/dL with a negative Coombs' test. Furthermore, peripheral blood smears showed fragmented red blood cells. Urinalysis revealed microscopic hematuria.

The patient was diagnosed as having TTP and treated with steroid pulse and plasma exchange (PE) therapy (40 mL/kg/d) for six consecutive days. The patient responded with elevations in the Hb to 8.0 g/dL. LDH decreased to 600 IU/L. Further serum analysis on admission showed a noticeable decrease in ADAMTS13 activity to <0.5% (normal, 70–120%), with the existence of anti-ADAMTS13 IgG autoantibody. Anti-ADAMTS13 IgG autoantibody was evaluated with the chromogenic ACT enzyme-linked immunosorbent assay (ELISA) with the Bethesda method in the Department of Blood Transfusion, Nara Medical University. One Bethesda unit is defined as the amount of inhibitor that reduces the enzymatic activity by 50% of the control value, and values >0.5 U/mL are considered significant (13, 14). Our patient showed markedly decreased ADAMTS13 activity (<0.5%) and tested positive for anti-ADAMTS13 IgG autoantibody (3.6 Bethesda U/mL) at the onset of TTP.

Viral serology study showed a positive result for CMV IgM. CMV was subsequently identified by a urine shell vial culture method and a plasma polymerase chain reaction test for CMV (PCR-CMV) demonstrated significant viremia with 7.0 × 10^5 copies/mL. Administration of intravenous ganciclovir (10 mg/kg/d) was initiated. Gancyclovir therapy was continued until viral loads were stable at around 1000 copies/mL and did not seem to further decline. His platelet counts, however, did not rise and the child required repeated platelet transfusions. A trial of intravenous immunoglobulin (IVIG) as well as a trial of systemic prednisone failed to induce a rise in platelet counts. Antiplatelet antibodies were negative. He also developed several episodes of gastroenteritis due to norovirus and methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia secondary to soft tissue infection or pneumonia, despite the monthly administration of prophylactic treatment with intravenous immunoglobulin. The presence of thrombocytopenia, small sized platelets, frequent potentially life-threatening infections and autoimmune disease led to the consideration of WAS. WASP expression was examined by flow cytometric analysis of intracellular WASP expression and a reduced expression level was detected (Fig. 1A). Western blot analysis of lysates from the normal control showed that WASP was normally expressed (66 kDa), but a truncated WASP was expressed in the patient (Fig. 1B). Sequencing of WASP genomic DNA identified a one-nucleotide (G) deletion at the position of exon 11, that cause a frameshift, resulting in the generation of a premature stop signal at codon 470 (Fig. 1C and 1D). This mutation has not been previously described. Immunological analysis of peripheral blood revealed normal percentages and numbers of $CD3^+$ T cells (1.35 × 10^9 cells/L), $CD19^+$ B cells (0.85 × 10^9 cells/L) and $CD16^+CD56^+$ NK cells (0.78 × 10^9 cells/L). Analysis of cytolytic activity against K562 target cells demonstrated a normal functional activity of the patient's NK cells compared with that from control.

Discussion

The 502-amino acid protein, WASP, consists of five functional domains: an N-terminal *Drosophila*-enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domain, a basic region (BR), a GTPase-binding domain (GBD), a polyproline-rich region (PRR) and a C-terminal verpolin cofilin homology domains/acidic region (VCA) domain (3) (Fig. 1D). Since the causative gene was first isolated and cloned in 1994(15), various unique mutations have been reported in the WASP gene, spanning all 12 exons. Here, we report a novel WASP gene mutation identified in a Japanese boy, that is, deletion of one nucleotide (G) in exon 11 (1345delG), which leads to a frameshift, resulting in a stop codon at amino acid 470. Most missense mutations are localized to the EVH1 domain, and a mutated WASP often cannot bind to WASP-interacting protein (WIP), leading to defective WASP expression (16). However, since 1345delG mutation causes the partial deletion of WASP in VCA domain, but still maintains an intact EVH1 domain for WIP binding, we can assume that the mutant WASP can bind to WIP and is relatively stable, which protects the truncated WASP from being degraded. But, due to the lack of the VCA area, the truncated WASP cannot combine with the actin-related protein (ARP) 2/3 complex, which plays a key role in cytoskeletal remodeling. WASP, in the active form, binds the ARP 2/3 complex, which gives rise to nucleation of actin filaments at the side of pre-existing filaments, thus creating a branching network of actin at the plasma membrane (8). The activity of the ARP2/3 complex was shown to contribute to a variety of cellular functions, including change of cell shape, motility, endocytosis, and phagocytosis (17).

While many thought that autoimmunity was more common in patients with complete WASP deficiency, recent reports show that autoimmunity can occur in both severe and attenuated cases of the disease (6). Antibody-mediated cytopenias are the most frequent manifestation of autoimmune reactions but various vascular and organ-based autoimmune processes have also been reported (18). Although 22–72% of reported WAS cases suffered from autoimmune disorders,

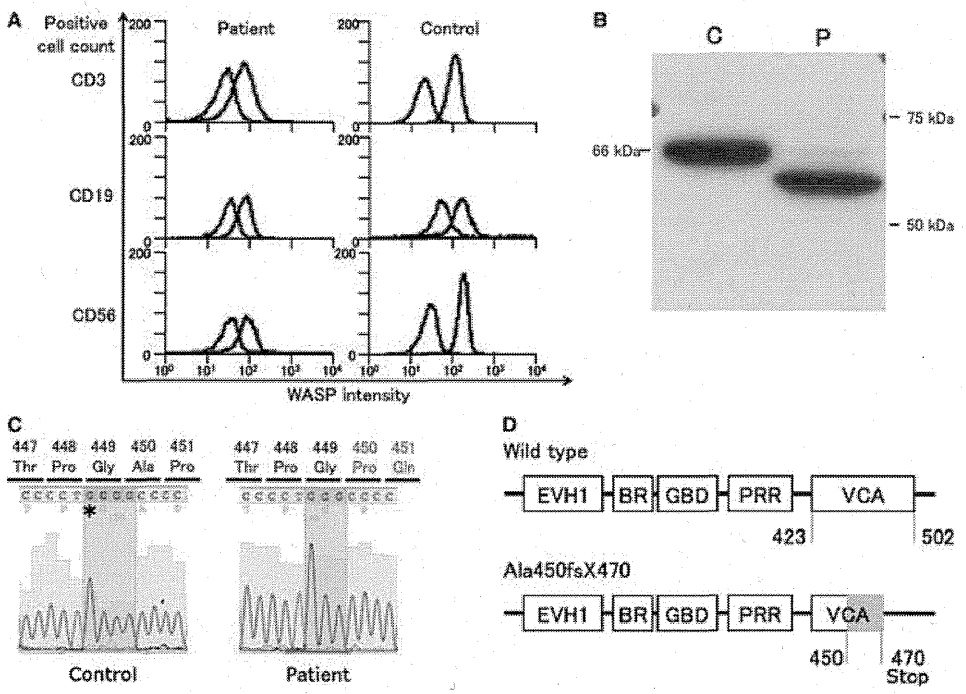

Figure 1 (A) Intracytoplasmic Wiskott–Aldrich syndrome (WAS) protein (WASP) expression analysis by flow cytometry. Histograms represent anti-WASP staining compared with isotype control in different lymphocyte subsets as indicated. (B) Anti-WASP Western blot analysis from peripheral blood mononuclear cells (PBMCs). The lysate from normal individual expressed WASP at a normal size (66 kDa), and a truncated WASP was expressed in the patient's PBMCs. C: normal control, P: patient. (C) Mutation analysis of the WASP gene. Electropherogram shows the deletion in exon 11 of the WASP gene. The position of the deletion is indicated by the asterisk on the wild-type sequence, and the changes of amino acids in the patient are shown. (D) Wild type and 1345delG-mutated WASP. EVH1, Ena/VASP homology 1 domain; BR, basic region; GBD, GTPase-binding domain; PRR, proline-rich region; VCA, verpolin cofilin homology domains/acidic region.

none of them developed TTP (8, 19). Why the present case developed TTP as an autoimmune disorder is not clear. Thrombotic microangiopathy (TMA) including TTP has been shown to occur in the setting of bacterial infections, viral infections, autoimmune diseases, malignancies, pregnancy related complications, and certain medications such as ticlopidine, cyclosporine, and tacrolimus (20). To date, there are several case reports of active CMV infection associated with TMA in both immunocompetent and immunosuppressed individuals. Although the exact pathogenesis by which CMV infection results in TMA is unknown, CMV has been shown to injure endothelial cells either by direct infection or indirectly by initiating an abnormal immune response (20, 21).

Thrombocytopenic purpura concurrently occurs in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren's syndrome, scleroderma, Still's disease, polymyositis, and myasthenia gravis (22). While the present case has no autoimmune disorders other than TTP, Monteferrante *et al* (23). presented a patients with WAS who developed SLE at the age of 12 yr. The definitive phenotype in patients with mutations in the WAS gene may manifest only late in life and never reach the medical literature (6). Nikolov *et al.* and Humblet-Baron *et al.* (24, 25) have found that older WASP deficient mice develop anti-nuclear and anti-dsDNA antibodies at much higher rates than isogenic controls with titers approaching those of other autoimmune-prone mouse strains. In WASP deficient mice over 6 months of age, Nikolov *et al.* (24) found circulating immune complexes, immune complex deposition in the kidney, and mild nephritis resembling the IgA nephropathy seen in some patients with WAS. As infants with WAS may not yet have developed the final clinical phenotype, careful observation for unexpected clinical phenotypes is warranted.

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LETTER TO THE EDITOR

Recipient seropositivity for adenovirus type 11 (AdV11) is a highly predictive factor for the development of AdV11-induced hemorrhagic cystitis after allogeneic hematopoietic SCT

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Late-onset hemorrhagic cystitis (HC) is one of the most troublesome complications in patients undergoing allogeneic hematopoietic SCT (HSCT). As adenovirus serotype 11 (AdV11) with striking tropism for the urinary system is a pathogen predominantly responsible for late-onset HC after allogeneic HSCT in Japan,[1,2] it is important to assess the risk of AdV-HC and to make a rapid diagnosis of HC for early intervention.

Sixty-nine patients who underwent the first allogeneic HSCT between April 2005 and December 2006 were enrolled before the start of preparative conditioning. Standard urinalysis was performed at least once a week from 2 weeks before HSCT up to 3 months post HSCT and at the outpatient clinic every 2 or 4 weeks thereafter until 1 year post HSCT. In this study, late-onset HC was defined as HC that occurred 10 days after completion of the preparative

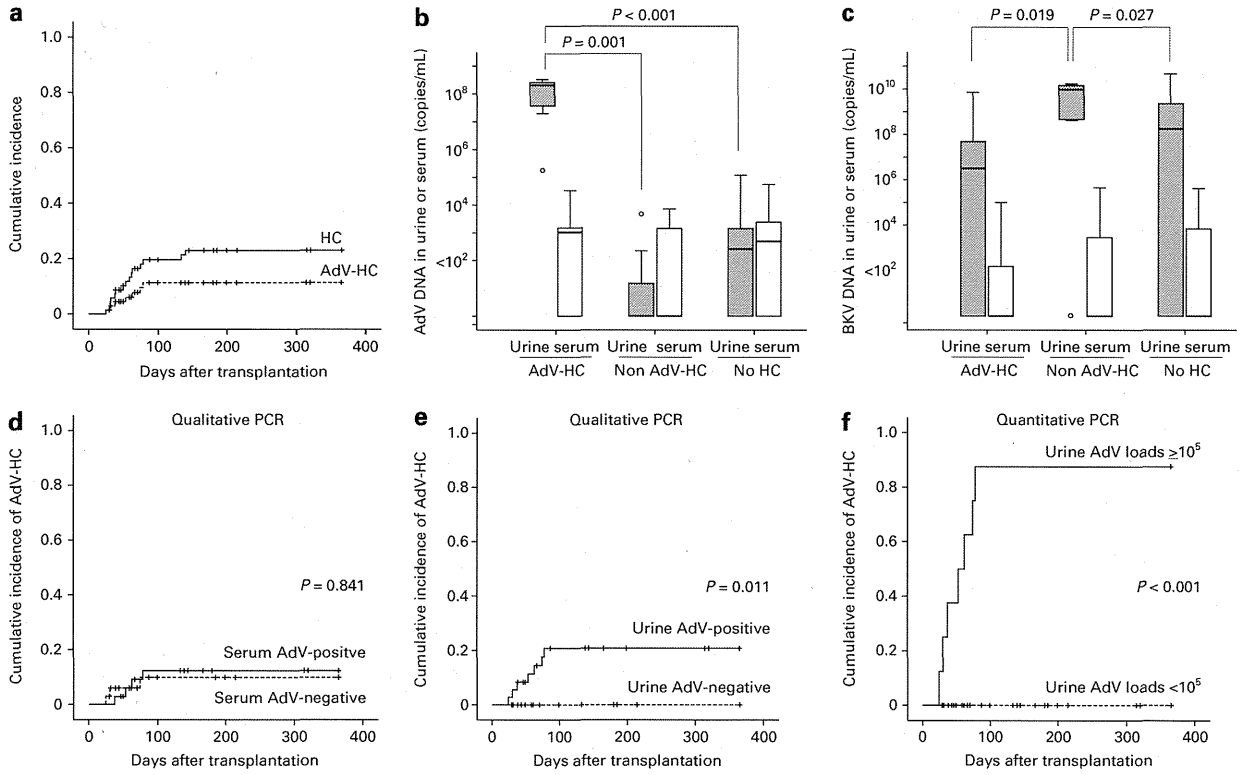

Figure 1. Cumulative incidence and viral loads of late-onset HC after allogeneic HSCT. (**a**) Cumulative incidence of late-onset HC and AdV-HC. In the Kaplan–Meyer curves, death without incidence of HC was defined as a competing event. The cumulative incidence of late-onset HC (solid line) over intervals from the start of allogeneic HSCT to the first day of hematuria was 23%, with a median interval after HSCT of 53 days (range: 24–139 days). The cumulative incidence of AdV-HC (dashed line) was 11%, with a median interval after HSCT of 53 days (range: 24–78 days). (**b**) Comparison of urinary and serum AdV loads among patients with HC from whom AdV was isolated by viral culture (AdV-HC), patients with HC from whom AdV was not isolated by viral culture (non-AdV-HC), and patients without HC (no HC). (**c**) Comparison of urinary and serum BKV loads among AdV-HC, non-AdV-HC and no HC group. The detection limit of the assay for both serum and urine was 1.0×10^2 copies/mL. To determine the significance of differences between two independent groups, the Mann–Whitney U-test was used. Only *P*-values showing statistical differences are presented. (**d–f**) Comparison of cumulative incidence of AdV-HC between qualitative PCR and quantitative PCR. The Kaplan–Meyer curves were compared using the log-rank test. (**d**) The cumulative incidence was 12.4% in patients with sera positive for AdV (solid line) and 9.9% in patients with sera negative for AdV (dashed line) according to the qualitative PCR. (**e**) The cumulative incidence was 20.8% in patients with urine positive for AdV (solid line) and 0% in patients with urine negative for AdV (dashed line) according to the qualitative PCR. (**f**) The cumulative incidence was 87.5% in patients with 1.0×10^5 copies/mL or higher of urine AdV loads (solid line) and 0% in patients with less than 1.0×10^5 copies/mL (dashed line) of urine AdV loads according to the quantitative PCR.

treatment. According to the published criteria,[3] we recorded grade 2 or higher HC as a clinically important complication in HSCT patients and performed viral cultures of their urine. As Akiyama *et al.*[2] reported that the viral culture is equivalent to PCR for diagnosis of AdV-HC, we classified HC into AdV-HC and non-AdV-HC based on the culture results. Among 69 subjects, 15 patients developed late-onset HC during the 1-year follow-up period (Figure 1a). Twenty-eight (40.6%) of 69 patients developed moderate or severe acute GVHD. Thirty-six patients (52.2%) were treated with corticosteroids for post-transplant complications such as engraftment syndrome and acute GVHD. The occurrence of acute GVHD was strongly associated with the incidence of HC according to the χ^2-test ($P<0.001$). Patients treated with corticosteroids developed HC more often than those not treated with corticosteroids, although this trend did not reach statistical significance ($P=0.064$). AdVs were cultured from the urine of 7 of

Table 1. Analysis of pre-transplant risk factors for development of late-onset HC: (a) univariate analysis (b) multivariate analysis

Variable	*Number of patients (n = 69)*	Number of patients with HC (AdV-HC)	P
Univariate analysis[a]			
Age[a]			
⩾16 years	27	9 (5)	0.061
<16 years	42	6 (2)	
Sex			
Male	45	12 (5)	0.174
Female	24	3 (2)	
Disease status[b]			
Advanced (non-CR)	27	8 (4)	0.203
Stable (CR or non-malignant)	42	7 (3)	
AdV11 serostatus of recipients[c]			
Positive	11	6 (6)	0.009
Negative	54	8 (1)	
Donor type[d]			
2- or 3-Ag mmRel or unrelated	48	13 (6)	0.104
Matched or 1-Ag mmRel	21	2 (1)	
Conditioning regimen[e]			
3-12Gy TBI-containing	56	11 (5)	0.458
No TBI	15	4 (2)	
Bu-containing	13	6 (2)	0.028
No Bu	56	9 (5)	
Cy-containing	46	8 (3)	0.216
No Cy	23	7 (4)	
Mel-containing	14	3 (2)	1.000
No Mel	55	12 (5)	
Flu-containing	31	7 (4)	0.878
No Flu	38	8 (3)	
ATG/ALG-containing	8	2 (1)	1.000
No ATG/ALG	61	13 (6)	
GVHD prophylaxis			
FK + MTX ± mPSL	50	10 (4)	0.745
CsA ± MTX	19	5 (3)	

Variables	*Unfavorable factors*	*Multivariate analysis*	
		Hazard ratio (95% CI)	P
Multivariate analysis[f]			
AdV11 antibody	Seropositive	7.87 (2.54 – 24.4)	<0.001
Sex	Male	4.54 (0.99 – 20.8)	0.051
Bu-containing	Used as conditioning	2.87 (0.94 – 8.77)	0.064
Donor type	2 or 3-Ag mmRel or unrelated	—	0.350
Age	⩾16 years	—	0.825

Abbreviations: AdV11, adenovirus serotype 11; ATG/ALG, anti-thymocyte/lymphocyte globulin; CI, confidence interval; FK, tacrolimus; Flu, fludarabine; HC, hemorrhagic cystitis; Mel, melphalan; mPSL, methylprednisolone; mmRel, mismatched relative. [a]Parameters from the patients' pre-transplant information were analyzed with the χ^2-test or Fisher's exact test. Statistical significance was defined as $P<0.05$. [b]Sixty patients had hematologic malignancies, four aplastic anemia, two primary immunodeficiencies and three metabolic disorders. [c]AdV11 serostatus was determined by a neutralizing antibody test using patient sera obtained before the start of preparative treatment. A result of 1:4 or higher was considered positive. Not tested in four recipients. [d]Sixteen patients underwent transplants from HLA-matched relatives, five from singe-antigen mismathced and eight from two- or three-mismatced relatives. Forty patients received grafts from unrelated donors (bone marrow in 30 and cord blood in 10). [e]The conditioning regimen was TBI + CY ± others in 40 patients, TBI + Mel ± others in 9 patients,TBI + Bu ± others in 7 patients. Bu + CY ± others in 3 patients, Bu + Flu ± others in 3 patients, non-TBI + non-Bu in 7 patients. [f]Parameters for which $P<0.2$ in the univariate analysis of pre-transplant information were applied to Cox regression model. Statistical significance was defined as $P<0.05$.

15 patients with late-onset HC and all strains were identified as AdV11. To examine the relationship between viral load and development of late-onset HC in patients undergoing allogeneic HSCT, we performed quantitative PCR for AdV and BK virus (BKV) in all serum and urine samples collected every 1 to 2 weeks after HSCT upto 180 days after HSCT. Primers and probe for identification of all serotypes of AdV and those for identification of BKV were designed, based on the reports described previously.[4–5] As presented in Figure 1b, the urine AdV loads at the onset of HC in the 7 patients with AdV-HC were markedly higher than the maximum values in the 8 patients with non-AdV-HC or the 54 patients without HC. There was no significant difference in the serum AdV loads among the AdV-HC, non-AdV-HC and no HC group. Lion *et al.*[6] presented the data that the incidence of AdV viremia in patients with AdV at above 1×10^6 copies/g of stool was significantly higher than in those with AdV levels in stool specimens below this threshold, suggesting that increase of stool AdV load predicts viremia. Accordingly, we investigated the data set for urine and blood. Among seven AdV-HC patients, four patients with AdV viremia (1.1×10^3 to 3.3×10^4 copies/mL) had 1.8×10^5 to 3.3×10^8 copies/mL of urine AdV loads, whereas 3 patients with no AdV viremia had 2.0×10^7 to 2.8×10^8 copies/mL of urine AdV loads. The cumulative incidence of AdV-HC was substantially different between qualitative PCR and quantitative PCR (Figures 1d–f). In particular, when AdV at 1.0×10^5 or higher copies/mL was detected in the urine, AdV-HC was diagnosed with 100% sensitivity, 98% specificity, 88% positive predictive value and 100% negative predictive value. On the other hand, qualitative PCR in urine samples displayed 100% sensitivity, 52% specificity, 19% positive predictive value, and 100% negative predictive value. Serial analyses in four of seven patients who developed AdV-HC revealed that adenovirurin reached $>1.0 \times 10^4$ copies/mL 1–2 weeks before the onset of HC. Accordingly, quantification of the urine AdV load may be more useful for diagnosing AdV-HC than qualitative PCR positivity.

As seven of eight non-AdV-HC patients had significant high urine BKV load between 4.3×10^8 and 1.7×10^{10} copies/mL (Figure 1c), most of the non-AdV-HC was considered to be BKV-associated HC. Among seven AdV-HC patients, three patients had concomitant BKV infections because of over a diagnostic viral load in urine (4.2×10^7 to 7.2×10^9 copies/mL) according to the criteria described by Cesaro *et al.*[7] The serum BKV load did not influence the development of HC. Therefore, BKV might be an alternative main cause of HC in Japan.

To identify factors predictive of the occurrence of late-onset HC, we first performed univariate analysis of the patients' pre-transplant information (Table 1a). Five factors (age, sex, recipient AdV11 serostatus, type of donor and conditioning regimen with or without Bu) showed $P<0.2$. Multivariate analysis revealed that recipient AdV11-seropositivity was the only significant risk factor (Table 1b).

HSCT-related AdV-HC is more frequent in Japan than in other countries (0–4%).[3,7,8] Several retrospective Japanese studies have reported risk factors including acute GVHD and chronic GVHD.[1,2,9,10] The influence of seropositivity for AdV was controversial.[1,2,9,10] In this study, we used a neutralizing antibody test to detect anti-AdV11 antibodies because this test is serotype-specific and can detect IgG antibodies for longer period after primary infection than can the complement fixation test. This prospective study revealed that the cumulative incidence of AdV-HC was 64% in the seropositive patients, but only 2% in the seronegative patients (log-rank test, $P<0.001$). Accordingly, recipient AdV11 serostatus is suggested to be the sole predictor of late-onset HC in Japanese allogeneic HSCT patients. Therefore, patients seropositive for AdV11 may be candidates for prophylactic anti-AdV treatment. It is likely that AdV-HC occurs in approximately 90% of allogeneic HSCT patients when the urine AdV load reached 1.0×10^5 copies/mL or more. Taken together with the finding of the time-course study, preemptive treatment may be recommended to begin when the urine AdV load reaches 1.0×10^4 copies/mL or higher.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

PBSC collection from family donors in Japan: a prospective survey

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Severe adverse events (SAE) and late hematological malignancies have been reported after PBSC donation. No prospective data on incidence and risk factors have been available for family donors so far. The Japan Society for Hematopoietic Cell Transplantation (JSHCT) introduced therefore in 2000 a mandatory registration system. It defined standards for donor eligibility and asked harvest centers to report any SAE immediately. All donors were examined at day 30 and were to be contacted once each year for a period of 5 years. Acute SAEs within day 30 were reported from 47/3264 donations (1.44%) with 14 events considered as unexpected and severe (0.58%). No donor died within 30 days. Late SAEs were reported from 39/1708 donors (2.3%). The incidence of acute SAEs was significantly higher among donors not matching the JSHCT standards ($P = 0.0023$). Late hematological malignancies in PBSC donors were not different compared with a retrospective cohort of BM donors (N:1/1708 vs N:2/5921; $P = 0.53$). In conclusion, acute and late SAEs do occur in PBSC donors at relatively low frequency but risk factors can be defined.

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Keywords: PBSC harvest (PBSCH); family donors; prospective study; acute adverse events; late health problems; predictive factors

INTRODUCTION

Allogeneic PBSC harvest has gained wide acceptance for hematopoietic SCT (HSCT). The stem cell harvest procedure is more convenient for both donors and medical teams,[1–3] the speed of post-transplant, hematological recovery is faster in recipients,[4–7] and outcomes are similar compared to that of BMT.[8–10] However, there have been occasional reports of mortalities[11–15] and severe complications such as splenic rupture.[16,17] Most of these severe adverse events (SAE) occurred with family donors, appeared as anecdotal or were based on retrospective analyses. No standardized or centralized reporting database was available 13 years ago.[18] Therefore, a prospective reporting study was initiated for family donors in Japan in the year 2000 to monitor the types and frequencies of adverse events potentially associated with PBSC donation, and to define factors associated with such events. We present here a comprehensive report summarizing the early adverse events (defined as within 30 days post donation) and late adverse events within 5 years post donation among 3264 consecutively pre-registered PBSC family donors from April 2000 to March 2005. The follow-up was completed in March 2010 and the data were analyzed as of September 2010. Furthermore, these PBSC donor data have been compared with the BM family donor data obtained via retrospective questionnaires shared with EBMT.[19]

MATERIALS AND METHODS

Study design

This was a prospective controlled study on all PBSC donations in Japan for a period of 5 years of recruitment and 5 years of additional follow-up. The prospective study was accompanied by a retrospective survey of an earlier cohort of patients transplanted with BM as stem cell source.

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JSHCT pre-registration mailings to institutes in 2000

JSHCT provided information to all hematology teams that were performing allogeneic PBSC transplants in 2000 or were interested in doing so. The package included: standards for donor eligibility; guidelines for G-CSF mobilization, harvest and storage of PBSC; informed consent form; donor pre-registration instructions including donor name, birth date, gender, relationship to recipient, agreement to annual health check, compliance with JSHCT standards, past/present illness, batch of G-CSF and preliminary information on recipient; donor follow-up procedure and acute SAEs report form. The registration period extended from 1 April 2000 to 31 March 2005 (5 years) and the annual long-term follow-up for 5 years was scheduled for each individual donor from April 2001 to March 2010. A day 30 short-term report had to be submitted in the fourth week after the harvest with the following information: (1) donor profiles, (2) laboratory data pre- and post donation, (3) dose of G-CSF and batch details, (4) harvested PBSC count and (5) any adverse events other than those urgently reported. A long-term report was sent by JSHCT to all donors who did consent to follow-up. It contained the following information: (1) current laboratory data and (2) any adverse events before the day of each health check. The participating transplant/harvest teams were obliged to report any adverse events to the JSHCT registration center via an emergency reporting system. Acute and late SAEs were defined as follows: (1) death, (2) events dangerous to life, (3) prolongation of hospitalization, (4) morbidity, (5) potential morbidity, (6) other events with levels equivalent to (1)–(5), (7) disease or abnormality inherited to offspring and (8) any malignancy ((7) and (8) were designated only for late events).

JSHCT eligibility criteria for PBSC family donors

The JSHCT did define formal standards for the eligibility of PBSC family donors. They were in part derived from blood donation standards, in part out of safety concerns: donor candidates should not have (1) allergy to G-CSF, (2) pregnancy, (3) cardiovascular risk factors defined as history of hypertension, coronary disease, cerebrovascular disease, diabetes mellitus or hyperlipidemia (4) splenomegaly determined by sonography, (5) hematological abnormality, (6) history of of interstitial pneumonitis, (7) history of of any malignancy, (8) ongoing heart, lung or renal disease requiring treatment, (9) ongoing autoimmune disease, (10) ongoing liver disease or (11) history of neurological disorders. Recommended donor age was between 10 and 65 years. Finally, each harvest team was required to have a third-party team to confirm the eligibility of each donor. The harvest team was free to choose a non-JHSCT-standard donor upon request of the family or the patient if no other donor was available; in any case they had to report the donor follow-up as well. No information was obtained on the number of donors rejected during the donor check-up evaluation or on the factors associated with such a decision.

Comparison with adverse events in BM family donors in Japan

To compare the frequency and the SAEs among PBSC donors to those of BM donors, a retrospective survey was conducted in collaboration between JSHCT members and EBMT for all BM donations between 1990 and 2004. The questionnaire items covered (1) any death within 30 days after donation of BM cells, (2) any SAE within 30 days after donation of BM cells and (3) any hematological malignancies (lymphoid/myeloid) at any time post donation of BM in recipients. These items were identical as reported earlier by the European Group for Blood and Marrow Transplantation EBMT.[19]

Statistical analysis

Correlation between groups was examined using the χ^2 test. Incidence of low-frequency events was compared using a Poisson regression analysis. Data were analyzed with STATA statistical software (Stata Cooperation, College Station, TX, USA). Predictive factors on PBSC donation outcomes within 30 days were examined by a logistic regression model. Factors included in the model were (1) donor profiles age (<19, 20–59 and >60 years), gender, body weight (<39, 40–69 and >70 kg), past and current health conditions and previous PBSC donation, (2) pre-and post-donation laboratory data, (3) total dose of G-CSF administered (<2499, 2500–2999, 3000–3499, >3500 μg, converted into dose of Filgrastim), (4) the occurrence of any adverse events such as thrombocytopenia, prolongation of hospitalized period, any clinical symptoms (bone pain, fatigue, headache, insomnia, anorexia, nausea and vomiting), splenomegaly and (5) numbers of mobilized CD34 + cells.

All statistical analyses were performed using the Statistical Analysis System (SAS 9.1, Cary, NC, USA).

RESULTS

Participation in the Japanese family donor PBSC pre-registration system

From 1 January 2000 to 31 March 2005, data on 3264 PBSC donations from 3188 donors (3114 with one, 72 with two and 2 with three donations) were reported to the registration system by 233 harvest teams (see Supplementary Information). This corresponds to the participation of 231 out of the 311 transplant teams that performed allogeneic HSCT during this time period (74.3%). The participating teams performed a total of 11 405 allogeneic HSCT during the same time period; hence, the proportion of PB donation concerned 28% of all allogeneic HSCT. Over the same period, the JSHCT patient registry independently reported 3262 PBSC transplants from family donors (data not shown). This confirms a close correspondence between the donors included in this survey and the actual total PBSC donations performed in Japan during this period. From the 3264 donations, 2873 (88.0%) day 30 check reports were submitted and analyzed. At the close of the projects in March 2010, 6233 reports of annual health checks had been submitted from 1708 donors. Of these 1708, 833 received all five consecutive annual health checks. The numbers of pre-registration, day 30 reports and the annual health-check forms are summarized in Figure 1.

Early SAEs

Out of 3264 PBSC donations, 47 donors (1.44%) were reported by the harvest teams to have experienced one or more SAEs either during the harvest or within the 30-day period as summarized in Table 1. The 47 events were classified by the JSHCT into three subgroups: (1) unexpected and severe (19; 0.58%), (2) transient, probably G-CSF-associated (9; 0.27%) and (3) transient, probably apheresis-associated (19; 0.58%). Some SAEs were potentially life-threatening (subarachnoid hematoma, interstitial pneumonitis), still all donors recovered. All SAEs were reported immediately as requested by the system. A comparison of the urgently reported SAEs with the standard day 30 reports revealed no inconsistencies or additional events.

Factors associated with early outcomes

The factors associated with early outcomes are summarized in Table 2. Risk factors for thrombocytopenia were higher total dose of G-CSF and older age. Risk factors for prolonged hospitalization were older age, low body weight, higher total dose of G-CSF, any past and present illness and previous stem cell donations. Risk

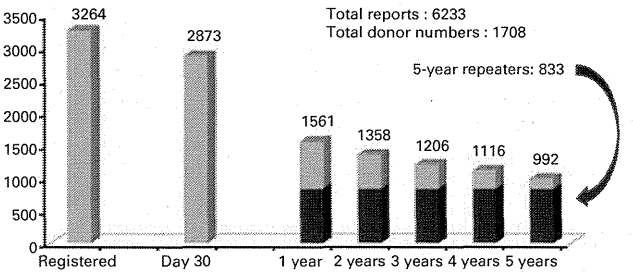

Figure 1. The cumulative numbers of pre-registered donors and Day 30 reports for 5 years, and the status of 5-year follow-up of each donor (April 2000—March 2010) are shown. The numbers of pre-registered donors, the numbers of donors whose day 30 reports were submitted and the numbers of donors who received annual health check at least once for their 5-year follow-up period are 3264, 2873 and 1708, respectively.

Table 1. Forty-seven acute severe[a] adverse events reported as emergencies to the JSHCT donor registration center between April 2000 and March 2005

	Onset	*Resolved*
Unexpected and severe[b]*: 19 (/3264 = 0.58%)*		
Angina attack with or without hypoxemia (4)	Days 2–4	Days 4–6
Deep vein thrombosis	Day 14	—
Ascites, pericardial effusion and general edema	Day 7	Day 9
Hemosputum	Day 3	Day 5
Subarachnoidal hematoma	Day 23	Day 48 (Ope)
Retroperitoneal hematoma/ Anemia	Day 4	Day 25 (Ope)
Gastric ulcer with bleeding	Day 8	Day 16
Interstitial pneumonitis (2)	Day 3	Day 6
	Day 25	Day 70
Cholangitis and gout attack	Day 2	Day 19 (Ope)
Fever and (or) Infection (5)	Days 2–7	Days 12–32
Disc herniation	Day 7	Day 62 (Ope)
Probably G-CSF-related, transient[b]*: 9 (/3264 = 0.27%)*		
Liver dysfunction (8)	Days 3–10	Days 11–36
Anorexia, nausea and vomiting	Day 4	Day 19
Probably apheresis-related, transient[b]*: 19 (/3264 = 0.58%)*		
Thrombocytopenia (1.8–6.6 × 104/mL) (13)	Days 2–6	Days 8–111
Vagovagal reflex (2)	Day 4	Days 4–5
Tetany	Day 4	Day 6
Hypesthesia of extremities	Day 4	Day 6
Hematoma of the leg	Day 7	Day 13
Migraine attack	Day 9	Day 10

Abbreviation: Ope, received surgical operation. ( ): case numbers. [a]Judged by harvest team. [b]Classified by JSHCT donor registration center.

factor for bone pain was any present illness. Female gender was the only risk factor for fatigue, headache, insomnia, anorexia or nausea. Younger age was a risk factor for vomiting. Risk factors for splenomegaly (>150% enlargement from baseline by abdominal sonography) were older age and higher total dose of G-CSF. Risk factors for lower CD34+ cell mobilization/donor body weight ($<2 \times 10^6$ CD34+ cells/kg) were age above 60 years (HR 2.55, $P<0.01$), female gender (HR 1.52, $P<0.01$) and previous stem cell donation (HR 3.10, $P<0.001$). Age below 20 years (HR 2.81, $P<0.001$) was the only parameter associated with higher CD34+ cell mobilization/donor body weight ($>9 \times 10^6$ CD34+ cells/kg).

Late events

A total of 6233 annual reports from 1708 donors were received by 31 March 2010. Hence, 52.3% of all donors had received the annual health check at least once during this 5-year period; 833 (25.5%) completed all five annual health checks. In total, 1223 donors (71.6%) reported no complaint while 485 donors (28.4%) reported one or more complaints. Of these, 108 (6.4%) donors had their complaints already before donation; 133 donors (7.8%) reported new but transient events (such as a traffic accident, common cold, hypertension, diabetes mellitus, surgical operation or pregnancy). Health problems that arose after donation and could have been related to the donation were reported by 243 (14.2%) donors. They were classified by JSHCT in 204 cases (11.9%) as non-malignant and non-significant diseases, in 26 (1.5%) as non-malignant but significant diseases, in 12 (0.7%) as non-hematological malignancies and in 1 (0.06%) as hematological malignancy.[20] Hence, 39 of 1708 donors (2.3%) were considered to have had severe complications that could have been related to the donation as judged by either the harvest teams or the JSHCT registration center. Classified as non-malignant but significant events were seven donors with thyroid dysfunction (10–34 mo), three with uterine fibroid (14–36 mo), two with rheumatoid arthritis (20, 23 mo), two with cerebral infarction (7, 33 mo) and one each with subarachnoidal hemorrhage, (9 mo), cataract (7 mo), ocular bleeding (33 mo), atopic dermatitis (12 mo), uveitis (20 mo), bronchial asthma (20 mo), ITP (27 mo), endometriosis (20 mo), mole (9 mo), cerebral aneurysm (24 mo), pancreatic cyst (53 mo) and IgA nephritis (44 mo). The 12 cases of non-hematological malignant diseases reported were 6 donors with breast cancer (4∼43 mo) and one each with gastric cancer (23 mo), uterus cancer (10 mo), brain tumor (6 mo), pharyngeal cancer (13 mo), lung cancer (54 mo) and prostatic cancer (55 mo). There was one case of hematological malignancy (0.06%) and one donor developed AML. It should be noted that one donor with a chronic myeloproliferative disorder at the time of donation (defined later), who developed acute myelogeneous leukemia 4 years after donation, was not included among the 39 cases.

Donor eligibility and frequency of severe acute and late events

Out of 3264 donors, 133 (4.07%) did not meet the eligibility criteria, 90 because of age (53 older and 37 younger than required by the standards) and 43 because of concurrent health problems. Follow-up with the annual health check was the same, for donors meeting or not meeting the standards at donation (4.3%, 74 donors). As indicated in Table 3, acute and late events tended to increase with age, although neither association was statistically significant. In contrast, early SAEs but not late events were clearly and significantly associated with concurrent health problems at the time of donation.

Comparison of adverse events between PBSC donation (prospective study) and BM donation (retrospective study) of family donors in Japan

To estimate the incidence of acute and late adverse events among BM family donors in Japan, questionnaires corresponding to those used by EBMT[19] were sent to 286 transplant teams belonging to JSHCT. A total of 191 teams (67%) responded with information from 5921 BM harvests from family donors performed between 1991 and 2003. Based on the HSCT Recipient Registry information, ∼89.7% of all related BMTs performed in Japan during the reporting period were represented. One of the 5921 donors, who died 1 year after BM donation following anoxia and brain damage during harvest, was counted as a death within 30 days following donation.[21] SAE within 30 days of donation occurred in 25 out of the 5921 (0.42%) donors (for details see Table 4). As for hematological malignancies, 2 donors developed AML after BM donation. The frequencies of adverse events among BM family donors was not significantly different from those following PBSC in terms of either 30-day mortality, frequency of SAE (unexpected SAE being adopted for PBSC donations) within 30 days or frequency of hematological malignancies.

DISCUSSION

PBSC donation is considered by many to be less stressful for a donor than BM donation.[22,23] Nevertheless, it involves other potential stress factors. These include G-CSF administration to healthy individuals, the short- and long-term effects of which remain insufficiently characterized.[24–27] Recent publications have reported that the administration of G-CSF can influence the blood coagulation system of healthy donors.[28–30] Some studies indicated genetic and epigenetic alterations in lymphocytes of healthy donors after G-CSF stimulation[31] while others could not identify such changes.[32,33] The leukapheresis procedure itself may be a

Table 2. Factors associated with adverse events after PBSC donation

Donor basic information	*Thrombo-cytopenia* 985/1074[a]	*Hospitalization >10 days* 208/2605[a]	*Clinical symptoms*							*Splenomegaly* 59/199[a]
			Bone pain 449/2370[a]	*Fatigue* 128/2691[a]	*Headache* 105/2713[a]	*Insomnia* 85/2734[a]	*Anorexia* 38/2781[a]	*Nausea* 29/2790[a]	*Vomiting* 12/2707[a]	
Age										
20–59	1	1	1	1	1	1	1	1	1	1
<19	0.64	0.98	0.75	0.87	0.92	n.o.	0.91	0.19	n.o.	0.78
60>	1.83***	2.16**	0.37	0.44	0.23	1.22	0.33	0.75	0.88	3.23**
Gender										
Female	1.14	1.37	1.02	1.92**	2.08**	2.33**	4.33**	2.89**	2.88	0.7
Body weight										
40–69	1	1	1	1	1	1	1	1	1	1
<39	0.88	2.18*	n.o.	0.64	1.65	1.4	1.86	5.24	9.20*	1.7
70>	0.89	0.68	1.07	0.41*	1.11	1.17	0.5	0.68	1.54	0.27*
Total dose of G-CSF administered										
<2499	1	1	1	1	1	1	1	1	1	1
2500–2999	1.44**	1.17	1.05	1.11	1.07	0.89	1.51	0.75	0.66	1.36
3000–3499	1.63***	1.52*	0.94	1.32	1.38	1.02	1.16	0.76	0.95	2.95*
3500>	1.88***	2.30**	0.89	1.44	1.11	1.7	0.7	1.36	0.44	1.36
Past health problems										
Yes	0.86	1.54**	1.15	1.07	0.92	1.45	1.27	1.1	2.49	1.11
Current health problems										
Yes	0.92	1.78**	1.37*	1.4	1.27	1.44	0.59	2.08	1.06	1.92
Episode of past HSC donation										
Yes	0.8	1.72*	1.11	0.86	1.05	0.68	1.61	0.98	1.4	0.14

*$P<0.5$, **$P<0.01$, ***$P<0.001$. [a]Numbers of present/absent (at clinical symptoms, present means moderate or severe symptoms and absent means none or mild symptoms.)

Table 3. Impact of age and JHSCT standards on acute and late events

	Age (years)				*JSHCT standards fulfilled (age: 10–65)*		
	0–9	*10–65*	*>65*	P-*value*	*Yes*	*No*	P-*value*
Early SAEs							
Yes	0	46	1	—	43	3	—
No	37	3128	52	—	3088	40	—
%	0	1.4	1.9	0.735	1.37	6.98	0.0023
Late SAES							
Yes	0	38	1	—	38	1586	—
No	30	1618	21	—	0	22	—
%	0	2.3	4.5	0.547	0	2.33	0.4693

Abbreviation: SAE, severe adverse event.

Table 4. Comparison of adverse events between PBSC harvest (prospective study) and BM harvest (retrospective study) in Japan

	PBSCH	*BMH*
Death within 30 days	0/3264	(1)[a]/5921 $P=0.99$
SAE within 30 days	19[b]/3264	25[c]/5921 $P=0.21$
Hematological malignancy	1[d]/1708	2[e]/5921 $P=0.53$

[a]Respiratory failure during spinal anesthesia for BMH, died after 1 year. [b]Unexpected SAE at PBSCH, see Table 1. [c]Respiratory failure (1), shock (1), malignant hyperthermia (1), lung edema (1), auricular Fibrillation (1), bradycardia (1), hypotension (2), hematoma (1), severe or prolonged pain of aspirated portion (9), chest pain (2), urethral damage (1), fever with infection (2), renal dysfunction (1), ECG abnormality (1). [d]AML. [e]AML × 2.

stress factor; more blood is processed and a longer time for harvest is needed[34] compared with a platelet collection, an apheresis procedure for which donor safety is relatively well established.

To evaluate the safety and risks of PBSC donation, JSHCT initiated a nation-wide pre-registration system followed by an annual health check for family donors. This included consecutive pre-registration for 5 years, emergency reports at any time (for both acute and late events), a formal day 30 report of the laboratory data post donation and an annual health check report for 5 years. Nearly 100% of the collected pre-registration forms and emergency reports were received on time and >80% of day 30 check reports were obtained. The collection rate of the annual health check reports was ~50%. The JSHCT eligibility criteria, specifically lack of donors to fulfill these criteria appeared to be predictive for the occurrence of severe acute adverse events. Still, the 19 donors with unexpected severe events ranged in their age between 10 and 65 years and had no health problems at the time of donation. Of interest, these 19 events were of cardiovascular (angina, thrombosis and so on), hemorrhagic (subarachnoid/retroperitoneal hematoma and so on.) or inflammatory nature (interstitial pneumonitis and so on). Other information and techniques, such as high-sensitivity CRP assay that might predict the presence of active cardiovascular disorders, should be tested to see whether these measures can identify patients at increased risk of severe acute adverse events.[35]

There were no mortality cases within 30 days in our cohort. Our numbers could still be too low. Mortality has been reported in about one death per 10 000–15 000 donations.[19] Still, we consider this absence of mortality to be in part a consequence of pre-registration and setting the eligibility standards for family donors. We cannot identify a single critical factor but pre-registration and nation-wide standards might raise awareness for potential risks in a harvest team. As evidence defines specific factors that define donors at increased risk for SAEs, the harvest team might then refuse harvest of unsuitable family donors despite their request.[36]

To compare the risk of PBSC donation to that of BM donation, the questionnaires shared with EBMT were sent to JSHCT member institutes. The results confirm that the incidence of deaths, unexpected SAEs within 30 days of donation or subsequent hematological malignancies were not different between PBSC and BM donors. There is a note of caution: events were characterized differently in the two cohorts, one was a prospective study (PBSC donation), one a retrospective study (BM harvest) and both were performed in different time periods (PBSC: 2000–2005; BM harvest: 1990–2004). A prospective follow-up system should also be applied for family BM donors.

The donor's safety is an essential part and prerequisite in allogeneic stem cell donation. For BM harvesting, an anesthesiologist usually assesses the suitability of the candidate donor and acts as a life-saving third-party expert for the hematology team. Furthermore, the harvest procedure is performed in a fully equipped operation room. In contrast, PBSC harvest can be performed by a hematology team by its own in an apheresis room; an objective risk assessment and risk management might be compromised. Allogeneic PBSC donation and transplantation are excellent medical procedures; donor safety remains an essential part in order to ascertain the future use of these techniques.[18,37] Our study has shown that the life-threatening SAEs can occur during or immediately after the donation process. These events are not erratic and risk factors can be identified. Tools are required to reduce the complication rate. Strict standards for donor eligibility and an independent third-party evaluation of donor's suitability might eliminate the conflict of interests of transplant physicians and increase donor safety. Both have been a *sine qua non* for unrelated donors in advanced blood and marrow donor bank systems such as NMDP[38]or DKMS,[39] and only a few unexpected SAEs have been reported. The same pre-donation approach and donor follow-up should become the standard-of-care for all HSCTs, from family or unrelated donors as well. It will serve to provide more accurate information about early and late effects of PBSC donation, which is needed now more than ever.[40,41]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

YK, KY and MH designed the study and wrote the paper. YM, HD, SA, MI, KK, SK and MT collected and organized the data. YK, RT, SS, SWK, KN, MH, KM and RS supervised the process of data collection. NH, MF and AK performed the statistical analysis. JH, NS, DN and AG consulted with the study concept and reviewed the results.

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Selective expansion of donor-derived regulatory T cells after allogeneic bone marrow transplantation in a patient with IPEX syndrome

Horino S, Sasahara Y, Sato M, Niizuma H, Kumaki S, Abukawa D, Sato A, Imaizumi M, Kanegane H, Kamachi Y, Sasaki S, Terui K, Ito E, Kobayashi I, Ariga T, Tsuchiya S, Kure S. Selective expansion of donor-derived regulatory T cells after allogeneic bone marrow transplantation in a patient with IPEX syndrome.

Abstract: IPEX syndrome is a rare and fatal disorder caused by absence of regulatory T cells (Tregs) due to congenital mutations in the Forkhead box protein 3 gene. Here, we report a patient with IPEX syndrome treated with RIC followed by allogeneic BMT from an HLA-matched sibling donor. We could achieve engraftment and regimen-related toxicity was well tolerated. Although the patient was in mixed chimera and the ratio of donor cells in whole peripheral blood remained relatively low, selective and sustained expansion of Tregs determined as CD4+CD25+Foxp3+ cells was observed. Improvement in clinical symptoms was correlated with expansion of donor-derived Tregs and disappearance of anti-villin autoantibody, which was involved in the pathogenesis of gastrointestinal symptoms in IPEX syndrome. This clinical observation suggests that donor-derived Tregs have selective growth advantage in patients with IPEX syndrome even in mixed chimera after allogeneic BMT and contribute to the control of clinical symptoms caused by the defect of Tregs.

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Key words: allogeneic hematopoietic stem cell transplantation – enteropathy – Forkhead box protein 3 – immune dysregulation – polyendocrinopathy – reduced intensity conditioning – regulatory T cells – X-linked syndrome

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Abbreviations: ALL, acute lymphoblastic leukemia; APC, allophycocyanin; ATG, antithymocyte globulin; BMT, bone marrow transplantation; CyA, cyclosporine A; DAB, 3, 3′-diaminobenzidine; DLI, donor leukocyte infusion; FITC, fluorescein isothiocyanate; GST, glutathione-S-transferase; GVHD, graft-vs.-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; IVIG, intravenous immunoglobulin; MLL, mixed lineage leukemia; PBMCs, peripheral blood mononuclear cells; PBSCT, peripheral blood stem cell transplantation; PE, phycoerythrin; PSL, prednisolone; RIC, reduced intensity conditioning; TBI, total body irradiation.

IPEX syndrome is primary immunodeficiency caused by the defects of regulatory T cells (Tregs). IPEX syndrome is often lethal in the first few months of life due to severe diarrhea associated with refractory enteropathy, infections, diabetes mellitus, dermatitis, and other autoimmune complications. This disorder is caused by mutations of Forkhead box protein 3 (*FOXP3*) gene located on chromosome Xp11.23. *FOXP3* encodes Forkhead box protein 3, which is essential for the development and maintenance of CD4+CD25+Foxp3+ Tregs (1, 2).

Established treatments for patients with IPEX syndrome include immunosuppressive therapy and allogeneic HSCT (3–6). Allogeneic HSCT serves as a curative therapy for patients with IPEX syndrome, and RIC regimens have been reported and resulted in better outcome than myeloablative conditioning regimen (7–11). In general, allogeneic HSCT with RIC regimen may increase the risk of rejection and mixed chimera. Some RIC regimens included the antibody against T lymphocytes such as alemtuzumab or ATG. However, these agents may increase the risk of viral reactivation after HSCT. To the best of our knowledge, only two cases of IPEX patients treated with allogeneic HSCT following RIC consisted of low-dose TBI instead of alemtuzumab or ATG have been reported (12).

Here, we report a patient with IPEX syndrome treated with RIC regimen consisted of fludarabine, cyclophosphamide, and low-dose TBI followed by allogeneic HSCT from an HLA-identical sibling donor. Although the patient was in mixed chimera, he was free from symptoms caused by the absence of Tregs. We could observe selective and sustained growth advantage of donor-derived Tregs and disappearance of anti-villin autoantibody in his serum, which was correlated with the improvement in refractory enteropathy.

Patient and methods

Patient

A Japanese male suffered from severe diarrhea at two months of age. He was diagnosed as IPEX syndrome by identifying a missense mutation of T1117G substitution in exon 10 of the *FOXP3* gene (13). We quantified CD4+CD25+Foxp3+ cells by flow cytometry, and positive cells were not identified at all in PBMCs. Autoantibodies examined were negative except anti-villin antibody in patient's serum. He was treated with immunosuppressive therapy of intravenous CyA and oral PSL. After complete remission was achieved, he was free from the symptom for six yr with oral low-dose CyA and PSL (14).

At the age of six, the patient suffered from severe diarrhea again and was referred to our hospital. Although he was treated with increased doses of CyA and PSL in addition to other immunosuppressive agents, these treatments were not effective enough to control his diarrhea completely. We next tried IVIG therapy, which resulted in the improvement in diarrhea, and we could taper immunosuppressive agents.

To control the disease without continuous immunosuppressive therapy, we considered to perform allogeneic BMT from an HLA-matched healthy sibling donor. The donor did not have the mutation in *FOPX3* gene. We used a RIC regimen consisted of 4 Gy (2 × 2 Gy) TBI (day 7), fludarabine at a dose of 30 mg/m^2 for five days (days 6 to day 2) and cyclophosphamide at a dose of 60 mg/kg for two days (days 3 and 2). Total nucleated bone marrow cells of 4.32×10^8/kg were transplanted. We selected CyA and short-term methotrexate as GVHD prophylaxis, and IVIG was continued weekly until autoimmune colitis was resolved.

Chimerism assay

Chimerism assay was performed by polymerase-chain-reaction-based assays analyzing polymorphic short tandem repeat markers (15). The chimerism was examined in each fraction of T cells, total lymphocytes, and granulocytes in bone marrow or peripheral blood. We evaluated the chimerism in bone marrow before day 100 and in peripheral blood after day 100, because we had similar results in both samples before day 100 in the patient and avoided frequent bone marrow aspiration after day 100.

Flow cytometry

PBMCs were stained with monoclonal antibodies of APC-conjugated human CD4, PE-conjugated human CD25, and FITC-conjugated human Foxp3 antibodies (BD Biosciences, San Jose, CA, USA) and analyzed by a FACSCanto II flow cytometer (BD Biosciences), as described previously (16).

Immunoblot analysis of anti-villin antibody

Anti-villin autoantibody in patient's serum was analyzed as described previously (17). Briefly, 500 ng of GST-villin recombinant protein (121 kD) was transferred to the membrane and incubated with diluted serum at 1:160. Anti-villin antibody bound to GST-villin was detected by horseradish peroxidase-conjugated antibody and DAB system.

Case report

Clinical improvement after RIC and allogeneic HSCT

The patient achieved an engraftment on day 11, and the last transfusion of platelets was on day 7 and that of red blood cells was on day 1. He was complicated with transient acute GVHD of the skin (grade I) on day 35 but this resolved without additional immunosuppressive therapy. He had no episodes of significant infection and other severe regimen-related toxicity during the course of RIC and allogeneic HSCT.

Severe and bloody diarrhea settled down on day 14 after engraftment. The patient was