IgD^-CD27^+ memory B cells were 77.01 ± 5.50% and 76.37 ± 5.63, 4.33 ± 1.02, and 5.23 ± 1.15%, 16.33 ± 3.86% and 16.64 ± 3.35%, respectively). Also, when we used IgM instead of IgD, the difference in percentages among each B cell population was barely recognized (Fig. 2*A*). Due to the known reduction in the total number of PB B cells in the aged, the increased levels in naive B cell percentages had no impact on the absolute number of naive B cells in the aged, whereas the absolute number of memory B cells, especially IgM memory B cells, significantly decreased (Fig. 2*B*).

These results show that circulating IgM memory B cells are strongly reduced in elderly people compared with those in normal adults.

B cell functions in elderly people

The declines of memory B cells in elderly persons led us to examine whether Ig productions were different from adults to elderly persons. We found similar levels of serum IgG in the elderly persons in the study compared with those of adults (1468 ± 35.5 mg/dl in elderly persons (n = 60) vs 1407 ± 3.8 mg/dl in adults (n = 60); not significant by Student's t test). PB MNCs isolated from the elderly produced only moderate levels of IgA, IgM, and IgG in response to stimulation with SAC and IL-2 compared with those taken from adults (Fig. 3*A*). To exclude effects by T cells and monocytes, we purified B cells by positive selection using CD19 microbeads. The highly purified B cells in adults produced substantial amounts of IgG, IgM, and IgA after being stimulated with SAC plus IL-2, whereas Ig syntheses in elderly persons were remarkably diminished (Fig. 3*B*).

We also investigated the generation of plasma cells from highly purified B cells in elderly persons and adults. We detected plasma cells by the expression of high levels of CD38 and low levels of CD20, which were identified morphologically as plasma cells (i.e., basophilic cytoplasms with pale Golgi zone and eccentric nuclei) (data not shown). After stimuli, the induction of the differentiation into the plasma cell was decreased in elderly persons compared with that in adults (Fig. 3, *C* and *D*). Statistical significance was found between the plasma-cell inductions in elderly persons and adults ($p < 0.05$) (Fig. 3*D*). These findings show that Ig syntheses and plasma cell differentiation in B cells of elderly persons fall and their B cells have similar characteristics of naive B cells.

Effect of pneumococcal polysaccharide vaccine

The marked reduction with aging of IgM memory B cells, which may predominantly produce anti-polysaccharide IgM, raised the possibility of the change of IgM memory B cell compartment in circulating B cells after pneumococcal polysaccharide vaccination. To investigate this idea, we examined the population of memory B cells known as IgD^-CD27^+ and IgM memory B cells before and after vaccination. Specific IgM Abs to pneumococcal polysaccharides were detected both in young adult and aged sera. OD levels of the pneumococcus-specific serum IgM Abs in elderly persons were two-thirds of that in young adults before vaccination, and an ~2-fold increase in OD was found in sera of both groups after vaccination (Fig. 4*A*). Interestingly, marginal levels of increase in IgM memory B cells, but not in IgD^-CD27^+ memory B cells, were recognized after the vaccination. Significance was found in the percentages of IgM memory B cells before and after vaccination (Fig. 4*B*). Thus, pneumococcal vaccination, at least some part, has an effect on IgM memory B cells, probably resulting in an elevation of anti-polysaccharide IgM.

Discussion

In this study, we investigated the effects of IgM memory B cells, which are proposed to provide the splenic marginal zone and be

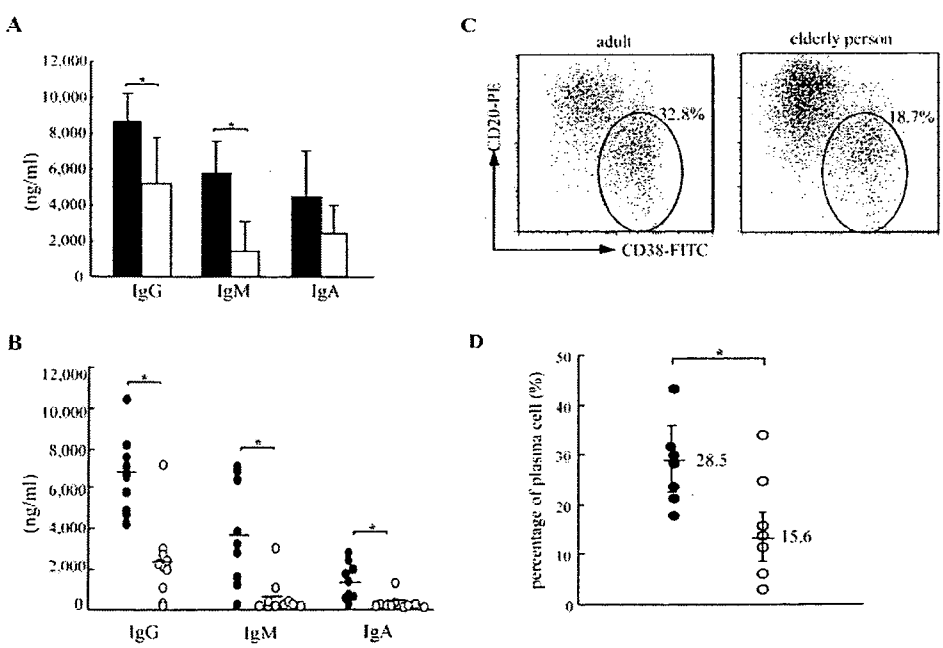

FIGURE 3. Ig synthesis and plasma cell differentiation by B cells. *A*, MNCs (10^5/well) from adults (n = 20) or elderly persons (n = 20) were stimulated with SAC and IL-2. Eight days after being stimulated, the supernatants were harvested, and their IgA, IgM, and IgG concentrations were measured by ELISA. Final concentrations of SAC and IL-2 were 0.01% and 50 ng/ml, respectively. ■, <65 years old; □, ≥65 years old. *B*, The amounts of IgG, IgA, and IgM secreted in supernatants of highly purified B cells cultured under the condition described in *A* were measured (●, <65 years old, n = 9; ○, ≥65 years old, n = 10). Results are shown as means ± SD. *, $p < 0.05$. *C*, B cells from an adult and an elderly person were incubated with SAC plus IL-2 for 8 days, and two-color analysis using anti-CD38-FITC and anti-CD20-PE was performed by flow cytometry. CD38 and CD20 were used as a surface marker of plasma cells and B cells, respectively. *D*, Percentages of the induced plasma cells. B cells in adults (●) and elderly persons (○) were incubated with SAC plus IL-2, and CD38-expressing cells were detected by flow cytometry. Values are the mean ± SD. *, $p < 0.05$.

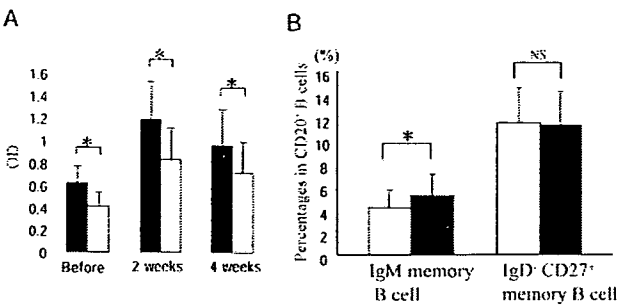

FIGURE 4. Effects of pneumococcal polysaccharide vaccine on production of IgM Ab specific for pneumococcal polysaccharide and IgM memory B cells. *A*, The levels of the concentration of IgM specific for pneumococcal polysaccharide in 20× dilution of sera were measured by ELISA in three young adults (■) and five old adults (□) before and after vaccination. Polysaccharide-specific IgM is indicated as scale in OD on the left *y*-axis. Results are shown as means ± SD. *, $p < 0.05$. *B*, Percentages of IgM memory B cells (IgD^+CD27^+ B cells) and IgD^-CD27^+ B cells in $CD20^+$ B cells in elderly persons before (□) and 4 wk after (■) vaccination. Values are shown as means ± SD ($n = 10$). *, $p < 0.05$.

responsible for the protection against encapsulated bacteria, on aged humoral immune defense. Memory B cells, in particular IgM memory, diminished markedly in the aged accompanied with reduction of plasma cell differentiation potency and Ig syntheses. In addition, levels of anti-pneumococcal IgM in sera of elderly persons were lower than those of young adults after pneumococcal vaccination.

In the aged immune system, the number of T cells and their response to Ag are declined (18). One well documented significant T cell change is the gradual shift from $CD45RA^+$ $CD4^+$ naive toward an increase in $CD45RO^+CD4^+$ T cells that represent activated or memory phenotype (19). However, the memory T cell population in aged persons is reported to be hyporesponsive against pathogens, and the loss of optimal IL-2 production may participate in the aging process and may represent the main Ag-independent defect in the $CD4^+$ T cells (20). In contrast to our knowledge of memory T cell functions in the elderly, it is not known whether there is an age-associated change in the naive and memory distribution in B cells. In regard to the change in the total B cell number, a reduction in the proportion of B cells in the PB has been found in elderly persons (21), and a decline in $CD19^+$ B cells during aging has been noticed in tonsillar lymphocytes (22). In contrast with our findings, Colonna-Romano et al. (23) reported that the percentage of $CD27^+$ B cells increase in the elderly. Surely, in our experiments, some elderly persons had the considerable percentages of IgD^-CD27^+ memory B cells, but extremely a little degree of IgM memory B cells. However, the percentages of $CD27^+$ B cells in total B cells range from 0 to 90 in 80- to 100-year-olds in this study. When B cells are separated by the expression of IgD and CD27, it is conceivable that their findings will become reliable. Surface expression levels of each Ig class on IgD^-CD27^+ memory B cells in young and elderly persons resembled (Fig. 1*C*), implying that IgM-only and each switched memory B cells diminished equally during aging. A statistically significant age-related increase in the serum levels of Ig isotypes (IgG and IgA but not IgM) and IgG subclasses (IgG1, IgG2, and IgG3, but not IgG4) was reported by Ginaldi et al. (18). Although the number of circulating B cells in elderly persons was barely reduced in our study (data not shown), their serum IgG was the same level. The discrepancy between the same serum IgG levels and decreased differentiation potency in plasma cells may be due to other factors such as circulating PB plasma cells and long-lived plasma cells. It is also reported that aging is associated with a decreased diversity in the Ab response reflected in the high-affinity Abs after immunization with the foreign Ag (4, 21). These findings are content with our data of the decrease in IgD^-CD27^+ and IgM memory B cells in elderly persons.

The most definitive marker of memory B cells is somatic hypermutation in the Ig V-region genes; through this process, the generation of highly diverse Abs with high affinity can be achieved (24). To be effective, memory B cells must be able to differentiate rapidly into plasma cells and to produce high-affinity Ag-specific Abs efficiently during the secondary immune response. Triggering via B cell Ig receptors by Ags, cytokines such as IL-2 and IL-10, and direct cell-to-cell contact between T and B cells play an important role in the differentiation into plasma cells. In regard to B cell Ig synthesis, although the possibility remains that functions itself of aged B cells are declined, a decrease in both of two $CD27^+$ memory B cell pools may result in the impaired differentiation into plasma cells and the diminished production of high-affinity Abs against pathogens in the elderly.

Bacteria such as *S. aureus* and *Homophiles influenza* are known to cause post-influenza pneumonia, but *S. pneumoniae* is the most prominent pathogen causing secondary bacteria pneumonia, especially in elderly people (25). However, *S. pneumoniae* is a common cause of pneumonia but not always the most common cause, because agents vary in different clinical populations. Regarding defense from *S. pneumoniae* infections, many components of the immune system are likely involved in the defense. B cells and Abs may be especially important for the defense from blood-borne infections. Recently, it was reported that increased susceptibility to secondary pneumococcal pneumonia is at least in part caused by excessive IL-10 production and reduced neutrophil function in the lung (26). In young children, the response to T-independent Ag has been reported to be defective with a high incidence of infections caused by encapsulated bacteria. The splenic marginal zone is especially well equipped for rapid humoral responses and is unique in its ability to initiate an immune response to encapsulated bacteria (27), suggesting the importance of the spleen for host immune defense toward encapsulated bacteria infections. Our data suggest that the remarkable age-related decline in IgM memory B cell compartment in elderly persons may have an influence on their humoral immunity. So, it is expected that strategies targeting the maintenance of the IgM memory B cell pool is valuable in the treatment of this condition.

IgD^-CD27^+ memory B cells can produce IgG, IgM, and IgA, whereas IgM memory B cells predominantly produce IgM (7, 28). A complete absence of the IgD^-CD27^+ memory B cell population, which results in severe susceptibility to infections, is found in some diseases involving primary immunodeficiency such as X-linked hyperIgM syndrome (29) and common variable immunodeficiency (30). Some patients of X-linked hyperIgM syndrome and common variable immunodeficiency have IgM memory B cell phenotypes with/without somatic hypermutation, and the presence of IgM memory B cells somewhat correlates with their clinical aspects (31). B cell proliferation can be triggered by polyclonal stimuli derived from microbial products, such as lipopolysaccharides or unmethylated single-stranded DNA motifs (CpG oligonucleotides), which stimulate B cells via TLR4 and TLR9, respectively (32). The sensitivity to polyclonl stimuli represents a key feature of human memory B cells (33). Although the exquisite sensitivity of switched memory B cells to bystander help may be instrumental in maintaining systemic IgG Ab levels, the capacity of IgM memory B cells to respond to CpG in the absence of cytokines could be instrumental in maintaining levels of natural Abs to bacterial Ags (34, 35). Given the findings that functions in the

aged spleen are declined during aging (12, 13) and the incidence of lethal infections by pneumococcal infections increases during aging, the spleen may be the central organ in defense against encapsulated bacteria infections.

In conclusion, besides that circulating IgM memory B cells correspond to splenic marginal zone B cells and they are provided from the spleen as reported recently (9), decline of splenic functions may reflect diminished numbers of aged IgM memory B cells. Effectiveness of pneumococcal polysaccharide vaccine in older adults on protection against pneumococcal infections may be associated with the increase and activation of circulating IgM memory B cells, resulting in rapid synthesis of anti-polysaccharide IgM Abs.

Disclosures

The authors have no financial conflict of interest.

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Application of HSVtk suicide gene to X-SCID gene therapy: Ganciclovir treatment offsets gene corrected X-SCID B cells

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Abstract

Recently, a serious adverse effect of uncontrolled clonal T cell proliferation due to insertional mutagenesis of retroviral vector was reported in X-SCID gene therapy clinical trial. To offset the side effect, we have incorporated a suicide gene into therapeutic retroviral vector for selective elimination of transduced cells. In this study, B-cell lines from two X-SCID patients were transduced with bicistronic retroviral vector carrying human γc chain cDNA and Herpes simplex virus thymidine kinase gene. After confirmation of functional reconstitution of the γc chain, the cells were treated with ganciclovir (GCV). The γc chain positive cells were eliminated under low concentration without cytotoxicity on untransduced cells and have not reappeared at least for 5 months. Furthermore, the γc chain transduced cells were still sensitive to GCV after five months. These results demonstrated the efficacy of the suicide gene therapy although further in vivo studies are required to assess feasibility of this approach in clinical trial.

Keywords: Gene therapy; X-SCID; γc chain; Retroviral vector; Suicide gene; HSVtk

X-linked severe combined immunodeficiency (X-SCID) is a disease characterized by the absence of humoral and cellular immunity due to mutations in the gene encoding the common gamma (γc) chain [1,2]. The γc chain is an essential component of the cytokine receptors for the interleukins 2, 4, 7, 9, 15, and 21 [3–5]. Cytokine signals through the γc chain are required for differentiation and proliferation of T cells, NK cells, and functional B cells, and abnormal signaling through the receptor results in the immunological defects. The X-SCID patients are at risk of severe, recurrent infections unless stem cell transplantation is performed [2]. The overall survival of X-SCID patients who received allogeneic bone marrow transplantation (BMT) from an HLA-identical sibling is more than 90% [6]. However, if a patient does not have a well-matched family donor, survival rate is ranging from 50% to 78%, owing to potential risks of severe complications such as graft-versus-host disease (GVHD), graft rejection, and slow T cell development [7,8].

Clinical trial of X-SCID gene therapy was initiated in France from 1999 [9]. Ten patients with typical X-SCID were treated between 1999 and 2002, and T cell reconstitution was observed in 9 out of 10 cases resulting in clinical benefits such as resolution of ongoing infections and leaving their protected environments [2,10]. In addition, most of the patients showed NK cell recovery and immunoglobulin production [2,9,10]. Similar results were observed in four patients treated by retrovirus-based gene therapy in England [11]. Overall, gene therapy is very efficient in correcting immunodeficiency.

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However, clonal T cell proliferation occurred in three out of eleven patients treated in France [12,13]. The primary cause was insertion of the provirus within the *LMO2* locus, leading to aberrant expression of LMO-2 in mature T cells and thereby uncontrolled proliferation. Aberrant LMO-2 expression is associated with a form of T cell acute leukemia in childhood [14]. In addition, a retrovirus-induced mouse lymphoma that contained insertions in both *LMO2* and *IL2RG* was reported [15]. Recently, myeloid sarcoma was observed in a non-human primate associated with a retrovirus vector insertion [16]. Although retrovirus-based gene therapy has shown promising results in correcting immunodeficiency, the risk and consequences of insertional mutagenesis by retroviral vector must be taken into consideration. One of the alternatives is to incorporate a suicide gene into a therapeutic retroviral vector to eliminate the lymphoproliferative disease due to insertional mutagenesis. The Herpes simplex virus thymidine kinase (HSVtk) gene in combination with ganciclovir (GCV) has been used in clinical trials to control T cell mediated GVHD after hematopoietic stem cell transplantation [17–19] and cancer gene therapies [20–22]. In this study, we demonstrated functional expression of the γc chain after the bicistronic retrovirus-mediated gene transfer and importantly, the selective elimination of γc chain positive cells after GCV treatment for a period of 5 months.

Materials and methods

X-SCID patients' B lines. Peripheral blood mononuclear cells (PBMCs) were obtained from two X-SCID patients with written informed consent. The cells were transformed with Epstein-Barr virus (EBV)-containing supernatant to establish B-cell lines as previously described [23]. Both patients had typical clinical presentation of X-SCID, and the patient 2 was described previously [24]. The laboratory findings of patient 1 revealed absence of own T cells and NK cells, and profound hypogammaglobulinemia despite normal numbers of B cells. Mutations in the γc chain of the patients are shown in Table 1. Sequencing analysis of the γc chain was performed according to the standard method [25].

Retrovirus vector and producing cell lines. The entire human γc chain cDNA was generated by polymerase chain reaction (PCR) from γc chain expression vector, pSRG1 [4]. The upstream primer 5′-CATTAGCGGC CGCGAAGAGCAAGCGCCATGTTG-3′ and the downstream primer 5′-GCCGCGGATCCGATGATTATCAACAGAAACT-3′ contain *Not*I and *Bam*HI sites, respectively. The HSVtk cDNA was generated by PCR from a retroviral vector SFCMM3 encompassing an HSVtk gene [18]. The upstream primer 5′-GAATTCGTTGATCCGCCGCCACCATGG-3′ and the downstream primer 5′-GAAGCGCATCGATCCTCGAGTTAAT TCTCAG-3′ contain *Nco*I and *Cla*I sites, respectively. The backbone vector pDΔNsamIRESEGFP, with intact splice donor and splice acceptor sequences for generation of subgenomic mRNA, has a long-terminal repeat (LTR) derived from Murine stem cell virus (MSCV). The enhanced green fluorescent protein (EGFP) cDNA is located downstream of the internal ribosome entry site (IRES) of the backbone vector. The EGFP fragment was excised from pDΔNsamIRESEGFP using *Nco*I and *Cla*I sites, and replaced by the HSVtk gene. The γc chain cDNA was inserted into the vector using *Not*I and *Bam*HI sites. MSCV LTR promoter of the resultant bicistronic vector, pD-γc/TK, drives the expression of both the γc chain and HSVtk genes simultaneously. The pD-γc/TK vector was co-transfected with VSV-G plasmid encoding the Vesicular stomatitis virus G glycoprotein into 293gp cells which were stably expressing transfected MoMLV *gag* and *pol* genes [26]. Conditioned medium from the transfected 293gp cells was collected, filtered, and used for transduction of the gibbon ape leukemia virus (GALV)-pseudotyped MoMLV producing cell line PG13 [27]. After expansion of the transduced PG13 cells for 96 h, the cells were stained with a PE-conjugated anti-human γc chain monoclonal antibody, TUGh4 (BD Bioscience, San Diego, CA) and analyzed by FACSCalibur (Becton Dickinson, San Jose, CA) as previously described [25]. The γc chain positive cells were enriched by magnetic sorting using anti-PE micro beads and Auto-MACS (Miltenyi Biotec, Germany).

Transduction of EBV-transformed BCLs from X-SCID patients. EBV-transformed BCLs established from two typical X-SCID patients were infected by co-culturing with irradiated (30 Gy) producer cells in the presence of 8 μg/ml protamine sulfate. Cells were infected for two cycles each lasting 48 h.

IL-2 induced Jak3 phosphorylation. The EBV-transformed BCLs were cultured in serum-free RPMI 1640 for 24 h in humidified 5% CO_2 at 37 °C. After washing, the cells were stimulated by addition of 100 ng/ml IL-2 for 15 min at 37 °C. Stimulated cells were centrifuged and then lysed in ice-cold buffer (50 mM Tris–HCl, 10 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate decahydrate, 10 mM sodium molybdate, 2 mM sodium orthovanadate, 2 mM PMSF, and 0.1% aprotinin). For Jak3 phosphorylation analysis, lysates were clarified by centrifugation and immunoprecipitated with a polyclonal antibody against Jak3 protein (Santa Cruz Biotechnology, Santa Cruz, CA). Bound proteins were resolved by SDS–PAGE and transferred to nitrocellulose membrane. The membrane was blotted with a monoclonal antibody against phosphotyrosine, 4G10 (Upstate Biotechnology, Lake Placid, NY). Bound primary antibodies were detected by the horseradish peroxidase-conjugated anti-mouse IgG antibody followed by enhanced chemiluminescence (ECL) detection reagent.

Sensitivity of transduced cells to GCV in vitro. The γc chain transduced BCLs were plated in 48-well plates at a density of 1×10^5 cells/well. The cells were treated with 0.1, 1.0, 10, 25, 50, and 100 μM GCV for 14 days. Control cells (without GCV) were grown under the same condition. Percentages of the γc chain positive cells were assessed by flow cytometric analysis, every two days for 18 days after addition of GCV.

Cell proliferation and viability. Non-transduced BCLs were treated with various concentrations (1.0, 10, 25, 50, and 100 μM) of GCV. The total cell numbers were counted each day for 7 days. Cell viability was evaluated by trypan blue staining simultaneously. The percentages of viable cells were average viability of three independent experiments' scoring of 100 cells.

Table 1
Characterization of γc mutations in patients

Patient No.	Mutated exon	Nucleotide alteration	Protein alteration	TUGh4 staining
1	3	336T → C	S108P	—
2	6	868G → A	R285Q	—

Results

Retrovirus vector mediated expression of γc chain

To generate the recombinant retroviral vector pD-γc/TK, a full-length human γc chain cDNA and the HSVtk gene were cloned into *Not*I/*Bam*HI and *Nco*I/*Cla*I sites of pDΔNsamIRESEGFP, respectively (Fig. 1). pD-γc/TK was co-transfected with VSV-G plasmid encoding the vesicular stomatitis virus G glycoprotein into 293gp cells. Subsequently, 293gp-derived retroviral vector supernatants were used to transduce PG13 GALV-pseudotyped packaging cells. Four days later, the γc chain positive PG13 cells

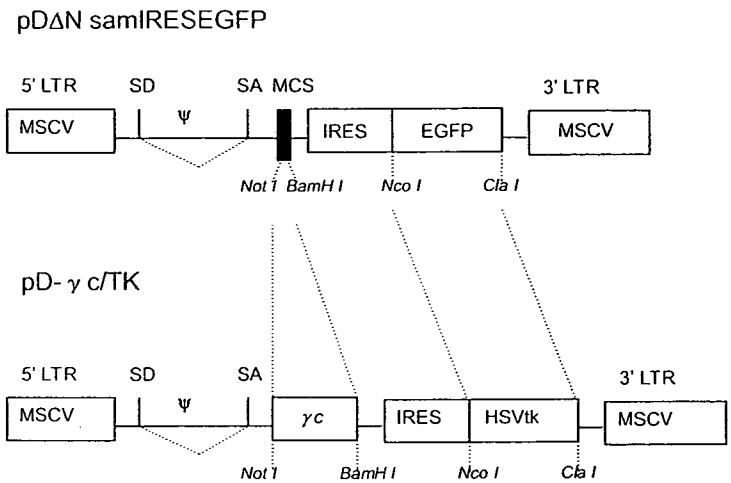

Fig. 1. Schematic representation of the pD-γc/TK vector. The backbone vector pDΔNsamIRESEGFP contains MSCV LTR with intact splice donor and splice acceptor sequences. The γc chain cDNA was inserted into multiple cloning sites (MCS) and EGFP fragment was replaced with the HSVtk gene. Characteristic sequences present in each vector are abbreviated as follows: ψ, packaging signal; MCS, multiple cloning sites; SD, splice donor; and SA, splice acceptor.

were sorted by Auto-MACS, and more than 90% of the sorted cells expressed the γc chain on the cell surface (data not shown). The resultant virus-producing PG13 cells were co-cultured with BCLs established from two typical X-SCID patients (P1 and P2). Clinical profiles and mutational analysis of the X-SCID patients are described in

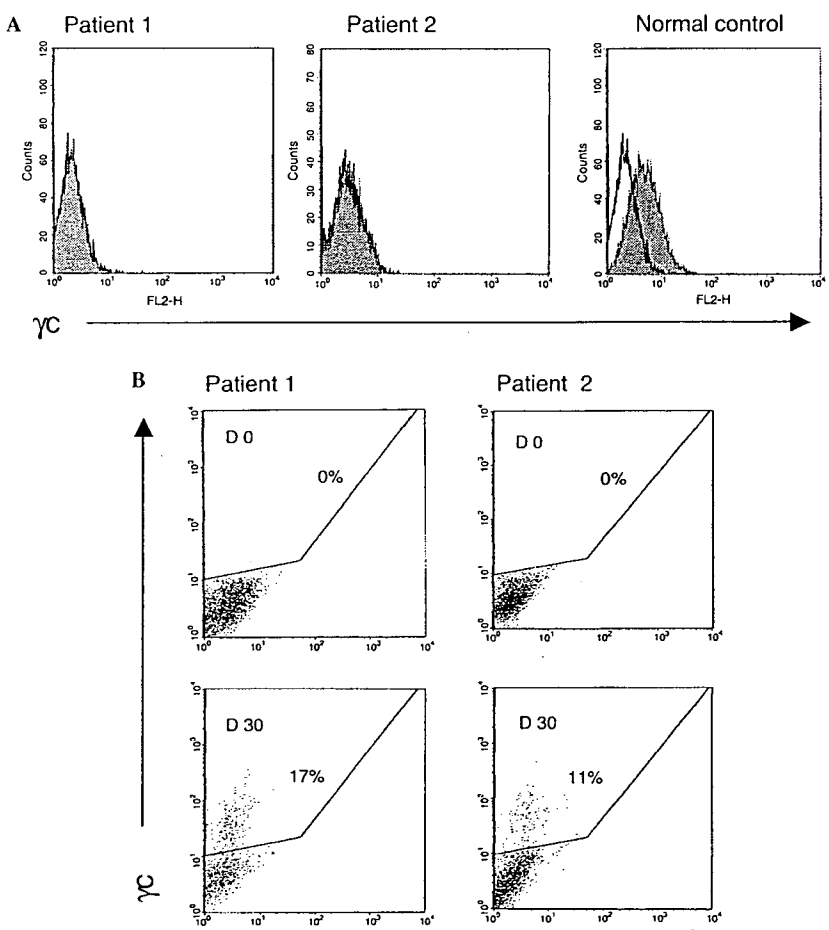

Fig. 2. Flow cytometric analysis of the γc chain expression on BCLs. (A) The γc chain expression profiles of the X-SCID BCLs and a normal control. (B) Expression of the γc chain was analyzed in transduced X-SCID BCLs immediately before (D0) and 30 days later (D30) after transduction.

Materials and methods, and Table 1. Before transduction, the γc chain was not detected on the cell surface of the X-SCID BCLs by flow cytometric analysis (Fig. 2A). One month after transduction, 17% and 11% of the X-SCID BCLs from P1 and P2 expressed the γc chain, respectively (Fig. 2B). Expression of the γc chain on the transduced BCLs was stable for more than five months (data not shown).

Functional analysis of the reconstituted γc chain

To determine whether the reconstituted γc chain could mediate intracellular signals, we examined tyrosine phosphorylation of Jak3 in response to IL-2 stimulation. Jak3 is the only tyrosine kinase known to directly associate with the γc chain and transduces signals through its phosphorylation [28,29]. Because strong constitutive tyrosine phosphorylation of Jak3 was observed in control EBV-transformed BCLs when cultured with 10% FCS, Jak3 tyrosine phosphorylation was analyzed in an optimized serum-deprived condition [30]. Under this condition, tyrosine phosphorylation of Jak3 was obvious by IL-2 stimulation in control BCLs and the γc transduced X-SCID BCLs, but not in the untransduced parental X-SCID BCLs (Fig. 3). These results demonstrated a functional reconstitution of the γc chain in X-SCID BCLs.

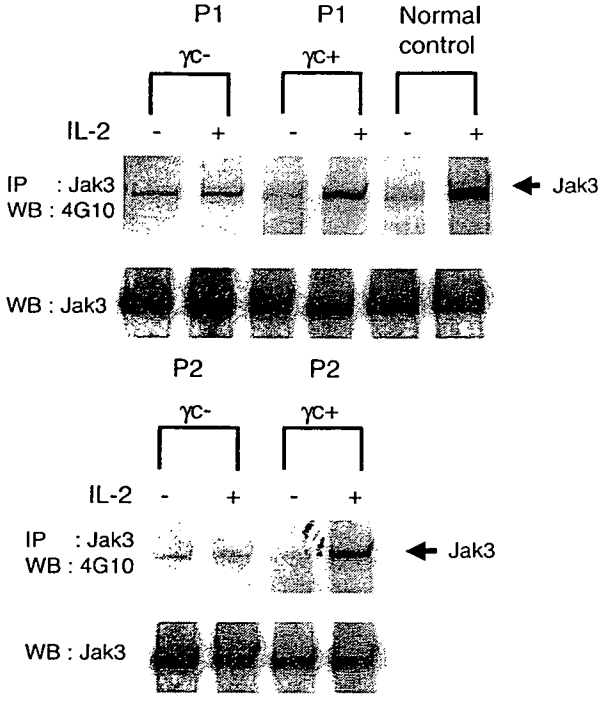

Fig. 3. Analyses of IL-2-induced Jak3 phosphorylation. The transduced and untransduced X-SCID BCLs, and a normal control were stimulated with 100 ng/ml IL-2 for 15 min, and immunoprecipitated with the Jak3 polyclonal antibody. The resultants were resolved by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antibody, 4G10.

Sensitivity of γc transduced BCLs to GCV treatment in vitro

The γc chain transduced X-SCID BCLs were cultured with various concentrations of GCV (0.1, 1.0, 10, 25, 50, and 100 μM) for 14 days. Every two days, aliquots of cells were stained with the anti-human γc chain mAb, TUGh4, to evaluate ratios of the γc chain expressing cells by flow cytometer. The γc chain positive cells were significantly decreased after 4-day culture with 10, 25, 50, and 100 μM GCV, and could not be detected within 14 days (Fig. 4A). In contrast, percentage of γc chain expressing cells in untreated BCLs was constant (data not shown). The γc expressing cells have not been detected at least for five months after cessation of GCV (Fig. 4B).

Toxicity of GCV to the non-transduced BCLs

To evaluate toxicity of GCV to the X-SCID BCLs, total cell numbers and viability were calculated each day after starting culture with various concentrations of GCV. Cell proliferation and viability of X-SCID BCLs were not affected in concentrations up to 25 μM of GCV. Whereas 50 μM GCV showed toxic effect on cell proliferation but not on cell viability. Both cell proliferation and viability were affected by 100 μM GCV (Fig. 5). Therefore, a range of GCV concentrations between 10 and 25 μM were preferable for this in vitro experiment because GCV had a suicide effect in transduced LCLs without any sign of cytotoxicity in untransduced cells.

Discussion

Retrovirus-based X-SCID gene therapy clinical trials were carried out in France and England [2,9–11]. The outcome was promising with successful immunologic reconstitution and clinical benefits in most of the patients. However, uncontrolled clonal proliferation of T cells occurred in three out of eleven patients treated in France about three years after gene therapy [12,13]. The T cell lymphoproliferation was driven by insertion of the retrovirus vector into *LMO2* locus, thereby triggering continuous and aberrant LMO-2 expression. Retroviral vectors are known to integrate into genome randomly and similar incidences of cancers due to insertional mutagenesis have been reported [16,31]. Therefore, new strategies designed to minimize the consequences of such events are needed. In this study, we have constructed a novel bicistronic vector carrying both therapeutic and suicide gene that enable selective elimination of uncontrolled proliferation of transduced lymphocytes.

The HSVtk/GCV approach has been used in several clinical trials. For example, HSVtk transduced donor T cells were infused to relapsed leukemic patient after allogeneic SCT to obtain maximum GVL effect and GCV administration. This method allows eradication of the gene modified T cells in case of GVHD [17–19]. In this trial,

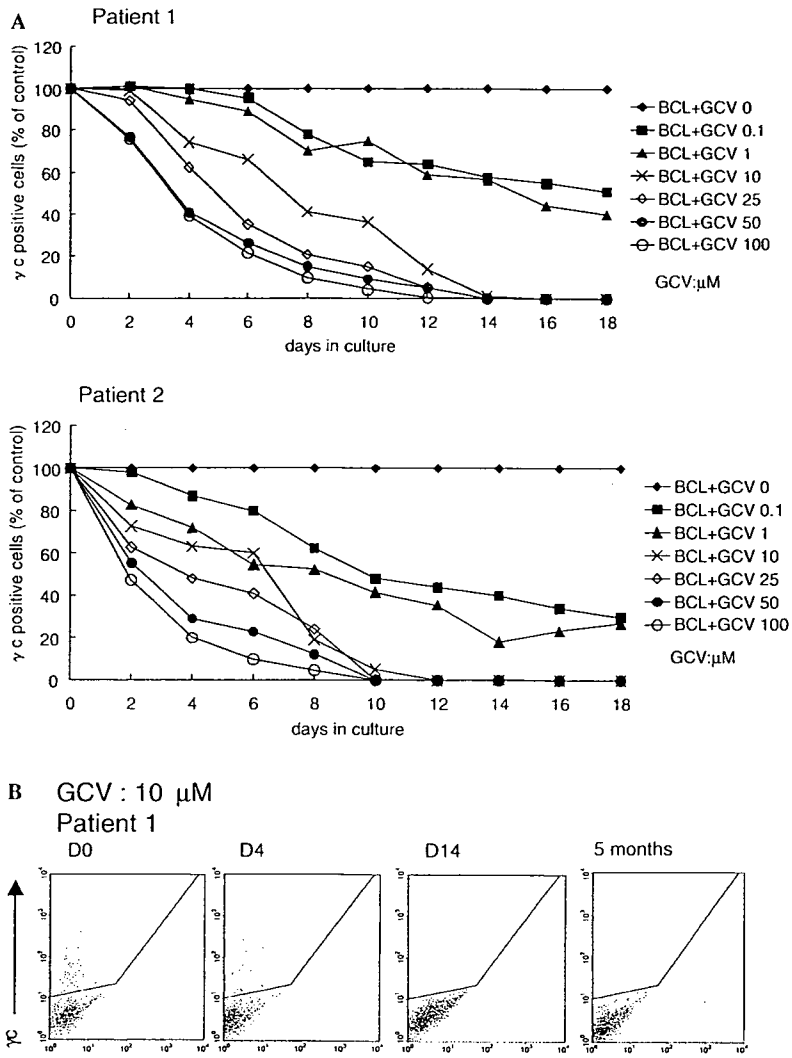

Fig. 4. Sensitivity of transduced cells to GCV treatment in vitro. (A) The γc transduced BCLs were cultured with various concentrations (0.1, 1.0, 10, 25, 50, and 100 μM) of GCV for 14 days. The transduced BCLs were grown without GCV as a control. The ratios of the γc chain expressing BCLs were expressed as relative percentages to referring control cells. (B) Representative profiles of the γc chain expression on the transduced X-SCID BCLs with 10 μM GCV.

the HSVtk transduced T cells have survived for 10 years after gene therapy and are still sensitive to GCV [32]. In this study, we applied the HSVtk/GCV system to X-SCID gene therapy as a fail-safe measure in case uncontrolled proliferation of gene transduced lymphocytes caused by insertional mutagenesis occurred. After transduction of X-SCID BCLs with the retrovirus vector carrying the γc chain and HSVtk genes, the transduced cells were treated with various concentrations of GCV in vitro. All transduced cells were eliminated at 10 μM GCV for 14 days. Concentrations of GCV required for elimination of HSVtk transduced cells were reported to vary among different cell lines [33]. Bonnekoh et al. [34] reported that 40 μM GCV is necessary to eliminate all the HSVtk transduced melanoma cells, whereas Verzeletti et al. [18] showed that HSVtk transduced lymphocytes were sensitive and completely eliminated at 1 μM GCV in vitro. Based on the studies mentioned above, the concentration of GCV is dependent upon the nature of cell line used. Therefore, we speculated that relatively higher concentration of GCV (10 μM) is required in this experiment due to the relatively higher GCV resistance of patients' B-cell lines compared with normal lymphocytes.

It is also important to evaluate cytotoxicity of GCV because it has severe side effects such as myelosuppression, immunosuppression, and renal failure. Crumpacker [35] reported pharmacokinetic analysis of intravenous administration of GCV. A typical clinical use (10 mg of GCV per kilogram per day in two divided doses) results in a peak serum concentration of 40 μM and a trough concentration of 3 μM. We have determined a range of GCV that can eliminate transduced BCLs without causing cytotoxicity in vitro. The determined range of GCV in our study was within the peak and trough level. If target cells were clon-

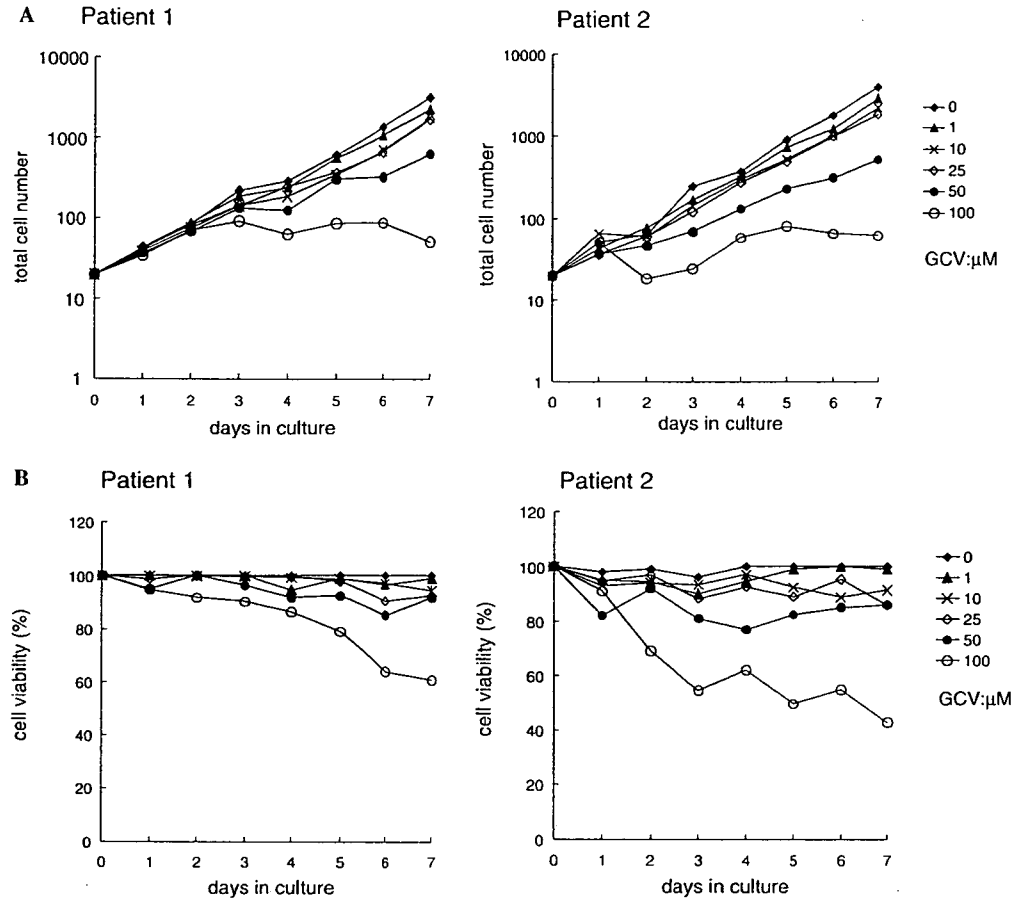

Fig. 5. Cell proliferation and viability. Cytotoxicity of GCV was evaluated by cell proliferation and viability in non-transduced BCLs. Cell proliferation and viability were calculated every day for 7 days. (A) Proliferation was estimated by plotting of total number of cells each day. (B) The percentage of viable cells was calculated by trypan blue exclusion.

ally expanded T lymphocytes due to retroviral insertion mutagenesis, lower concentrations of GCV would be effective because previous report demonstrated that HSVtk transduced lymphocytes were completely eliminated at 1 μM of GCV in vitro [18].

Escape mechanisms of suicide gene such as chromosomal deletion and silencing of HSVtk gene can influence the outcome of GCV treatment [36]. Silencing of HSVtk gene is a serious problem because it results in continuous cell proliferation even after GCV treatment. To overcome gene silencing, the MSCV LTR-based vector used in this experiment is engineered to resist de novo methylation [37–39]. The LTR differs from the MoMLV LTR by nine point mutations and deletion of one of the 75-bp repeats in the U3 region [37]. These changes enhance transcriptional activation and prevent transcriptional suppression. In addition, to ensure the co-expression of the γc chain and HSVtk genes, IRES element was employed in the bicistronic retroviral vector because it permits translation of two genes simultaneously from a single transcriptional unit. With the modified MSCV LTR-based bicistronic vector, pD-γc/TK, the transduced X-SCID BCLs expressed the γc chain stably and remained sensitive to GCV for more than five months.

Host immune response might be generated against the HSVtk suicide gene, leading to elimination of the transduced cells [18]. However, the possibility that HSVtk would induce immune reaction after gene therapy is less likely due to profound impairment of cellular and humoral immunity in X-SCID patients. Furthermore, HSVtk is recognized as a self-antigen because T cell progenitor cells had already expressed the gene prior to thymus migration.

One might argue that all stem cells carrying the γc chain gene will be eliminated with the HSVtk/GCV approach. However, we hypothesized that a small proportion of resting hematopoietic stem cells can escape from GCV treatment because only proliferating cells are sensitive to GCV. The escaped hematopoietic stem cells would reconstitute the immune system, whereas the actively proliferating cells caused by insertional mutagenesis are killed after GCV treatment.

The result of the present study using a novel therapeutic bicistronic retrovirus vector carrying both causative and suicide gene is encouraging. Currently, we are testing the efficacy and safety feature of this bicistronic retroviral vector in an X-SCID murine model.

Acknowledgments

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HUMAN MUTATION 27(4), 370–375, 2006

RESEARCH ARTICLE

A Second-Site Mutation in the Initiation Codon of *WAS* (*WASP*) Results in Expansion of Subsets of Lymphocytes in an Wiskott-Aldrich Syndrome Patient

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Wiskott-Aldrich syndrome (WAS) is caused by mutations in the gene encoding WAS protein (WASP). Recently, somatic mosaicism caused by reversions or second-site mutations has been reported in some inherited disorders including WAS. In this article, we describe somatic mosaicism in a 15-year-old WAS patient due to a second-hit mutation in the initiation codon. The patient originally had a single-base deletion (c.11delG; p.G4fsX40) in the *WAS* (*WASP*) gene, which resulted in a frameshift and abrogated protein expression. Subsequently, a fraction of T and natural killer (NK) cells expressed a smaller WASP, which binds to its cellular partner WASP-interacting protein (WIP). The T and NK cells were found to have an additional mutation in the initiation codon (c.1A>T; p.M1_P5del). The results strongly suggest that the smaller WASP is translated from the second ATG downstream of the original mutation, and not only T cells but also NK cells carrying the second mutation acquired a growth advantage over WASP negative counterparts. To our knowledge, this is the first report describing somatic mosaicism due to a second-site mutation in the initiation codon of any inherited disorders. Hum Mutat 27(4), 370–375, 2006.

INTRODUCTION

The Wiskott-Aldrich syndrome (WAS; MIM#s 301000, 277970) is an X-linked recessive immunodeficiency characterized by thrombocytopenia, eczema, and recurrent infections, and increased susceptibility to lymphoid malignancies and autoimmune disorders [Aldrich et al., 1954]. WASP, the product of the gene mutated in *WAS*, is expressed in all hematopoietic cells including $CD34^+$ stem cells, platelets, lymphocytes, neutrophils, macrophages, and dendritic cells [Derry et al., 1994; Kawai et al., 2002; Wengler et al., 1995]. It plays an important role in signal transduction pathway for cell growth and cytoskeletal organization through its interaction with many cellular partners such as WASP interacting protein (WIP), Grb2, Btk, Itk, Nck, and Arp2/3 complex [Badour et al., 2004; Ramesh et al., 1997]. Among them, WIP binds to the NH_2-terminal EVH1 (WH1) domain of WASP, where most of the missense mutations of WAS patients are located [Jin et al., 2004]. In addition, interaction of WIP with WASP has been demonstrated to be involved in regulation of T cell activation [Badour et al., 2004].

Recently, somatic mosaicism has been reported in growing numbers of inherited disorders including WAS [Ariga et al., 1998, 2001; Hirschhorn, 2003; Holzelova et al., 2004; Jin et al., 2004; Lutskiy et al. 2005; Wada et al., 2001, 2002, 2003]. Somatic mosaicism due to reversions or second-site mutations resulting in compensatory functional changes is caused by several different mechanisms. The mechanisms include intragenic recombination, mitotic gene conversion, second site compensating mutation, DNA slippage, and site-specific reversion of a mutated nucleotide to normal. Functional recovery by somatic mosaicism becomes evident when mutation corrected cells have a selective growth advantage. This rare event may modify the clinical phenotype of the disease, leading to atypical and unexpectedly mild presentations [Hirschhorn et al., 1996; Lutskiy et al., 2005; Pittis et al., 2004; Stephan et al., 1996; Wada et al., 2001]. In this article, we describe the somatic mosaicism of a 15-year-old WAS patient due to a second-site mutation in the initiation codon. The patient originally had a single-base deletion (c.11delG; p.G4fsX40) in the *WAS* gene, which results in a frameshift and abrogates protein expression [Sasahara et al., 2000, 2001]. Seven years after

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chemotherapy for Hodgkin disease, WASP expression was detectable in a fraction of T and natural killer (NK) cells carrying the second-site mutation (c.1A>T; p.M1_P5del). Thus, it was suggested that cells carrying the second-site mutation had expanded due to selective growth advantage over the WASP-negative cells.

MATERIALS AND METHODS

Case Report

The patient was a 15-year-old male without a clear family history. Thrombocytopenia was noticed at 1 month of age and thereafter, eczema and recurrent infections were remarkable. At 8 years of age, he had complications and persistent cough due to pulmonary hilar lymph node swelling. From the result of hilar lymph node biopsy, he was diagnosed with Hodgkin disease and received chemotherapy and local radiotherapy [Sasahara et al., 2000, 2001]. The patient has been in complete remission since then. His platelet count was in the range of 6,000–15,000/μl. The serum immunoglobulin (Ig) was as follows: IgG 1,466 mg/dl; IgA 239 mg/dl, and IgM 25 mg/dl; the lymphocyte profile was CD3 70.6%, CD4 22.4%, CD8 42.8%, CD19 15.9%, and CD56/16 5.1%. In recent years, episodes of respiratory infections were gradually reduced, although severe eczema and thrombocytopenia persisted.

Cell Preparations

Peripheral blood mononuclear cells (PBMCs) from heparinized blood samples were isolated by Ficoll-Paque™ (Amersham Pharmacia, Uppsala, Sweden) density centrifugation. The cells were fractionated into $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells by positive magnetic immunoselection using automated magnetic cell sorting (autoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously [Kumaki et al., 2001]. Each fraction contained 96.5% $CD4^+$ cells, 91.5% $CD8^+$ cells, 82.5% $CD19^+$ cells, and 83.3% $CD56^+$ cells (data not shown). The Epstein-Barr virus (EBV)–transformed B cell lines and Cos7 cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640) and Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco BRL, Rockville, MD) at 37°C in 5% CO_2, respectively.

Flow Cytometric Analysis of WASP

Intracellular staining with a mouse anti-human WASP monoclonal antibody (mAb), 5A5, was performed as previously described [Kawai et al., 2002]. Briefly, cells were fixed in 4% paraformaldehyde and then permeabilized in 0.1% Triton X-100. Subsequently, these cells were reacted with 10 μg/ml of 5A5. The stained cells were immediately analyzed on a FACSCalibur (Beckton Dickinson, Franklin Lakes, NJ) flow cytometer. WASP fluorescence was assayed on $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ lymphocytes selected by fluorescent mode vs. side scattered light (SSC) gate.

Sequencing Analysis of Genomic DNA

Blood samples were collected from the patient and his mother with informed consent after approval of the Tohoku University Ethics Committee. Genomic DNA was isolated from fractionated PBMCs or the EBV-transformed B cell lines using the QIAamp Blood Kit (Qiagen, Chatsworth, CA). The *WAS* gene was amplified using primer pairs encompassing each exon–intron boundary [Sasahara et al., 2000]. The fragment including the *WAS* exon 1, in which the patient's mutation was located, was PCR amplified with a primer pair of W-2 and W-496c (Table 1) [Zhu et al., 1995]. Sequencing was performed directly on the PCR products as previously described [Sasahara et al., 2000].

Analysis of T-Cell Antigen Receptor Vβ Repertoire Diversities

To determine T-cell antigen receptor (TCR) diversities, we examined TCR Vβ repertoire distributions by flow cytometry and CDR3 size distribution by an automated sequencer and GeneScan software, as described previously [Wada et al., 2001, 2005]. Peripheral blood samples were stained with appropriate phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) with specificity for TCR Vβ 1-24 (Beckman Coulter, Fullerton, CA), and analyzed with a FACSCalibur flow cytometer. TCR Vβ expression was represented as a percentage of $CD4^+$ or $CD8^+$ cells for each family. Next, total RNA was extracted from $CD4^+$ and $CD8^+$ T cells, and first-strand cDNA was generated from 2 μg of total RNA with random hexanucleotides and RAV-2 reverse transcriptase (Takara Bio Inc., Shiga, Japan). Each TCR Vβ fragment was amplified with one of the 24 Vβ-specific primers and a 6-fluorescein phosphoramidite (6-FAM)-labeled Cβ primer recognizing both Cβ1 and Cβ2. One microliter of 1:10 diluted PCR products was mixed with 12 μl of deionized formamide and 0.5 μl of size standard and heated at 95°C for 3 min. The size distribution of each fluorescent PCR product was determined by electrophoresis in the Performance Optimized Polymer 4 on the Applied Biosystems 310 automated sequencer, and data was analyzed by GeneScan software (Applied Biosystems, Foster City, CA).

Expression Plasmid Construction and Transfection

The wild-type WASP cDNA (a generous gift from Dr. J Derry; Immunex Corp, Seattle, WA) was cloned into an expression vector, pcDNA3 (pcDNA/WASP). Vectors containing the mutant cDNAs were generated by site-directed mutagenesis as described elsewhere [Kumaki et al., 2001]. Briefly, a pcDNA3 vector containing c.11delG mutation of WASP (pcDNA/WASPdelG) was constructed using a template vector pcDNA/WASP with a primer pair of dG-5′ and dG-3′ (Table 1). Next, pcDNA3 vector containing both c.1A>T and c.11delG mutations (pcDNA/WASP2m) were constructed using a template vector pcDNA/WASPdelG with a primer pair of A35T-5′ and A35T-3′ (Table 1). Sequences of the mutant cDNAs were confirmed by comparing with a reference cDNA sequence (GenBank accession: NM_000377). For the expression of WIP, pcDNA/WIP-GFP was used. The expression vectors were transiently transfected into Cos7 cells using FuGene™ 6 (Roche Diagnostics, Mannheim, Germany). WASP and WIP expression in Cos-7 cells were detected by immunoblotting using anti-WASP mAb 5A5, and mouse anti-GFP monoclonal antibody for WIP (Becton Dickinson), respectively. Immunoprecipitation and Western blotting were performed according to the standard procedures [Kumaki et al., 2001].

TABLE 1. Primers used for sequencing and mutant generation

Primer	Sequence (5′→3′)
dG-3′	CCCAATGGGAGGAAGGCCCCG
dG-5′(2)	CCCCACTCATGGTGCTTTCTC
W-2	GCTTCGCCAGAGAAGACAAG
W-496c	TCCACTTTGCCTCTGATTCC
A35T-5(3)	CTCTAGAGAAAGCACCTTGAGTGGGGCCCAATG
A35T-3(3)	CATTGGGCCCCACTCAAGGTGCTTTCTCTAGAG

RESULTS

Expression of WASP in a Fraction of the Patient's T and NK Cells

We first analyzed WASP expression in PBMCs from the patient by intracellular staining with an anti-WASP mAb, 5A5 [Kawai et al., 2002]. Expression of WASP was detected in about half of the $CD4^+$ T cells and most of the $CD8^+$ T cells, and in a quarter of the $CD56^+$ NK cells, but not in $CD19^+$ B cells and $CD14^+$ monocytes by flow cytometric analysis (Fig. 1; and data not shown). Absolute cell counts of WASP positive $CD4^+$, $CD8^+$, and $CD56^+$ cells were 254, 1,288, and 73/μl, respectively. To further characterize the WASP expressed in a fraction of T and NK cells, Western blot analysis was performed. The size of the WASP was slightly smaller than that of healthy controls (Fig. 2A).

Presence of a Second-Site Mutation in the Initiation Codon

The patient originally had a single-base deletion (c.11delG) in exon 1 of *WAS* gene as described previously [Sasahara et al., 2000, 2001]. This mutation resulted in a frameshift and abrogated protein expression. Seven years after chemotherapy for Hodgkin disease, expression of WASP was observed in a fraction of T and NK cells. To determine whether the original mutation was still presented in the WASP expressing cells, genomic sequencing was performed on magnetically sorted $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells. We demonstrated that the c.11delG mutation was present in all of the cells, whereas an additional mutation in the initiation codon (c.1A>T) was detectable only in $CD4^+$, $CD8^+$, and $CD56^+$ cells, but not in $CD19^+$ cells (data reviewed, but not shown). Genomic DNA from his mother contained only the original mutation but not the second-site mutation (data not shown). Therefore, we hypothesized that the de novo second-site c.1A>T point mutation mitigates the effects of the original mutation by initiating alternate translation downstream of the original mutation site (Fig. 3).

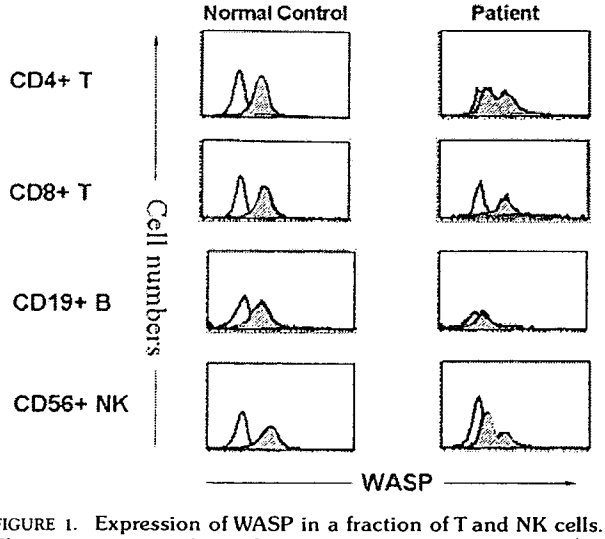

FIGURE 1. Expression of WASP in a fraction of T and NK cells. Flow cytometric analysis of WASP expression in gated $CD4^+$ T cells, $CD8^+$ T cells, $CD56^+$ NK cells, and $CD19^+$ B cells from a normal control (left panel) and the WAS patient (right panel). Histograms present the staining profile of intracytoplasmic fluorescence with isotype-matched control (empty histogram) and 5A5 mAb (hatched histogram). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

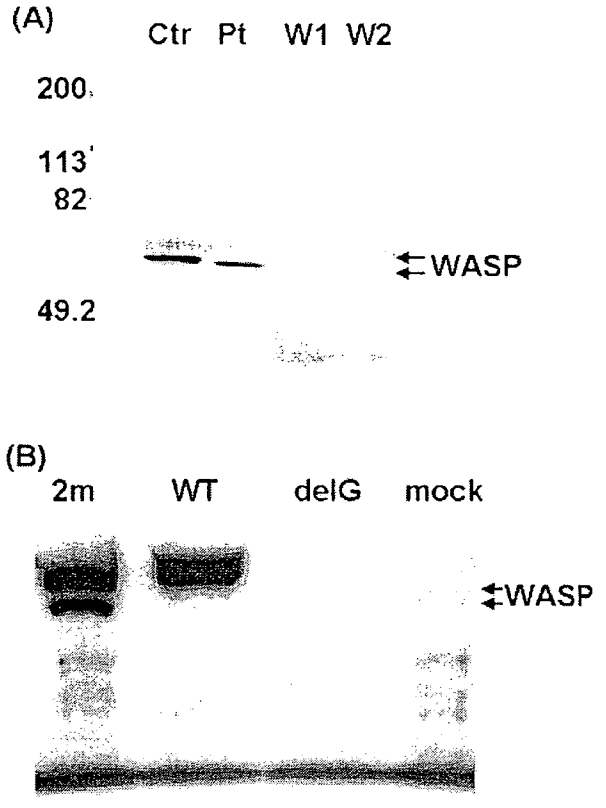

FIGURE 2. A smaller WASP is expressed in the patient's PBMCs and transfected cells. **A:** Expression of a smaller WASP in the patient's PBMCs. PBMCs from a normal control (Ctr), the patient (Pt), a WAS patient with a reversion in a limited T cell fraction (W1), and a typical WAS patient (W2) were lysed and separated by SDS-PAGE followed by immunoblotting with an anti-WASP mAb, 5A5. Arrow and arrowhead indicate the wild-type WASP and a smaller WASP, respectively. **B:** The second-site mutation resulted in expression of a smaller WASP in vitro. Cos7 cells were transfected with pcDNA/WASP (WT), pcDNA/WASPdelG (delG), pcDNA/WASP2m (2m), or the vector control (mock). Expression of WASP in the transfected cells was assessed by Western blot.

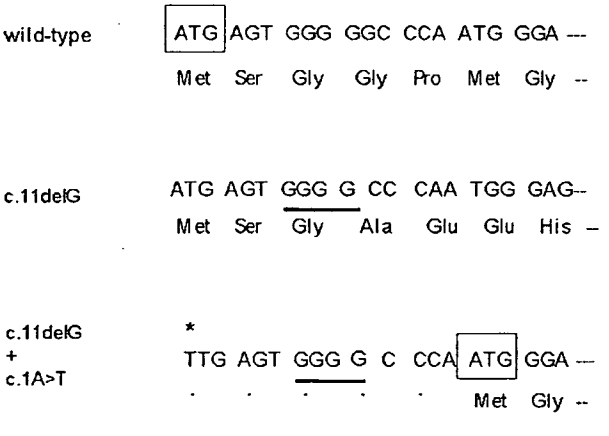

FIGURE 3. Schematic representation of the mutant WASP. A bar and an asterisk indicate the original mutation, c.11delG, and the second-site mutation, c.1A>T, respectively. The original mutation resulted in a frameshift after codon 4 and a premature stop codon at codon 44. The second-site mutation in the initiation codon alters translation initiation from the second ATG locates downstream of the original mutation.

Significant Skewing of TCR Vβ Usage

To determine clonal dominance or restriction within T lymphocyte populations, we analyzed by flow cytometry the entire TCR Vβ repertoire in $CD4^+$ and $CD8^+$ T cells of the patient and found significant skewing of TCR Vβ usage (data not shown). In addition, CDR3 distributions were markedly skewed and most of the repertoires were undetectable or with only few peaks (Fig. 4). These results demonstrated that the smaller WASP expressed in this patient's T cells is not enough to reconstitute normal TCRβ repertoire, although it confers growth advantage over WASP negative T cells.

The Mutant WASP Was Expressed and Associated With WIP

To prove that WASP produced due to the c.1A>T mutation was translated in cells, we examined the expression of WASP in transiently transfected Cos7 cells. As shown in Figure 2B, 5A5

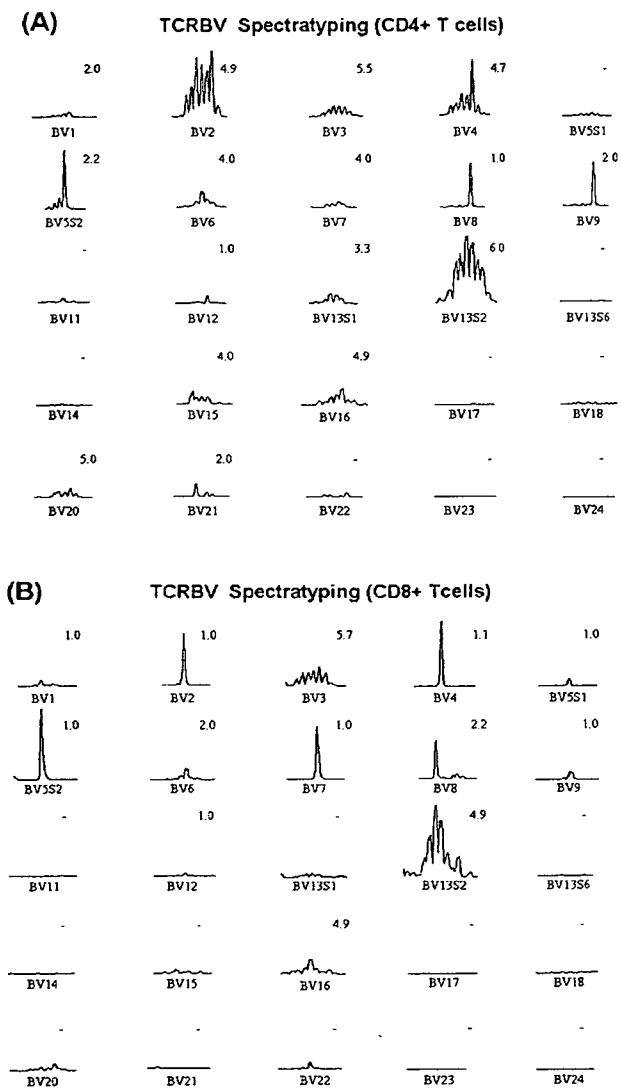

FIGURE 4. **Analysis of TCRβ gene rearrangement. CDR size distributions of TCRVβ subfamilies within $CD4^+$ (A) and $CD8^+$ (B) T cells. Each TCRVβ fragment was amplified from cDNA with 1 out of 24 Vβ-specific primers. The size distribution of PCR products was determined by an automated sequencer and GeneScan software.**

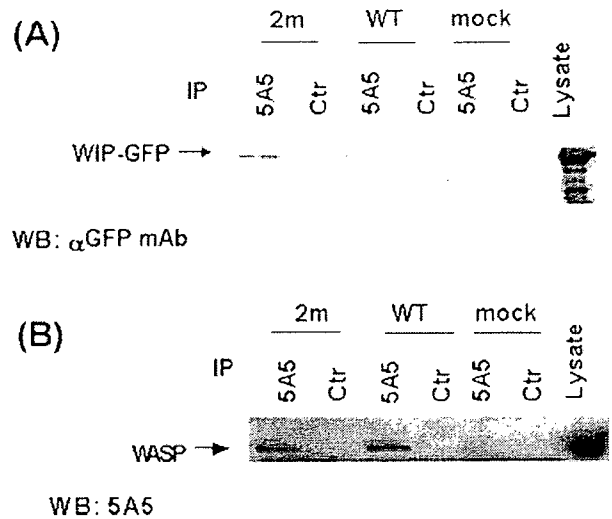

FIGURE 5. **Association of the WASP with WIP. Cos7 cells were transfected with pcDNA/WASP (WT), pcDNA/WASP2m (2m), or the vector control (mock) together with pcDNA/WIP-GFP. Lysates from transiently cotransfected Cos7 cells were immunoprecipitated with 5A5 or isotype-matched control (Ctr). The immunoprecipitates were sequentially immunoblotted with anti-GFP mAb (A) and anti-WASP mAb 5A5 (B).**

recognized the mutant WASP carrying the original and second-site mutations but not the original mutation alone. The expression level was comparable to that of the wild type, although the size was slightly smaller, demonstrating that the mutant WASP is efficiently translated and expressed. The result is compatible with the result of Western blot using the patient's PBMC (Fig. 2A). From deduced amino acid sequence of the mutant WASP carrying the original and second mutations, translated WASP lacks N-terminal 5 amino acids. In addition, a much smaller band was also detected in the mutant WASP but not in the wild-type WASP (Fig. 2B). The much smaller band might result from an alternative splicing or from the third ATG that locates 112 nucleotides downstream of the initiation codon.

Because WIP binds to the NH_2-terminal WH1 domain of WASP and plays an important role in regulation of T cell activation, association of the mutant WASP with WIP was evaluated. For this purpose, we transiently transfected Cos7 cells with a vector encoding the wild-type WASP or the mutant WASP carrying both mutations, together with WIP-GFP. The lysates of the transfected cells were immunoprecipitated with 5A5 or a control antibody followed by immunoblotting with anti-GFP antibody and 5A5. WIP was coprecipitated with the wild-type WASP and the mutant WASP carrying both mutations (Fig. 5A). In contrast, WIP was not detected in control mAb immunoprecipitates (Fig. 5A). The membrane was reprobed with 5A5 to confirm the presence of the transfected wild-type WASP or the mutant WASP (Fig. 5B). The results demonstrated that WIP efficiently interacted with not only the wild-type WASP but also the mutant WASP. Therefore, the smaller WASP found in our patient can associate with WIP, which is required for stabilization of WASP and TCR-induced activation.

DISCUSSION

Somatic revertant mosaicism resulting from back mutations or second-site mutations has been reported in growing numbers of genetic diseases [Ariga et al., 1998, 2001; Hirschhorn, 2003;

Holzelova et al., 2004; Jin et al., 2004; Lutskiy et al., 2005; Wada et al., 2001, 2002, 2003]. The mechanisms responsible for somatic reversion of inherited disorders include intragenic recombination, mitotic gene conversion, second site compensating mutation, DNA slippage, and site-specific reversion of a mutated nucleotide to normal by an unknown mechanism [Hirschhorn, 2003].

Here we describe a unique second-site mutation in a WAS patient; we hypothesized that the second-site mutation in the initiation codon (ATG to TTG) resulted in alternative translation initiation from the second ATG that is located downstream of the original single nucleotide deletion (c.11delG) [Kozak, 1987; Sasahara et al., 2001]. The second-site mutation was observed in a fraction of the patient's T and NK cells, leading to expression of a smaller WASP. Genomic DNA from his mother contained only the original mutation but not the second-site mutation. In addition, the second-site mutation was not detected in the patient's PBMCs prior to the onset of Hodgkin disease [Sasahara et al., 2001, 2002]. Thus, we conclude that the lymphocytes carrying the second-site mutation originated de novo. Taken together, it was suggested that a precursor cell carrying the second-site mutation acquired growth advantage over T and NK cells that did not express WASP, resulting in expansion of cells expressing the smaller WASP. In this context, it is noteworthy that CD4/CD8 ratio changed from 0.89 to 0.39 before and 3 years after the chemo- or radiotherapy, respectively, corresponding to $CD8^+$ cell numbers of from 500 to 600/μl to 1200–1400/μl. In contrast, $CD4^+$ and $CD56^+$ cells remained at similar levels of 500–700/μl and 200–300/μl, respectively. We speculate that the growth of $CD8^+$ cells carrying the second-site mutation is faster than that of the $CD4^+$ cells, resulting in a skewed CD4/8 ratio. The result is accordance with our observation that the mutant WASP was expressed by most of the $CD8^+$ cells, but by only half of $CD4^+$ cells. However, the patient's use of the TCR Vβ subfamilies was markedly skewed, similar to those of older WAS patients [Wada et al., 2005]. The oligoclonal pattern of TCRVβ usage of the patient suggests that the smaller WASP expressed is not sufficient to reconstitute normal TCRβ repertoire although it confers growth advantage over WASP negative T cells. Because the second-site mutation was detected only in samples after the chemo- or radiotherapies, it is possible that the mutation might be elicited by these therapies. Functional analysis of the smaller WASP demonstrated that it has a capacity to associate with WIP in vitro, which is responsible for WASP stability and for recruitment and activation of WASP in T cell signaling [Konno et al., 2004; Ramesh et al., 1997]. Our novel observation of somatic mosaicism suggests that WASP function is maintained even if a portion of N-terminal end of WASP is lacking. An earlier report described a mutation of the initiation codon that inactivates the first in-frame translation start site results in a mild form of disease with a residual protein suggesting a downstream ATG takes over and still produces a functional protein [Pittis et al., 2004]. This finding is compatible with our observation. Interestingly in our case, functional WASP is expressed both in T cells and NK cells. The result differs from previous studies, in which revertant WASP was expressed only in either T cells or NK cells [Konno et al., 2004; Lutskiy et al., 2005]. To our knowledge, this is the first report describing somatic mosaicism due to a second-site mutation in the initiation codon of inherited disorders.

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Human Tyrosine Kinase 2 Deficiency Reveals Its Requisite Roles in Multiple Cytokine Signals Involved in Innate and Acquired Immunity

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Summary

Tyrosine kinase 2 (Tyk2) is a nonreceptor tyrosine kinase that belongs to the Janus kinase (Jak) family. Here we identified a homozygous Tyk2 mutation in a patient who had been clinically diagnosed with hyper-IgE syndrome. This patient showed unusual susceptibility to various microorganisms including virus, fungi, and mycobacteria and suffered from atopic dermatitis with elevated serum IgE. The patient's cells displayed defects in multiple cytokine signaling pathways including those for type I interferon (IFN), interleukin (IL)-6, IL-10, IL-12, and IL-23. The cytokine signals were successfully restored by transducing the intact Tyk2 gene. Thus, the Tyk2 deficiency is likely to account for the patient's complex clinical manifestations, including the phenotype of impaired T helper 1 (Th1) differentiation and accelerated Th2 differentiation. This study identifies human Tyk2 deficiency and demonstrates that Tyk2 plays obligatory roles in multiple cytokine signals involved in innate and acquired immunity of humans, which differs substantially from Tyk2 function in mice.

Introduction

The majority of helical bundled cytokines transduce their signals via the Janus kinase (Jak) family kinases that are constitutively associated with cytokine receptors. Four mammalian Jak proteins have been described to date: Jak1, Jak2, Jak3, and Tyk2 (Ihle, 1995; Liu et al., 1998; O'Shea et al., 2002). When cytokines bind to their corresponding receptors expressed on the cell surface, receptor-associated Jaks are activated, and they in turn phosphorylate both cytokine receptors and neighboring Jaks. Phosphorylation of specific tyrosine motifs in the cytokine receptors provides docking sites for signal transducers and activators of transcription (STAT), and STATs recruited to the receptor are phosphorylated by the Jaks. Upon phosphorylation, STATs dimerize via their Src homology domain-2 (SH2) domains and translocate to the nucleus, where they activate different genes. Recent studies have demonstrated that several inhibitory mechanisms are involved in the regulation of the Jak-STAT pathway to prevent it from overactivation, including SOCS (suppressor of cytokine signaling) proteins that are induced through the Jak-STAT pathway to make a negative feedback loop (Alexander and Hilton, 2004).

The functional importance of the Jaks was first investigated in studies that used cell lines defective in cytokine signaling and was subsequently confirmed by the establishment of Jak-deficient mice (Velazquezet al., 1992; Muller et al., 1993; Neubauer et al., 1998; Nosaka et al., 1995; Parganas et al., 1998; Park et al., 1995; Rodig et al., 1998). *Jak1*$^{-/-}$ mice die perinatally and exhibit profound defects in lymphoid development (Rodig et al., 1998). Analyses of cells from *Jak1*$^{-/-}$ mice demonstrated that Jak1 is essential for signaling by type I and II IFN, in agreement with prior studies that used human mutant cell lines. *Jak2*$^{-/-}$ mice show embryonic lethality due to failure of erythropoiesis, while *Jak3*$^{-/-}$ mice suffer from severe combined immunodeficiency in accord with observations for human Jak3 deficiency (Macchi et al., 1995; Russell et al., 1995). These findings clearly demonstrated the nonredundant roles of Jak1, Jak2, and Jak3 in signaling through the corresponding cytokine receptors.

Tyk2 is the first member of the Jak kinase family to be isolated and was originally described as essential for type I IFN signaling in a human fibroblast cell line (Firmbach-Kraft et al., 1990; Velazquez et al., 1992). However, the establishment of Tyk2-deficient mice revealed that Tyk2 was not absolutely essential for type I IFN signaling in mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). *Tyk2*$^{-/-}$ mice displayed a lack of responsiveness to a small amount of IFNα, but a high concentration of IFNα could fully transduce its signals even in the absence of Tyk2. Although Tyk2 is a component of several other cytokine signaling pathways, including those of IL-6 and IL-12 (Bacon et al., 1995; Ihle, 1995; Stahl et al., 1994), in *Tyk2*$^{-/-}$ mice the IL-6-mediated signals are essentially normal and IL-12 signaling is partially impaired. Thus, the function of Tyk2 in mice appears to be compensated for in vivo, partly by other kinase(s), most likely other Jak(s). Whether the discrepancy in the stringency of Tyk2 requirement in cytokine signaling between the human cell lines and the mutant mice can be attributed to the difference between established cell lines and primary cells or the species difference as observed in several primary immunodeficiencies such as the Btk, BLNK, or λ5 deficiency has been unclear (Conley et al., 2000; Minegishi et al., 1998, 1999b).

In the present study, we investigated immunological abnormalities in a patient who had been clinically diagnosed with hyper-IgE syndrome (HIES). HIES is a unique primary immunodeficiency, characterized by recurrent skin abscesses, pneumonia, and highly elevated serum IgE (Grimbacher et al., 2005). Its etiology is totally unknown even though the autosomal dominant or recessive inheritance has been reported (Grimbacher et al., 1999a; Renner et al., 2004). The patient investigated in this study showed susceptibility to various microorganisms including virus, fungi, and mycobacteria and suffered from atopic dermatitis with elevated serum IgE. Peripheral blood cells from the patient showed almost complete defects in both IL-12 and IFNα/β signaling, prompting us to identify a homozygous mutation of Tyk2 in this patient, a signaling component shared by both cytokines. The patient's cells also displayed severe defects in IL-6 and IL-10 signaling, in contrast to previous observations in Tyk2-deficient mice. Transduction of the intact Tyk2 gene rescued the patient's cells from the cytokine signaling defects. Thus, the present study identified human Tyk2 deficiency as a type of primary immunodeficiency displaying the phenotype of the autosomal recessive HIES accompanied by susceptibility to intracellular bacterial infection and highlighted a unique and indispensable role played by Tyk2 in the innate and acquired immune responses in humans.

Results

Both IL-12 and IFNα Signaling Pathways Are Defective in T Cells from a Patient with HIES

We investigated, from an immunological point of view, a 22-year-old Japanese male who had been clinically

diagnosed as HIES. According to the HIES scoring system developed by the NIH group (Grimbacher et al., 1999b), the patient scored 48 points, similar to those with typical HIES (see Experimental Procedures). He showed complex clinical manifestations, including susceptibility to various types of microorganisms (virus, intracellular and extracellular bacteria, and fungi), atopic dermatitis-like skin inflammation starting from 1 month of age, and a high amount of serum IgE (2100 IU/ml). He has suffered recurrently from otitis media, sinusitis, pneumonias, skin abscesses, oral candidiasis, molluscum contagiosum, and herpes simplex infection in skin and mucosa. He also experienced an episode of Bacille Calmette-Guerin (BCG) infection at 22 months and nontyphi salmonella gastroenteritis at 15 years of age. The numbers of T, B, and NK cells in the peripheral blood were all within the normal range, and neutrophils showed normal functions in laboratory tests (data not shown). The patient's parents are consanguineous, indicating a possible recessive hereditary disorder. Because the patient experienced an infection with BCG, we first analyzed the genes encoding IL-12 p40, IL-12Rβ1, IFNGR1, IFNGR2, and STAT1, which have been identified as responsible genes for primary immunodeficiency with susceptibility to mycobacterial infection (Casanova and Abel, 2004). No mutation was detected in any of the corresponding cDNAs isolated from the patient (data not shown).

We next examined whether the patient had any defect in IL-12 signaling. When peripheral blood mononuclear cells (PBMCs) from the patient were stimulated with mitogens, IL-12 production was normal (data not shown). On the other hand, $CD4^+$ T cells isolated from the patient's PBMCs failed to produce detectable amounts of IFNγ when stimulated with IL-12 plus IL-18, a potent IFNγ inducer for T cells (Figure 1A). The failure could not be attributed to a total defect in the machinery for IFNγ production, because the patient's T cells produced considerable amounts of IFNγ in response to a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin, although the amount was a bit lower than normal (Figure 1A). Furthermore, IL-18 signaling appeared to be intact in the patient's T cells, as assessed by IκBα degradation in response to IL-18 stimulation (Figure 1B), implying a defect in the IL-12 signal. Indeed, the patient's T cells showed almost no response to IL-12 in contrast to those from a normal subject, as judged by the tyrosine phosphorylation of STAT4, a component of the IL-12 signaling pathway (Figure 1C). Interestingly, the patient's T cells also failed to respond to IFNα (Figure 1C). This finding was in sharp contrast to human IL-12Rβ1 deficiency, the only known human defect in IL-12 signaling, in which STAT4 phosphorylation is defective in response to IL-12, but its phosphorylation is intact in response to IFNα (Fieschi et al., 2004).

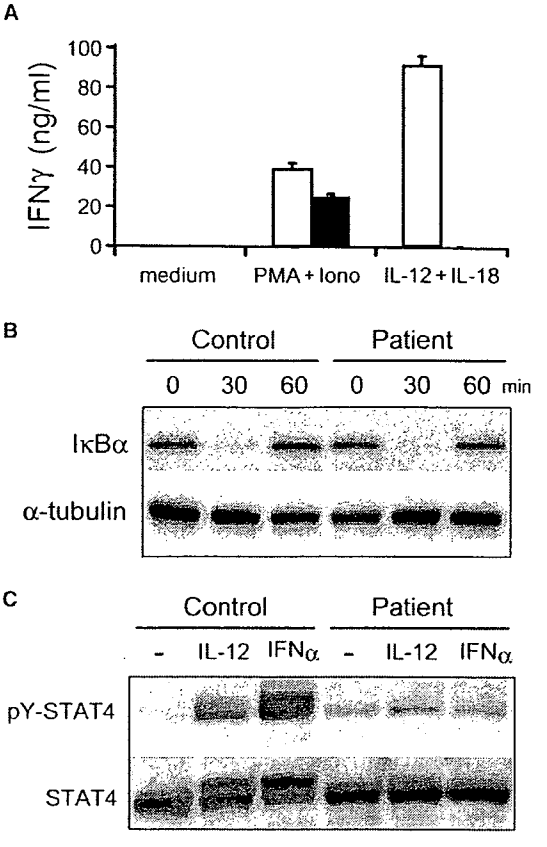

Figure 1. Impaired Responses to IL-12 and IFNα in a Patient with HIES

(A) The patient's T cells were defective in IFNγ production in response to IL-12 and IL-18. The concentration of IFNγ in culture supernatants was measured after $CD4^+$ T cells from a healthy control and the patient were cultured for 24 hr with medium alone, a combination of PMA and ionomycin, or a combination of IL-12 and IL-18. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations.

(B) The patient's T cells had intact IL-18 signaling. IκBα and α-tubulin proteins were detected by immunoblotting at the indicated time points after the T cells from a control subject and the patient were stimulated with IL-18.

(C) The patient's T cells had defects in both IL-12 and IFNα signaling. Tyrosine 694-phosphorylated and total STAT4 proteins were detected by immunoblotting after the T cells from a control subject and the patient were stimulated with IL-12 or IFNα for 15 min. Results are representative of at least three independent experiments.

A Homozygous Mutation in the Patient's Tyk2 Gene

The defect in both IL-12 and IFNα signaling strongly suggested an abnormality in one or more molecules involved in a signaling pathway shared by both cytokines. STAT4 was a good candidate; however, STAT4 was expressed at a normal amount in the patient's T cells (Figure 1C), and no sequence alteration was found in the coding region of the STAT4 gene (data not shown). The second candidate we investigated was Tyk2, because a mutant human cell line unresponsive to IFNα turned out be deficient in Tyk2 (Velazquez et al., 1992), even though Tyk2 appeared to be not absolutely essential for IL-12 and IFNα signals in mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). Sequencing of the patient's cDNA and genomic DNA coding for Tyk2 revealed a homozygous deletion of four nucleotides, GCTT, at nt 550–553, that was not found in 50 unrelated healthy individuals (Figures 2A and 2B). This deletion resulted in a frame-shift mutation from codon 70 to 89 and generated a premature stop codon at aa 90 in the 1187 amino acid-long Tyk2 protein (Figure 2B). The predicted truncation of Tyk2 deletes most of the protein, including