Besides immunodeficiency, half of ADA-SCID patients show metaphyseal dysplasia including costochondral abnormalities, which are identified by X-ray examination [Figure 2]. In some cases, elevations of liver enzymes are observed, and neurological abnormalities such as movement disorder, nystagmus, and sensorineural deafness are also although infrequent complications [28,29].

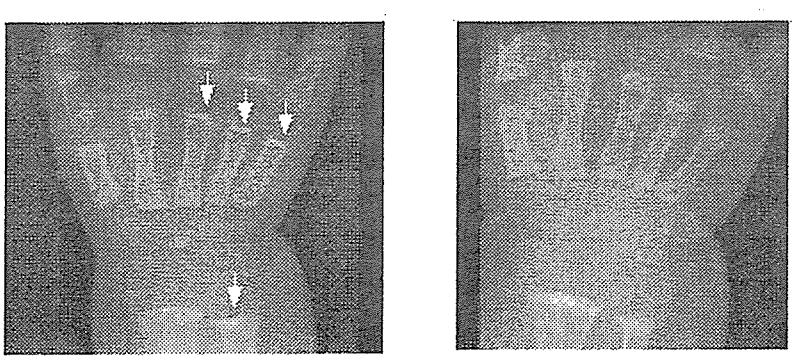
Table 2. ADA deficiency; range of phenotype.

1. SCID: Show profound lymphopenia at birth. Diagnosed under 1 year old.
2. Delayed onset: Show rapid clinical deterioration with diagnosis between one to 10 years.
3. Late onset: Show slow clinical deterioration with diagnosis after the first decade.
4. Partial deficiency: Show normal immune function. Red blood cells show a lack of ADA activity and elevated toxic metabolites.

Table 3. ADA mutant alleles grouped by activity observed following expression in *E. coli* Sϕ 3834.

Allele group	Mutations	ADA activity expressed percent of wild type (*range*)
0	Deletions, nonsense	0
I	H15D, H17P, G74V, G74D, A83D, R101L, R101Q, R101W, P104L, L107P, G140E, R149W, R156C, R211H, G216R, E217K, R235Q, S291L, A329V, E337del	0.015 ± 0.02 (*0.001 to ~0.07*)
II	V129M, R156H, V177M, A179D, Q199P, R253P	0.11 ± 0.04 (*~0.06 to 0.17*)
III	G74C, P126Q, R211C	0.42 ± 0.19 (*0.27 to 0.63*)
IV	R142Q, R149Q, A215T, G239S, M310T	8.3 ± 11.3 (*1.03 to 28.2*)
spl	Splicing	Variable

Hershfield, M.S. [10]



A B

Figure 2. Abnormal metaphyseal dysplasia observed in ADA deficiency A; Before PEG-ADA therapy; arrows indicated abnormal metaphyseal cupping B; Four months after PEG-ADA therapy.

Atypical carriers

We recently found atypical ADA-SCID carriers in two different families (I and II) [30]. Although their clinical and immunological status looked normal, ADA activity in their peripheral blood lymphocytes was as low as that seen in ADA-SCID patients in the same family. However, the toxic metabolite of dAXP in RBCs was only slightly elevated in contrast to high level in the patients. To solve this riddle, we sought third ADA mutation(s) relevant to these carriers, other than those mutations already detected in the patients. Consequently, we found M310T in the carriers (patient's mother and sibling) of family I, and G239S in the carriers (patient's mother) of family II. Using in vitro expression activity of mutant ADA alleles revealed M310T and G239S showed 1.0% and 1.5% of wild type ADA activity, respectively [30]. Thermal stability studies also revealed that these ADA mutants were very thermal sensitive and fragile. Accordingly, we diagnosed them as suffering from partial ADA deficiency. Interestingly, the patient of family I received hematopoietic stem cell transplantation using her carrier sibling as a donor with the successful outcome [31].

Reversion case

Although rarely observed, there are an increasing number of reports of somatic mosaicism due to reversion back to normal from the inherited mutations in selected human genetic diseases [32]. In general, reverted cells would gain growth/survival advantage over other cells, making a significant population in vivo. Consequently, these cases have been discovered as having a clinically milder than the expected course, or the presence of a mixture of both phenotypically normal and abnormal cells. The first ADA-SCID reversion cases were detected and shown by Hirschhorn et al. [33]; they reported ADA-SCID patients with life threatening disease early in life, without receiving any therapy including ADA enzyme replacement (see below section). To their surprise, their clinical status improved over time and they were alive at 12 and 18 years, respectively. Consistent with their unusual clinical course, the level of their toxic metabolite (dATP), was usually extremely high in ADA-SCID patients, but in these cases was only minimally elevated. In one of the patients, they proved that a maternally transmitted ADA mutation had reverted to normal in an established B cell line or in DNA purified from blood cells. We observed the same reversion of inherited mutational defects in two ADA-SCID patients [34]. The revertant cells were identified as from the patients' origin, and either of parents' mutation was reverted, respectively. Interestingly, we could detect a small revertant population only prior to the initiation of PEG-ADA therapy, indicating that the therapy would exert a drastic reduction in toxic metabolites and elimination of the selective advantage for reverted cells. We considered that these findings hold significant implications regarding the

prospects for stem cell gene therapy for ADA deficiency (see below section). Recently, a second site de novo mutation that resulted in the restoration of ADA activity was reported [35]. The original mutation was homozygous leading to abnormal, aberrant splicing. The second mutation was a small deletion, that suppressed the aberrant splicing, and was detected in some T cells in an ADA-SCID patient with an atypical course. Interestingly during PEG replacement, a decline in revertant T cell number, was observed, just as we had experienced [34].

Diagnosis of ADA deficiency

Any patient suspected to have SCID, including severe lymphopenia and hypogammaglobulinemia should also be considered to have ADA-SCID. In general, ADA-SCID patients have a much more profound lymphopenia than those with other type of SCID, and costchondral abnormality could be detected. Diagnosis of ADA will be made by measurement of ADA activity in RBCs or fibroblasts. Concomitant measurement of toxic metabolites; dATP, dAXP in RBCs will also be helpful. Finally, diagnosis of ADA deficiency will be confirmed by mutation analysis in the ADA gene itself.

Treatment

Without curable treatments, most ADA-SCID patients die within the first year of life. In case where an HLA-matched sibling is available, hematopoietic stem cell transplantation (HST) from such a family member is recommended as the fist line of treatment. A good outcome is expected after engraftment and reconstitution of immune system in most cases, although less than a quarter of patients will have an HLA-identical sibling. Alternative therapy would be HST from other sources (haploidentical donor or cord blood), ADA enzyme replacement therapy and gene therapy. The draft of optional therapy for ADA-SCID at present is shown in Figure 3.

Hematopoietic stem cell transplantation (HST)

As described above, the best therapy for ADA-SCID at present is HST from an HLA-identical sibling [36, 37, 38]. The results of HST form either parents is generally considered not as good as that of an HLA-matched sibling. Recently, the cord blood bank system has been developed and HST using cord blood stem cells is available now for patients with various diseases including SCID. The results of increasing numbers of cord blood stem cell transplantation need to be urgently and critically evaluated.

The residual immune function in ADA-SCID patients varies between different cases depending on the genotype, therefore, it remains controversial whether prior myelo-suppressive conditioning is needed or not, and by how

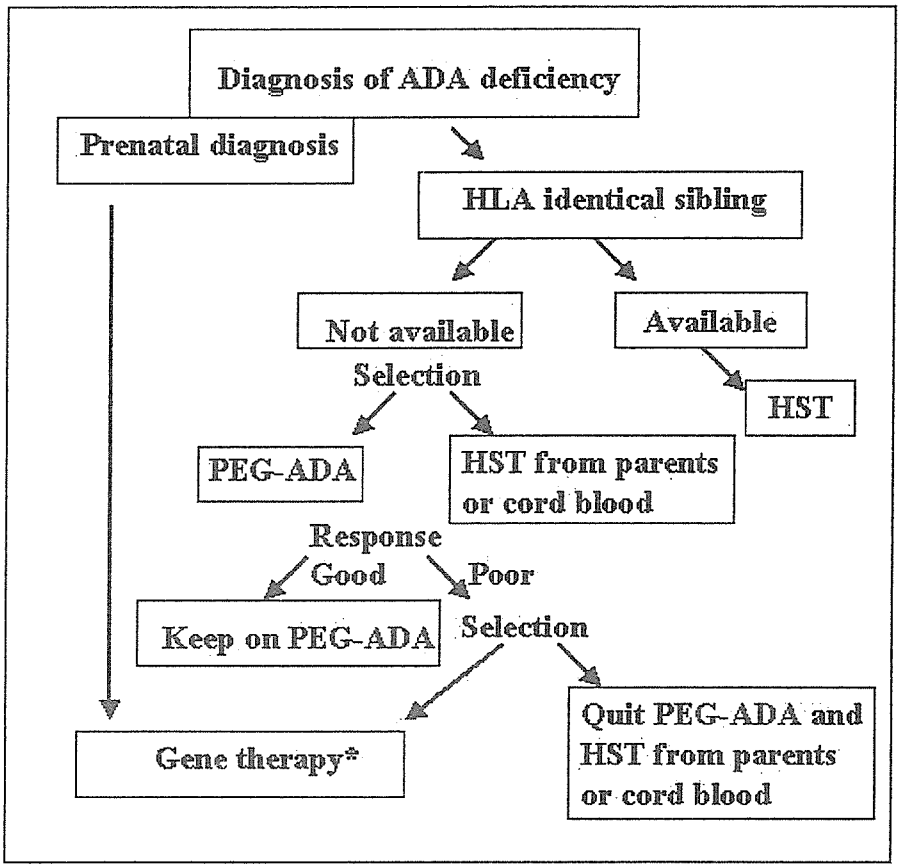


Figure 3. The draft of optional therapy for ADA-SCID. *: PEG-ADA should be suspended for stem cell gene therapy.

much if needed at all. Many complicating factors; genotype, source of donor, infused number of cells, patient's condition, etc. will have important effects on the treatment outcome.

ADA enzyme replacement

The first attempt at treatment by ADA enzyme replacement used frozen irradiated normal RBC and was reported as successful in 1976 by Polmar et al. [39]. They reported infusion of 4 week-interval, the clinical and immunological benefits were observed for 17 months. However, these benefits obtained by RBC infusions were demonstrated to be limited only in a fraction of ADA deficient patients [40, 41]. In 1987, Hershfield et al. reported the successful treatment of ADA-SCID patients with polyethylene glycol modified bovine intestinal ADA (PEG-ADA). PEG-ADA therapy; administered subcutaneously once weekly, this has been accepted as a safety and effective alternative therapy for ADA deficiency. Both clinical and immunological improvement were observed in more than 100 patients with ADA deficiency [42]. It would be indicated for ADA deficient-patients, who lack an HLA-identical donor and are at too high a risk for HST from their parents.

Gene therapy

In 1990, the first authorized clinical gene therapy trial was performed on patients with ADA-SCID [43]. The technique employed the retrovirus vector LASN, and peripheral blood T cells were targeted for gene transduction. The target cells were collected and gene-transduced *in vitro*, and then infused back to the patients. The procedure was repeated for 11-12 cycles at near monthly intervals. PEG-ADA was combined with the gene therapy. Consequently, the therapy was demonstrated to be safe, and some clinical benefits were observed. It was notable that the number of gene-transferred lymphocytes had unexpectedly persisted in some patients for more than 10 years [44]. We observed similar clinical benefits through a similar trial in 1995 [45]. To obtain a permanent therapeutic effect with gene therapy, some groups started several trials using hematopoietic stem cells (HSC) as a target in the early 1990's [46, 47, 48]. For safety, all the cases were also treated with the PEG-ADA replacement therapy. As a result, no beneficial clinical effects were observed. The reason for these unsuccessful gene therapy results has been understood after the recent opposing results reported from two groups. The NIH group's trial; HSC gene therapy using an improved system, concomitant with PEG-ADA replacement, has failed [49]. In contrast, an Italian group's trial; HCS gene therapy without PEG-ADA and under non-myeloablative conditioning, showed good results [50]. Thus, it seems clear that the concomitant PEG-ADA replacement spoiled the selective advantage of gene-transduced HSCs to outgrow the other non-transduced major HSCs, and eventually resulted in ineffective outcome. However, it remains unclear if the impact of conditioning without a PEG-ADA treatment is essential for a successful outcome. The non-myeloablating conditioning should be avoided, unless it is genuinely indispensable. We have performed HSC gene therapy for two ADA-SCID patients after the cessation of PEG-ADA without the use of the conditioning. Although complete immune reconstruction has not thus far been achieved in the patients, they have had an ordinary life without PEG-ADA approximately for two years [51]. Longer follow up studies using large number of ADA deficient patients, which have been treated by gene therapy with or without conditioning is needed to determine the optimum protocol.

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Successful treatment of congenital pulmonary alveolar proteinosis with intravenous immunoglobulin G administration

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Successful treatment of congenital pulmonary alveolar proteinosis with intravenous immunoglobulin G administration

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Abstract: The authors report a female patient with congenital pulmonary alveolar proteinosis (PAP). She had two brothers who died from the same disease. BAL did not improve her progressive respiratory failure. After intravenous immunoglobulin G (IVIG) administration for complicated hypogammaglobulinemia, she recovered from respiratory failure. The efficacy of IVIG was confirmed by recovery from deterioration in respiratory status and improvement in chest CT findings on two separate occasions. Subsequently, the patient remains free from respiratory symptoms for more than 3 years on an ongoing regimen of monthly IVIG. She had no surfactant protein (SP) B deficiency. Alveolar macrophages (AM) obtained from her BAL fluid were small and showed decreased phagocytotic activity. Immunostaining revealed weak expression of PU.1 in her AM, a key protein in AM maturation. All nucleotide sequences of granulocyte-macrophage colony stimulating factor (GM-CSF), GM-CSF-receptor and PU.1 were normal. Endotoxin-induced GM-CSF release from peripheral mononuclear cells (PMNC), and proliferation of PMNC in response to GM-CSF were normal. In addition, an antibody against GM-CSF, as seen in adult patients with idiopathic PAP, was not detected in the serum or BAL fluid. Although the patient's PMNC secreted only small amounts of IgG and IgM, an EB virus-derived cell line of her B cells secreted IgM as much as normal control cells. In a flow cytometric study, IgM was expressed on the cell surface. In conclusion, an abnormality in a single gene may have decreased secretion of immunoglobulin from the B cells and the AM phagocytotic activity in the patient.

Key words: alveolar macrophage, granulocyte-macrophage colony-stimulating factor, hypogammaglobulinemia, infant, pulmonary alveolar proteinosis.

CASE

The patient was a female born after 39 weeks of gestation and with a birthweight of 2290 g to non-consanguineous healthy parents. She had three male siblings, two of whom had died of congenital pulmonary alveolar proteinosis (PAP). Her first brother died of respiratory failure at 91 days of age after airway lavage with artificial lung surfactant and exchange transfusion. The onset of respiratory symptoms was at 39 days of age and the diagnosis of PAP was established at autopsy. The third brother also showed respiratory symptoms at 39 days of age. His respiratory failure was progressive. There was no surfactant protein (SP) B deficiency in the BAL fluid. He died of renal failure at 163 days of age after steroid administration, NO inhalation, ECMO and bone marrow transplantation from his father. The sequence of granulocyte-macrophage colony-stimulating factor (GM-CSF) in his genomic DNA was normal. The autopsy showed PAP and systemic CMV infection. Common features in the current patient and both of her affected brothers were splenomegaly and leukocytosis without abnormality in cell differentials. The remaining brother has been entirely healthy and is now 10 years old.

The current patient was born in June 2001 at the Maternity and Perinatal Care Center, Sapporo, Japan,

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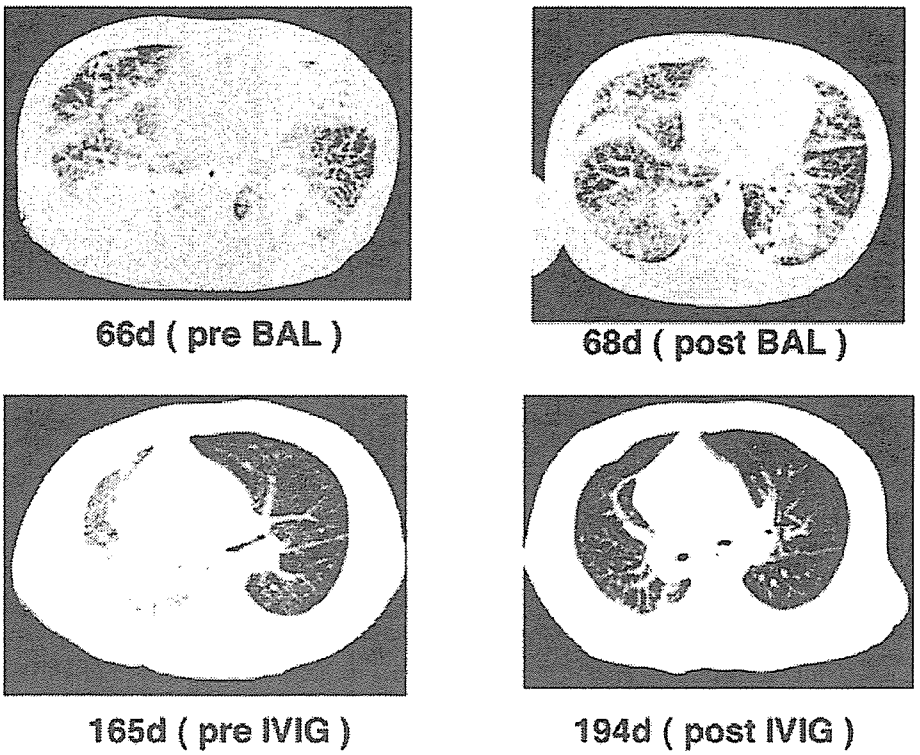

Figure 1 Chest CT findings of the patient. Only partial improvement in the chest CT can be observed after the BAL at 66 days of age. Oxygen status is improved after intravenous immunoglobulin G (IVIG) with BAL at 109 days of age. Between 165 and 194 days of age, only IVIG administration was performed, and apparent improvement in chest CT can be observed.

because of the high risk of hereditary respiratory disease. She showed no respiratory symptoms at birth. Weekly CXR examinations were performed, and at 42 days of age, progressive diffuse consolidation was first observed on her CXR. Then, at 67 days of age, BAL was performed for the diagnosis of PAP. Her oxygenation status gradually deteriorated, and at 87 days of age she needed supplemental oxygen.

She had previously received intravenous immunoglobulin G (IVIG) for a fever (40°C) that was unresponsive to antibiotics at 26 days of age. She showed a generalized skin rash, positive CRP (6.12 mg/dL) and an increased WCC (maximum 82 000/μL) in peripheral blood. She recovered after administration of 180 mg/kg IVIG.

The BAL fluid obtained from her right lung at 66 days was milky white in appearance and contained flocculent precipitates, which stained positively with periodic acid-Schiff stain. BAL fluid concentrations of SP-A and B were markedly elevated at 10 000 ng/mL and 700 ng/mL, respectively, but their ratio was normal, consistent with the diagnosis of PAP. The acidophilic sediments stained strongly with PE10 (anti-SP-A antibody). There were many small alveolar macrophages (AM) which stained weakly with PE10. The concentrations of GM-CSF and macrophage colony stimulating factor (M-CSF) in the BAL fluid were 17.7 pg/mL and 70 840 pg/mL, respectively. Bacterial and viral studies for pathogens including CMV were negative. She developed gradually progressive respiratory failure and a therapeutic BAL on the right lung was performed at 96 days, without improvement in her oxygen-dependent status. The serum IgG level decreased gradually to 102 mg/dL by 102 days. Serum IgA and IgM levels were below 2 and 23 mg/dL, respectively, throughout. At 109 days, a second therapeutic BAL on her left lung was performed with simultaneous IVIG administration for prophylaxis against infection. Surprisingly, her respiratory failure rapidly improved over the next 7 days to the stage where she no longer required supplemental oxygen. The efficacy of IVIG was subsequently confirmed by response to the re-treatment without therapeutic BAL at 165 days after the recurrence of respiratory impairment and consolidation seen on chest CT imaging (Fig. 1). Following administration of high-dose IVIG at 210 days, she was discharged. Subsequent administration of IVIG for recurrent respiratory distress at 262 days was also effective (Fig. 2). She has been free from respiratory symptoms and displays normal neurological development under an ongoing monthly regimen of IVIG. Her splenomegaly has fluctuated concordantly with her respiratory impairment, enlarging during periods of severe impairment, but her leukocytosis has persisted unchanged throughout.

METHODS

Informed consent for therapy and all studies were obtained from the parents. All genetic and cytological studies were approved by the Ethical Committee of Hokkaido University Hospital.

To determine the phagocytic activity of AM, AM isolated by plastic plate adhesion were suspended in RPMI/10% FCS and harvested in a 4-well chamber slide (LabTek Chamber, Nalge-Nunc, Wiesbaden, Germany). After incubation for 2 h at 37°C, cells were incubated with 0.5% PE-labeled latex beads (Sigma-Aldrich, St. Louis, MO, USA) for 30 min, washed with

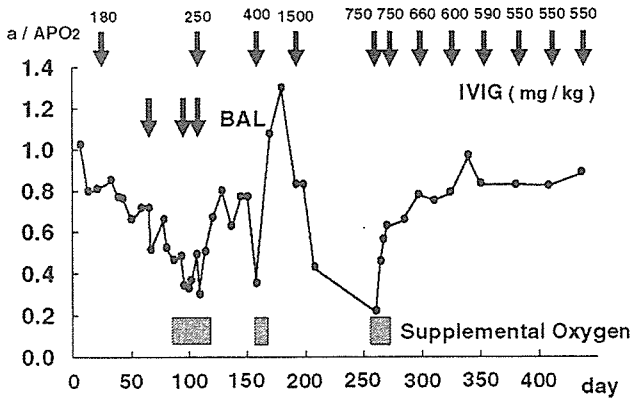

Figure 2 Time course of oxygenation status of the patient. The vertical axis shows the ratio of arterial to alveolar oxygen tension (a/APO_2). Three sessions of BAL showed no therapeutic effect on the oxygenation status. After intravenous immunoglobulin G (IVIG) at 109 days of age, the oxygenation status of the patient improved. Subsequently, deterioration in the oxygenation status was resolved twice with IVIG at 165 and 262 days of age, respectively. The patient has been free from respiratory symptoms under ongoing monthly IVIG administration.

phosphate-buffered saline (PBS) three times, and fixed in 4% paraformaldehyde at 4°C for 15 min. Counterstaining was performed with Syber green (Sytox-green, Dojindo, Kumamoto, Japan) diluted 3000-fold. The percentage of phagocytosed AM was enumerated under confocal laser microscopy.

AM isolated by adherence were fixed in a 4% paraformaldehyde/0.1 M phosphate buffer pH 7.2 for 15 min at 4°C. Cells washed twice with PBS were enumerated with hemocytometry, and cytocentrifuge sediments were prepared. Immunohistochemical staining for PU.1 on the cytocentrifuge samples was performed using rabbit polyclonal anti-PU.1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and alkaline phosphatase labeled antirabbit IgG (Promega, Madison, WI, USA). Color development was done using a BCIP/NBT substrate (DakoCytomation, Glostrup, Denmark).

Genomic DNA was prepared from peripheral white blood cells. From cells in the BAL fluid, RNA was purified and stored as c-DNA. Each nucleotide sequence of GM-CSF, GM-CSF-receptor β chain, M-CSF, M-CSF-receptor and PU.1 was examined on genomic DNA and c-DNA. The DNA/c-DNA was amplified with polymerase chain reaction using specific primers. The amplified fragments were purified and then directly sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and the automated ABI 373 A DNA sequencer (Perkin-Elmer, Boston, MA, USA).

Endotoxin-induced GM-CSF release from peripheral mononuclear cells (PMNC) was measured following the previously reported methods.[1] Briefly, PMNC (1×10^6/mL) from the patient and a healthy volunteer were cultured with or without 2.5 μg/mL of LPS (*Escherichia coli* 055:B55, Sigma, St. Louis, MO, USA) for 24 h. The concentration of GM-CSF as well as M-CSF in supernatant was measured using a commercially available ELISA kit, Human GM-CSF (M-CSF) ELISA kit (R & D Systems, Minneapolis, MN, USA).

Table 1 Excretion of gammaglobulin

Origin	Cells	PHA	IgG (ng/mL)	IgM (ng/mL)
Patient	PMNC	+	59	8.3
Patient	PMNC	–	25	19.5
Control	PMNC	+	246	89
Control	PMNC	–	788	639
Father	CL	–	162	1650
Mother	CL	–	5130	1030
Healthy brother	CL	–	3720	992
Patient	CL	–	7.9	1430
Control 1	CL	–	1360	1540
Control 2	CL	–	1010	2360
Control 3	CL	–	134	2850

CL, EB-virus derived B-cell lines; PMNC, peripheral mononuclear cells.

The CD-14-positive PMNC were purified with anti-CD-14 antibody coated beads and cultured with GM-CSF and M-CSF. The proliferation of the cells was observed with light-microscopy.

An antibody against GM-CSF in serum and BAL fluid was measured with the method previously reported.[2]

PMNC and EB virus-transformed lymphoblastoid cell lines were cultured with/without PHA in RPMI/10% FCS under a 50% CO_2 atmosphere for 72 h.[3,4] The concentrations of IgG and IgM in supernatant were measured using a commercially available ELISA kit, Human IgG and IgM, ELISA Quantitation Kit, respectively (Bethyl Laboratories, Montgomery, TX, USA).

The distribution of surface antigen of PMNC from the patient was examined with flow cytometry system, EPICS XL-MCL (Beckman Coulter, Fullerton, CA, USA).

RESULTS

The current patient's AM were small and showed decreased phagocytotic activity (data not shown). Expression of AM PU.1 was weak in immunostaining, in contrast, PU.1 expression on the PMNC was normal (data not shown). All nucleotide sequences of GM-CSF, GM-CSF-receptor β chain, M-CSF, M-CSF-receptor and PU.1 were normal. Endotoxin-induced GM-CSF and M-CSF release from PMNC, and proliferation of PMNC in response to GM-CSF and M-CSF were normal (data not shown). The concentrations of anti-GM-CSF antibodies in her serum and in the BAL fluid of the third brother were 1.62 (normal <3) μg/mL, and 0.076 (normal <0.1) μg/mL, respectively.

Although, her PMNC excreted only small amounts of IgG and IgM, EB virus-transformed lymphoblastoid cells secreted IgM as much as control (Table 1).

In flow cytometry study, IgM was expressed on 20% of cells. The expression of the M-CSF-receptor was normal.

DISCUSSION

The majority of cases of congenital PAP are transmitted in an autosomal recessive manner. Mutations in genes encoding SP-B, GM-CSF or its receptor have been reported.[5–7] The present case is unlikely to be due to these known disorders for several reasons. First, the normal ratio of SP-B to SP-A excludes SP-B deficiency. Second, the nucleotide sequences of GM-CSF and the GM-CSF-receptor β chain, endotoxin-induced GM-CSF release from PMNC, and proliferation of PMNC in response to GM-CSF were all normal. In addition, an antibody against GM-CSF, as seen in adult patients with idiopathic PAP, was not detected in the serum or BAL fluid. Finally, although congenital PAP, with[8] or without hypogammaglobulinemia, is generally lethal without bone marrow or lung transplantation, PAP in the current case has been completely controlled by ongoing IVIG. This is the first report of a patient with congenital PAP successfully treated by IVIG. Although the M-CSF level in the BAL fluid was high, no abnormal findings concerning M-CSF or M-CSF-receptor were observed.

It has been reported that PU.1 is an essential protein in the process of AM maturation under GM-CSF stimulation.[9] The expression of PU.1 in the AM of the current patient decreased, but the nucleotide sequence of PU.1, as well as its expression on PMNC, was normal.

The patient and her two brothers may have the same genetic disorder. The gene may be essential in both process of maturation of AM and gammaglobulin secretion from B cells. Loss of function of the gene may cause pulmonary alveolar proteinosis. The gene may be located in the pathway from GM-CSF-receptor to PU.1.

Why the IVIG improved the lung condition of the patient remains unknown. IgG may have some effect in maintaining the homeostasis of the lung surfactant system. If hypogammaglobulinemia had decreased the phagocytotic activity of AM, this may be a reasonable clinical approach for premature infants who have small pool sizes of lung surfactant and low levels of serum IgG.

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Two sibling cases of hydrops fetalis due to alloimmune anti-CD36 (Nak^a) antibody

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Summary

Two female sibling cases, who were born to a CD36 deficient mother, were presented with Coombs' test-negative hydrops. The alloimmune anti-CD36 (Nak^a) antibody was accidentally found in the mother's serum after an episode of anaphylactic shock with thrombocytopenia, which occurred in an individual receiving fresh frozen plasma prepared from the mother's donated blood. The mother was then diagnosed as having type II CD36 deficiency, lacking CD36 on both platelets and monocytes, while both of her daughters were CD36 positive. Analyses of the CD36 gene revealed that the mother was a compound heterozygote for the CD36 gene mutation with a novel C→T transition at nt 1366 in exon 12, corresponding to Arg386Trp, and a known 12bp deletion at nt 1438–1449 in exon 13. On the other hand, both patients, who showed half the normal level of CD36 on platelets and monocytes, were heterozygote with one mutation at Arg386Trp. The anti-CD36 antibody in the mother seemed to be responsible for the hydrops fetalis observed in her daughters, because the IgG isolated from the mother's serum showed suppressive effects on the CFU-E colony formation of CD34$^+$ cells from a control donor. This is the first case report of hydrops fetalis caused by an alloimmune anti-CD36 antibody.

Keywords

Platelet immunology, gene mutations, immunity (auto-)

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Introduction

CD 36 is a glycoprotein expressed in various human cells, including platelets, monocytes, erythroblasts, capillary endothelial cells, mammary epithelial cells and adipocytes (1). CD 36 interacts with a variety of ligands and is believed to play important roles in fatty acid metabolism. The anti-CD36 antibody has been used in the separation of fatal nucleated erythroblasts from maternal blood for the purpose of non-invasive prenatal genetic screening.

CD 36 deficiency is divided into two subgroups according to phenotype; neither platelets nor monocytes express CD 36 in type I deficiency, while in type II deficiency CD 36 is expressed only on monocytes and not on platelets. The incidences of type I and type II deficiency in the Japanese population have been reported as 1.0% and 5.8%, respectively (1). Platelet CD 36 deficiency (i.e., both type I and type II whole) is common in Asians and African Americans, whereas it is very rare in Caucasians (1). CD36 deficiency has been recognized as possibly leading to CD36 isoimmunization and causing refractoriness to platelet transfusion. Neonatal alloimmune thrombocytopenia caused by the anti-CD36 antibody has also been reported (2).

Hydrops fetalis is generally classified into two groups by the results of Coombs' test: immunne and non-immune hydrops. Here we present two female sibling cases of immune hydrops fetalis with negative Coombs' tests, who were born to a mother with CD36 deficiency having the alloimmune anti-CD36 (Nak^a) antibody. This is the first case report of hydrops fetalis caused by the alloimmune anti-CD36 antibody.

Cases

Case 1

A Japanese girl, now 9 years old, is the first daughter of a healthy mother indicated later. At 18 weeks of gestational age, ultra-

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sonography revealed that she had ascites, and the mother was admitted to our hospital at 24 weeks 3 days of gestational age. Fetal anemia (Ht 10%, Hb 3.2g/dl) and hypoproteinemia (less than 1.0g/dl) was revealed from umbilical cord centesis. Although its cause was unknown (negative Coombs' test, negative Parvo B19 infection, etc.), fetal therapies for emergent severe anemia, such as intraperitoneal RBC transfusion and albumin replacement, were performed. At 30 weeks 3 days of gestational age, the first patient was delivered by emergent caesarian section. She weighed 2420g at birth with her RBC count was 120×10^4 /μl, Hb 4.5 g/dl, Ht 13.4%, reticulocyte 62.1%, and platelet count 47×10^3 /μl. An RBC transfusion (total 109 ml) and several bloodlettings were performed to improve her anemia and congestive heart failure. After approximately a week of supportive care regimens such as conventional mechanical ventilation, abdomiocentesis, thoracentesis and intravenous infusions, generalized edema disappeared and did not recur. Her minimum body weight was 1420g. Her maximum serum total bilirubin concentration was 14.5mg/dl. She was discharged on her 94th day of age without any complications, and has had no sequelae to date.

Case 2

A Japanese girl, now 8 years old, is the second daughter of the mother of case 1. She was also noted to have ascites by ultrasonography from 25 weeks of gestational age. Since the clinical course was very similar to that of case 1, she was delivered by emergency caesarian section at a gestational age of 29 weeks 1 day. Her body weight at birth was 1920g. Her RBC count was 99×10^4 /μl, Hb 3.9 g/dl, Ht 13.6%, reticulocyte 130.6‰, and platelet count 108×10^3 /μl. Instead of RBC transfusion and several bloodlettings, a single-volume exchange transfusion with 200ml RBC was performed under isovolemic conditions. After approximately a week of supportive care regimens, generalized edema had disappeared and never recurred. Her minimum body weight was 1000g, and her maximum serum total bilirubin concentration was 12.4mg/dl. She was discharged on the 162nd day of age without any apparent sequelae, and remains sequelae-free to date.

At this point, because of the negative Coombs' test, we considered that both subjects were suffering from non-immune hydrops due to fetal anemia resulting from the same but unknown etiology.

The mother of the two cases

The mother of the two cases is Japanese, and was born to consanguineous parents (first cousins) in 1970. She had been apparently healthy and had no history of receiving blood transfusion. Five years after delivery of her second daughter, she donated blood to the Japan Red Cross Society. Fresh frozen plasma prepared from her donated blood was transfused to a female patient undergoing neurological surgery. Immediately after transfusion, she went into anaphylactic shock with her platelet counts decreasing from 200×10^3 /μl to 20×10^3 /μl. After a screening test for anti-platelet antibodies, the anti-CD36 (Nak^a) antibody was detected from the mother's plasma. On the basis of this report, we formulated the following hypotheses for the mechanism of hydrops fetalis in the two subjects and investigated the possible etiology.

- The mother has CD36 deficiency, whereas both her daughters are CD36-positive.
- An anti-CD36 alloimmune antibody transferred from the mother is responsible for hydrops fetalis observed in her daughters.

Materials and methods

Materials

Venous blood from both daughters and their mother were obtained by informed consent.

1. Studies for characterization of CD36 deficiency

Analysis for CD36 expression by flow cytometry

CD36 expression on monocytes and platelets was determined by flow cytometry as previously described (1). Briefly, venous blood was taken and placed into tubes containing EDTA-2K. For CD36 expression on the cells, 25μL of platelets (1×10^5/μL) or mononuclear cells (4×10^4/μL) were incubated with 10μL of FITC labeled anti-CD36 monoclonal antibody FA6–152 (Immunotech, Marseilles, France) for 30min at 4°C. For mononuclear cells, 10μL of PE labeled anti-CD14 monoclonal antibody RMO52 (Immunotech, Marseilles, France) was added prior to incubation to identify monocytes. After washing with 10mM EDTA-PBS, the mean fluorescence intensity of the platelets or the monocytes was evaluated by flow cytometry (FACS Calibur, Becton-Dickinson, San Jose, CA). Monocytes were analyzed under the gating condition of CD14 positive cells.

To evaluate the reactivity of the anti-CD36 alloimmune antibody in the mother, 25μL of platelets (1×10^5/μL) or mononuclear cells (4×10^4/μL) either from the daughters, the mother or a normal control were incubated with 10μL of the mother's serum for 30min at 4°C. After washing twice with 10mM EDTA-PBS, the cells were incubated with 20μL of 1:100 diluted PE labeled anti-human IgG (Jackson, West Grove, PA) for 30min at 4°C.

Mutation analysis of the CD36 gene

Genomic DNA

Genomic DNA was extracted from whole EDTA-blood, using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and subjected to sequencing for all exons. Each exon including a part of the flanking introns was amplified by PCR and directly sequenced with a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) as previously reported (1).

cDNA sequence

Total RNA was extracted from whole EDTA-blood, with a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany), and then subjected to reverse transcription and polymerase chain reaction (RT-PCR), using the primer set of 5'-GTATGCAAGT CCTGATGTTTC-3'(forward; nt 1251–1271 in the exon 11) and 5'-TCACCAATGGTCCCAGTCTC-3'(reverse; nt 1462–1481 in the exon 14), with the help of a One Step RNA PCR Kit (Takara, Otsu, Japan). The obtained PCR product was directly sequenced with the 310 Genetic Analyzer.

2. CFU-E colony assay

Cytokines

IL-3 and stem cell factor (SCF) were provided from Kirin Brewery Co Ltd. (Tokyo, Japan), and erythropoietin (EPO) was from Chugai Pharmaceutical (Tokyo, Japan).

CFU-E colony assay

Peripheral blood $CD34^-$ cells were isolated from normal control and differentiated into CFU-E according to the protocol described previously (3, 4). Briefly, peripheral blood mononuclear cells separated from the buffy coat by Ficoll-Hypaque were centrifuged with a 10% bovine serum albumin (BSA) cushion to remove platelets, and then processed using a nylon-fiber syringe system to deplete monocytes. $CD34^+$ cells were separated by immunomagnetic beads coated with a CD34 monoclonal antibody (Nihon DINAL, Tokyo, Japan) and cultured at 37°C in 5% CO_2 with Iscove's Modified Dulbecco's Medium (IMDM; Gibco BRL, US) supplemented with 10 ng/mL of IL-3, 10 ng/mL of SCF, 2 U/mL of EPO, 1% BSA, 50 μM of 2-mercaptoethanol (2-ME) and 30% fetal calf serum (FCS), which was designated as complete medium. After 7 days, the cultured cells were cryopreserved and stored in liquid N_2 until used.

The stored cells were thawed and pre-cultured in IMDM containing 2 U/ml of EPO, 1% BSA, 50 μM of 2-ME and 30% FCS overnight and then washed with cold PBS containing 0.1% BSA. This process was critical to recover the ability of the CFU-E colony formation of the stored cells to the level equivalent to that before storage. Subsequently, the washed cells were suspended in complete medium at a concentration of 6×10^3/ml in the presence of IgG from the mother's serum purified by protein A column, normal human IgG (Jackson ImmunoResearch Laboratory Inc, USA) or monoclonal anti-CD36 antibody, or in the absence of these antibodies. The concentration of IgG purified from the mother's serum was adjusted to 5, 20 and 80 μg/ml and that of monoclonal anti-CD36 antibody to 2μg /ml. One ml aliquot of cell suspension was placed in the 35ml suspension culture dish in duplicate and incubated at 37°C in 5% CO_2 for 7 days. Then, the number of CFU-E colonies which consisted of 8 ~ 49 cells were counted on an inverted microscope (5). Unfortunately, these experiments were performed only three times for practical reasons, so that we could not analyze the results statistically.

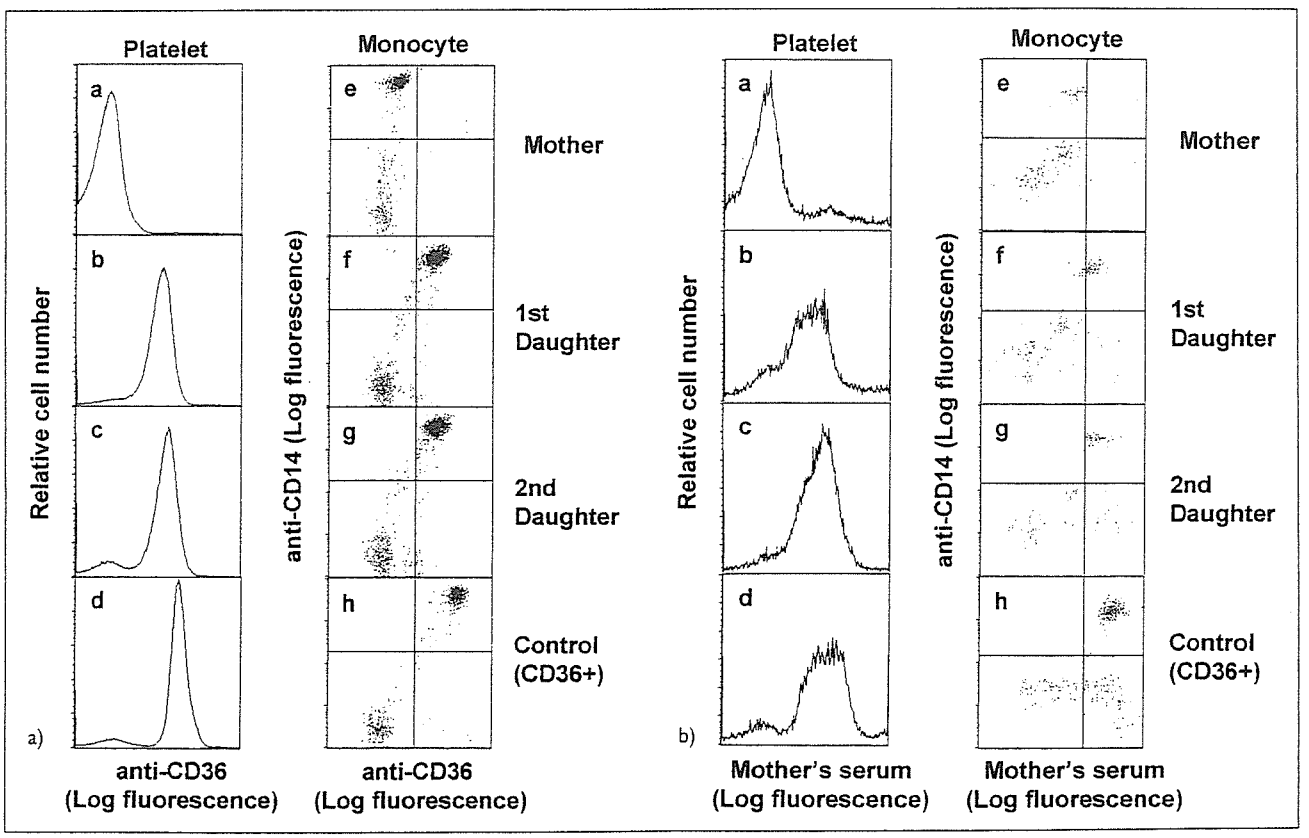

Figure 1: Flow cytometric analyses of CD36 on the platelets (a-d) and the monocytes (e-h), Mother (a, e), 1st daughter (b, f), 2nd daughter (c,g), and CD 36 positive control (d, h); CD36s on platelets are illustrated as a histogram and those of monocytes are illustrated as a cytogram, and monocytes are analyzed under the condition of the CD14 positive gating region (upper half region of each profile). Note that the mother's platelets and monocytes both lacked reactivity with the monoclonal anti-CD36 antibody, and both her daughters were CD36 positive, although the intensity of their CD36 expression on platelets and monocytes appeared half that of the normal control. **b:** Flow cytometric analyses of the mother's serum on the platelets and the monocytes. Note that the reactivity of the mother's serum to platelets and monocytes was quite similar to that of the monoclonal anti-CD36 antibody.

Results

1. Studies for characterization of CD36 deficiency

Analysis of CD36 expression by flow cytometry

The mother was confirmed as having type I CD36 deficiency, since her platelets and monocytes both lacked reactivity with monoclonal anti-CD36 antibody. Both her daughters were CD36 positive, although the intensity of their CD36 expression on platelets and monocytes appeared to be half the normal level compared with the normal control (Fig. 1a, and Table 1). The reactivity of the mother's serum to platelets and monocytes was quite similar to that of monoclonal anti-CD36 antibody (Fig. 1b, Table 1).

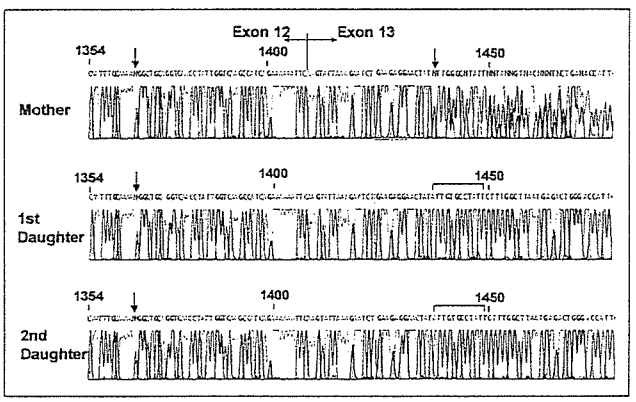

Figure 2: The cDNA sequence of exon 12 and 13 of the CD36 gene. Note that a novel C→T transition at nt 1366 is present in exon 12 in all the three subjects (arrows), and a known 12bp deletion at nt 1438–1449 in the exon 13 is present in the mother but not in the daughters (arrow).

Table 1: Flow cytometric analysis of CD36 on platelets and monocytes (mean fluorescence intensity).

	Flow cytemetric analysis of CD36 on platelets and monocytes (Mean fluorescence intensity)			
	CD36 monoclonal antibody		Mother's serum	
Specimen	Platelet	Monocyte	Platelet	Monocyte
Mother	5,09	42,93	15,25	393,35
1st Daughter	92,07	369,64	96,55	607,59
2nd Daughter	98,66	395,44	159,45	921,47
control (CD36+)	241,58	1258,16	210,95	2059,99

Table 2: Results of the CFU-E colony assay (colony counts).

	Results of the CFU-E colony assay (Colony counts)							
	Control	Monoclonal Ab	5 μg / ml		20 μg / ml		80 μg / ml	
			IgG	Mother	IgG	Mother	IgG	Mother
Exp. 1	141	74	118	67	119	87	106	65
Exp. 2	44	28	49	31	48	29	45	26
Exp. 3	115	110	121	118	122	98	139	110

Analysis of the CD36 gene mutation

The genomic DNA and cDNA analyses revealed that the mother was a compound heterozygote with a novel C→T transition at nt 1366 in exon 12, corresponding to Arg386Trp, and a known 12bp deletion at nt 1438–1449 in exon 13 (6). Both her daughters were heterozygote with one mutation of Arg386Trp (Fig. 2). No other mutation was detected in any exons of the CD36 gene or any exon-intron junctions of the subjects.

2. CFU-E colony assay

IgG from the mother exhibited some suppression of CFU-E colony formation to the same level as the monoclonal anti-CD 36 antibody in all three different concentrations (Table 2). No significant suppression was observed with the equivalent level of control IgG.

Discussion

Here we report the first sibling cases of hydrops fetalis caused by a maternal alloimmune anti-CD36 antibody. Mutation analysis revealed that the mother was a compound heterozygote of the CD36 gene mutation (including a novel mutation of Arg386Trp) with phenotype of type I CD 36 deficiency. The two daughters, whose levels of CD36 on platelets and monocytes were half those of normal levels, were heterozygote with one mutation at Arg386Trp. This finding is consistent with the fact that Arg386Trp is a mutation responsible for CD36 deficiency, not a polymorphic substitution. In fact, a C→T transition at nt 1366 in exon 12 of the CD36 gene has not been reported in JSNP database (7).

Because of negative Coombs' test and no recurrence of anemia after the therapy, we first suspected that the two daughters were suffering from non-immune hemolytic anemia or congenital defects in fetal red blood cell synthesis. Therefore, the sequences of the Hb-γ gene of the sisters were studied, but we did not find any abnormalities (results not shown).

Fortunately, an accidental episode of anaphylactic shock in an individual who received fresh frozen plasma from the mother's donated blood led us to the correct diagnosis. There have been several case reports (up to 15 cases) of alloimmune thrombocytopenia caused by the anti-CD36 antibody (2) including a case of neonatal anemia which probably resulted from bleeding with alloimmune thrombocytopenia (8). However, no cases of hydrops fetalis due to maternal anti-CD36 antibody have been reported. If there had been extraordinary high titer or high potential for complement activation of the antibody in our case, those findings might have provided some explanations for the differences of severity. Further investigations are needed in these points.

Regarding the daughters in our study, it seems very likely that the maternal anti-CD36 antibody was related to the observed hydrops fetalis. Erythroblasts are known to be CD36-positive, whereas mature erythrocytes are CD36-negative (9). Therefore, it is understandable that the Coombs' test was negative in both sisters. However, the precise mechanism of severe fetal anemia (hydrops fetalis) remains to be determined. The results of the CFU-E colony assay indicated that anti-CD 36 antibodies affected the erythropoiesis *in vitro*. Thus, the anti-CD36 antibody

would have effects on the growth and the differentiation of erythroblasts in the daughters, both of whom showed extreme reticulocytosis at birth for unknown reasons. These findings are very different from that of erythroblastic synartesis, an autoimmune dyserythropoiesis, whose auto-antigen was unknown but a molecule on the membrane of erythroblasts has been proposed and one candidate antigen was CD36 (10). This autoimmune dyserythropoiesis exhibits severe anemia with reticulocytopenia. The Coombs' test was positive in patients with erythroblastic synartesis. In addition, a bone marrow smear from case 1 did not show abnormal aggregation of erythroblasts (data not shown), which was a characteristic feature of erythroblastic synartesis. Thus, we concluded that the mechanism for anemia in erythroblastic synartesis seemed close to but different from our cases. One possibility is that the mechanism of severe anemia in our cases might depend on hemolysis from complement activation *in vivo*, not on the direct blockage of erythropoiesis by antibodies that we showed by CFU-E colony assay *in vitro*. Another possibility is that gene expression of CD36 in erythroblasts might have a large deviation and fewer-expressed erythroblasts could pass through the "gate" of the anti-CD36 antibody. Further investigations are also needed to clarify these aspects.

Although the hydrops of our cases seemed to result from immunogenic anemia of erythroblasts, this immune process could not be detected by routine immunological methods such as the Coombs' test. It is of note that a negative Coombs' test could not exclude the possibility of peculiar immune hydrops fetalis like our cases. There should be a significant number of cases of hydrops due to the same mechanism among those of unknown etiology because CD36 deficiency is not rare in some ethnic groups. We propose that examination of maternal CD36 expression on platelets or monocytes, or evaluations of anti-CD36 antibody in maternal serum should be considered in screening for hydrops fetalis.

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Gene Therapy for Primary Immunodeficiency Diseases: Recent Progress and Misgivings

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Abstract: The progress of clinical gene therapy trials during the last two decades has been remarkable, and its application has also expanded into various fields of human diseases. Among them, hereditary diseases such as the primary immunodeficiency diseases (PID) were considered suitable candidates for gene therapy because the therapeutic strategy was very simple, therefore, effective gene therapy may be obtained without significant difficulty compared to other more complex diseases such as cancer. Indeed, the first clinical gene therapy trial was safely performed and was in part, effective for adenosine deaminase (ADA) deficiency patients, a type of severe combined immunodeficiency diseases (SCID). However, because of certain unforeseen obstacles, it took approximately 10 years until the first curative effects were obtained for gene therapy in patients with X-linked SCID (X-SCID). Here, I review and discuss the background and historical events leading up to PID gene therapy, the safety issues, which unexpectedly arose after the successful report, and finally I will attempt to predict the future trends in this form of gene therapy.

INTRODUCTION

Gene therapy is a promising clinical option for treating patients with genetic diseases, which might be cured by hematopoietic stem cell transplantation (HST). In 1990, the first authorized clinical gene therapy trial was performed in patients with severe combined immunodeficiency disease (SCID) with defects in adenosine deaminase (ADA) [1]. This disease had been considered as a suitable candidate for the gene therapy. However, a method of gene transduction to hematopoietic stem cells was not established at that time; therefore, peripheral T lymphocytes were chosen as the target cells in this trial instead of the hematopoietic stem cells. A huge number of retroviral gene-transduced cells using the vector LASN were repeatedly transfused into the patients. These trials were also combined with ADA enzyme replacement therapy; which had the effect of masking the clinical effects produced by the gene therapy. While the safety issues involved in gene therapy using retroviral vectors were initially approved; the risk of insertional mutagenesis, which may cause activation of some oncogenes, resulting in the development of uncontrolled cell proliferation, were discounted. It was concluded that these serious adverse events might theoretically be possible, but would practically be negligible. Since the first gene therapy trial, a number of clinical trials involving gene targeting of hematopoietic stem cells have been performed for patients with PID with great expectations [2-6]. However, due to unexpected obstacles, it took approximately 10 years until the first curative effects of the gene therapy were obtained in patients with X-linked SCID (X-SCID) [7]. Sustained clinical effects were obtained in 9 of 11 patients receiving this therapy, however, two patients from these 9 patients developed leukemia-like adverse events 30 and 34 months respectively after the therapy [8]. These events are now forcing gene therapy researchers to investigate the precise mechanisms involved in these events, and to properly re-establish a safety protocol for hematopoietic stem cell gene therapy. Thus, the next generation of gene therapy clinical studies has started.

RECENT TECHNICAL ADVANCES IN HEMATOPOIETIC STEM CELL GENE THERAPY USING RETROVIRAL VECTORS

Previous problems in stem cell gene therapy were supposed due to the low efficiency of gene transduction, and restricted expression of the transduced-gene; therefore, the majority of effort has been made toward solving these problems. Various combinations of cytokines were studied to induce activation but not differentiation of hematopoietic stem cells [9-11]. Those cytokines include stem cell factor (SCF), IL-3, IL-6, soluble IL-6 receptor, thrombopoietin (TPO), Flt-3 ligand and TGF-β, and several of them were selected for the suitable combination. Consequently the efficacy of stem cell gene transduction has improved together with the presence of recombinant fibronectin, which helps the target cells and vector to co-localize [12]. The design of the retroviral vector has also been improved to provide higher performance in transduction efficiency and gene expression. We are now performing the clinical trials for hematopoietic stem cell gene therapy in 2 ADA deficient patients, using an advanced retroviral vectors; GCsapM ADA (MPSV) [13]. The differences in the characteristics of this vector compared with the previous vector LASN are summarized as follows. The selective marker *Neo* gene is removed from the vector construct to make the structure simpler. The vector possesses both splice donor and acceptor sites for the generation of subgenomic mRNA. Instead of using a promoter with the long terminal repeat (LTR) derived from murine leukemia virus (MLV), that of the myeloproliferative sarcoma virus (MPSV) is used. These

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alterations result in an amplification of gene expression and prevent silencing effects of the vector *in vivo*. In addition, the packaging cell lines have been changed from PA317 (amphotrophic) to PG13 (gibbon ape leukemia virus envelope), which is expected to improve the transduction rate of the retroviral vector within hematopoietic stem cells.

NATURAL GENE THERAPY

Although this is considered to have happened in very rare instances, there are increasing reports of somatic mosaicism observed in patients with genetic diseases, some of which result from *in vivo* reversion of an inherited mutation to normal [14]. In some cases of PID with reversion, the patients gradually show a milder phenotype, compared with other patients with the same diseases. The number of cells with the reversion is closely correlated with the clinical improvement of the patient. Therefore, this phenomenon is referred to as natural gene therapy. These events were reported in patients with ADA deficiencies [15, 16], X-SCID [17] and Wiskott-Aldrich syndrome (WAS) [18, 19]. In general, the reversions originally occurred in a single cell *in vivo*. Therefore, to expand in the general cell population *in vivo*, the reverted cells should possess a growth or survival advantage over the other cells. The specific cell lineage in which a revertant occurs is also important in hematological cells. This is due to the fact that in PID, the normal immune system functions depend on multiple T or B cell repertories to exhibit. Thus, these findings have significant implications for stem cell gene therapy prospects in PID. Expecting these conditions to require only small numbers of gene corrected cells to be infused to provide a therapeutic effects in the present gene therapy trials, a selective advantage of the gene corrected cells might be an important requirements for success. These examples suggest that ADA-SCID, X-SCID and WAS are good candidate diseases for hematopoietic stem cell gene therapy at our present level technical of expertise. We have previously reported interesting findings in an ADA deficient patient. We had found a small population of T cells with a mutation reversion in her blood before polyethylene glycol modified bovine ADA (PEG-ADA) replacement therapy; however, we were never able to detect these cells after the replacement [16]. The PEG-ADA seems to interfere with some selective advantage of the reversion cells *in vivo*, and this then conceals these cells behind the large numbers of PEG-ADA-dependent, defective T cells. The merits and problems encountered using PEG-ADA in ADA deficient patients will be discussed later.

PID AS SUITABLE CANDIDATES FOR GENE THERAPY

In general, some PID, which can be treated by HST, may also be a good candidate for the hematopoietic stem cell gene therapy. Among various PID, however, successful gene therapy results have thus far been confirmed in only two diseases, X-SCID and ADA deficiency. Several hematopoietic stem cell gene trials for chronic granulomatous disease showed no positive clinical effect but also a very low frequency of gene transduced cells *in vivo*. A list of possible PID candidate disease for stem cell gene therapy and the present status of any ongoing therapy is shown in Table 1.

1. ADA Deficiency

ADA is a purine salvage enzyme, which catalyses the deamination of adenosine and deoxyadenosine to inosine and doxyinosine, respectively. In 1972, an association between ADA deficiency and severe immunodeficiency was accidentally detected [20]. Extensive studies subsequently revealed that molecular defects in the ADA gene could cause an autosomal recessive form of SCID [21, 22]. ADA is ubiquitously expressed and a lack of ADA leads to the accumulation of deoxy ATP and deoxy adenosine, which has toxic effects on certain cells especially lymphocytes *via* a number of mechanisms. In general, ADA deficient patients have a more profound lymphopenia ($T^-B^-NK^-$ SCID) than other types of SCID, although milder types have also been reported. Hematopoietic stem cell transplantations (HSTs) from HLA-identical siblings are highly successful, however HLA-mismatched HSTs do not have good prognosis [23]. Regular injection of PEG-ADA is an alternative choice of therapy for ADA deficient patients [23]. It is safe and effective, although the effects in some cases are partial and temporal.

ADA deficiency was the first form of SCID whose etiology was molecularly elucidated. The ADA gene was mapped to chromosome 20q13.2-q13.11, cloned and sequenced in 1980 [24, 25]. ADA deficiency had been considered as the best candidate for gene therapy for various reasons, and indeed, the first authorized clinical gene therapy trial was performed for patients with ADA deficiency in 1990.

1) The First Clinical Trial of Gene Therapy

In 1990, the first authorized clinical gene therapy trial was performed using patients with ADA-SCID [1]. The technique employed the retroviral vector LASN, and peripherally circulating, mature T cells were targeted for gene transduction. Peripheral mononuclear cells were purified for *in vitro* culture, activated with anti-CD3 and IL-2, and the gene was transduced by retroviral vector LASN and the cells infused back into the patients. The therapy was repeated 11 times for the first case, and 12 times for the second case. In these cases, PEG-ADA was combined with the gene therapy; therefore, the evaluation of clinical effect obtained by only the gene therapy was unknown. However, it was reported that this therapy could be performed without any serious side effects, and some clinical benefits were suggested compared with the patient status before the gene therapy. It was notable that the numbers of gene-transferred lymphocytes has been unexpectedly persisting in the patient for more than 10 years [26]. Similar clinical benefits and long lasting transgene-containing peripheral T cells were observed in our similar trial in 1995 [27].

2) Clinical Gene Therapy Trials Targeting for Hematopoietic Stem Cell in the Early 1990's

To obtain a positive therapeutic effect with gene therapy, some groups started several trials using hematopoietic stem cells as the main ADA gene transfer target [2-5]. The target cells used were CD34+ cells in peripheral blood, cord blood and bone marrow. For safety all the cases were also treated with the PEG-ADA replacement therapy. Although cells carrying the vector were detected after the therapy, they were

Table 1. Candidate Primary Immunodeficiency Diseases for Gene Therapy

Disorders	Gene defected	Present status of affairs	Possible problem
SCID			
ADA-deficiency	ADA	Clinical trial	Need conditioning?
X-SCID	γc	Clinical trial	Leukemia like episodes
Jak 3 deficiency	Jak-3	Clinical trial	Based on over expression?
RAG-1/2 deficiency	Rag-1/2	Animal model	Based on unregulated expression?
IL-7 Ra deficiency	IL-7Ra	Cell level	Based on aberrant expression?
CD45-deficiency	CD45	Animal model	Need various isoforms?
Leukocyte defects			
Chronic granulomatous disease	gp91-phox, p47-phox p67-phox p22-phox	Clinical trial Animal model Cell level	No growth advantage No growth advantage No growth advantage
Leukocyte adhesion defect I	CD18	Clinical trial	No growth advantage
Severe congenital neutropenia/ Cyclic neutropenia	ELA2	not yet	Based on over expression?
Hermansky-Pudlak syndrome-2	AP3B1	Animal model	Substitute use of G-SCF for AP3B1
Other immunodeficiency			
Wiskott-Aldrich syndrome	WASP	Animal model	Based on over expression?
X-linked agammaglobulinemia	Btk	Animal model?	Based on aberrant expression?
X-hyper IgM	CD40 ligand	Animal model*	Based on unregulated expression
ZAP-70 deficiency	Zap 70	Animal model	Based on over expression?

*: developed T cell lymphoproliferative disorders

at only trace levels, and no beneficial clinical effects were observed. The reason for these unsuccessful results may be in part due to the low transduction rate of the gene into the target cells, but now it is understood that the concomitant PEG-ADA replacement therapy might spoil the selective advantage of these gene transduced CD34+ cells to outgrow the other non-transduced hematopoietic and progenitor cells.

3) Two Opposite Results Using Hematopoietic Stem Cell Gene Therapy for ADA Deficient Patients in the 21st Century

After the improvement of retroviral vectors and the techniques for hematopoietic stem cell gene transduction, two groups reported contrasting results. An Italian group reported the successful results [28]. They performed their gene therapy in two ADA deficient patients, who did not receive any PEG-ADA replacement therapy, under the non-myeloablative conditioning. In contrast, the National Institute of Health group (NIH, USA) reported that no clear clinical effects were observed using an improved hematopoietic stem cell gene therapy system used in combination with PEG-ADA replacement [29]. Thus, it seems clear that the concomitant PEG-ADA replacement should be discontinued prior to gene therapy. However, it remains unclear that the impact of conditioning together with a PEG-ADA discontinuance is essential for a successful outcome. A discontinue in the use of PEG-ADA alone increases the risk of serious infectious episodes in patients; therefore, the non-myeloablative conditioning should be avoided unless it is genuinely indispensable. Now our group in Hokkaido University Hospital, Japan, in collaboration with Dr. Fabio Candotti and coworkers at NIH, USA, is starting hematopoietic stem cell gene therapy for 2 ADA deficient patients after the cessation of PEG-ADA 3-4 weeks prior to the therapy without the use of conditioning. We think that the conditioning may not be necessary if a sufficient number of gene transduced stem cells are introduced into the patient, when this cell population should grow well under the conditions where there is a positive growth advantage. These results will be reported in the near future.

2. X-SCID

X-SCID is the most common type of SCID accounting for approximately 45% of all SCID patients [30]. It is clinically characterized by patients that lack T and NK cells, but possess a normal or increased number of defective B cells in their peripheral blood. The gene responsible for X-SCID; *IL2RG* was identified in 1993 [31]. Extensive studies revealed that the *IL2RG* sequence encodes a common gamma chain (γc) shared by the cell surface receptors for