Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2012.07.008.

References

[1] Castori M, Sinibaldi L, Mingarelli R, Lachman R, Rimoin D, Dallapiccola B. Pachydermoperiostosis: an update. Clin Genet 2005;68:477–86.
[2] Rimoin D. Pachydermoperiostosis (idiopathic clubbing and periostosis): genetic and physiologic considerations. N Engl J Med 1965;272:923–31.
[3] Touraine ASG, Golé L. Un syndrome ostéodermopathique: la pachydermieplicaturée avec pachypé riostose des extrémités. Presse Med 1935;43:1820–4.
[4] Bergmann C, Wobser M, Morbach H, Falkenbach A, Wittenhagen D, Lassay L, et al. Primary hypertrophic osteoarthropathy with digital clubbing and palmoplantar hyperhidrosis caused by 15-PGHD/HPGD loss-of-function mutations. Exp Dermatol 2011;20:531–3.
[5] Diggle C, Carr I, Zitt E, Wusik K, Hopkin R, Prada C, et al. Common and recurrent HPGD mutations in Caucasian individuals with primary hypertrophic osteoarthropathy. Rheumatology (Oxford) 2010;49:1056–62.
[6] Seifert W, Beninde J, Hoffmann K, Lindner T, Bassir C, Aksu F, et al. HPGD mutations cause cranioosteoarthropathy but not autosomal dominant digital clubbing. Eur J Hum Genet 2009;17:1570–6.
[7] Sinibaldi L, Harifi G, Bottillo I, Iannicelli M, El Hassani S, Brancati F, et al. A novel homozygous splice site mutation in the HPGD gene causes mild primary hypertrophic osteoarthropathy. Clin Exp Rheumatol 2010;28:153–7.
[8] Tariq M, Azeem Z, Ali G, Chishti M, Ahmad W. Mutation in the HPGD gene encoding NAD+ dependent 15-hydroxyprostaglandin dehydrogenase underlies isolated congenital nail clubbing (ICNC). J Med Genet 2009;46:14–20.
[9] Uppal S, Diggle C, Carr I, Fishwick C, Ahmed M, Ibrahim G, et al. Mutations in 15-hydroxyprostaglandin dehydrogenase cause primary hypertrophic osteoarthropathy. Nat Genet 2008;40:789–93.
[10] Yuksel-Konuk B, Sirmaci A, Ayten G, Ozdemir M, Aslan I, Yilmaz-Turay U, et al. Homozygous mutations in the 15-hydroxyprostaglandin dehydrogenase gene in patients with primary hypertrophic osteoarthropathy. Rheumatol Int 2009;30:39–43.
[11] Shigematsu Y, Niizeki H, Nozaki M, Sasaki R, Horikawa R, Seki A, et al. A case of pachydermoperiostosis. Rinsho Hifuka 2010;64:751–4.
[12] Tanese K, Wakabayashi A, Yamamoto K, Miyagawa S, Imanishi N. Compiete form of pachydermoperiostosis: case report. Rinsho Hifuka 2010;64:221–4.
[13] Niitsuma K, Hatoko M, Tada H, Tanaka A, Yurugi S. A case of pachydermoperiostosis treated with plastic surgery using tissue expander. J Jpn Soc Plast Reconstr Surg 2004;24:548–53.
[14] Nakahigashi K, Otsuka A, Doi H, Tanaka S, Okajima Y, Niizeki H, et al. Prostaglandin E2 increase in pachydermoperiostosis without 15-hydroprostaglandin dehydrogenase mutations. Acta Dermatovenereol, in press.
[15] Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 2009;25:1754–60.
[16] DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011;43:491–8.
[17] Robinson J, Thorvaldsdottir H, Winckler W, Guttman M, Lander E, Getz G, et al. Integrative genomics viewer. Nat Biotechnol 2011;29:24–6.
[18] Schuster V. Molecular mechanisms of prostaglandin transport. Annu Rev Physiol 1998;60:221–42.
[19] Zhang Z, Xia W, He J, Zhang Z, Ke Y, Yue H, et al. Exome sequencing identifies SLCO2A1 mutations as a cause of primary hypertrophic osteoarthropathy. Am J Hum Genet 2012;90:125–32.
[20] Seifert W, Kuhnisch J, Tuysuz B, Specker C, Brouwers A, Horn D. Mutations in the prostaglandin transporter encoding gene SLCO2A1 cause primary hypertrophic osteoarthropathy and isolated digital clubbing. Hum Mutat 2012;33:660–4.
[21] Nomura T, Chang HY, Lu R, Hankin J, Murphy RC, Schuster VL. Prostaglandin signaling in the renal collecting duct: release, reuptake, and oxidation in the same cell. J Biol Chem 2005;280:28424–29.
[22] Neufang G, Furstenberger G, Heidt M, Marks F, Müller-Decker K. Abnormal differentiation of epidermis in transgenic mice constitutively expressing cyclooxygenase-2 in skin. Proc Natl Acad Sci USA 2001;98:7629–34.
[23] Weinberg E, Topaz M, Dard M, Lyngstadaas P, Nemcovsky C, Weinreb M. Differential effects of prostaglandin E(2) and enamel matrix derivative on the proliferation of human gingival and dermal fibroblasts and gingival keratinocytes. J Periodontal Res 2010;45:731–40.
[24] Busch J, Frank V, Bachmann N, Otsuka A, Oji V, Metze D et al. Mutations in the prostaglandin transporter SLCO2A1 cause primary hypertrophic osteoarthropathywith digital Cubbing. J Invest Dermatol, in press.

Please cite this article in press as: Sasaki T, et al. Identification of mutations in the prostaglandin transporter gene *SLCO2A1* and its phenotype–genotype correlation in Japanese patients with pachydermoperiostosis. J Dermatol Sci (2012), http://dx.doi.org/10.1016/j.jdermsci.2012.07.008

Eur J Pediatr
DOI 10.1007/s00431-011-1644-x

ORIGINAL ARTICLE

Diagnosis and treatment trends in mucopolysaccharidosis I: findings from the MPS I Registry

Kristin D'Aco · Lisa Underhill · Lakshmi Rangachari · Pamela Arn · Gerald F. Cox · Roberto Giugliani · Torayuki Okuyama · Frits Wijburg · Paige Kaplan

Received: 8 November 2011 / Accepted: 29 November 2011

Abstract Our objective was to assess how the diagnosis and treatment of mucopolysaccharidosis I (MPS I) have changed over time. We used data from 891 patients in the MPS I Registry, an international observational database, to analyze ages at symptom onset, diagnosis, treatment initiation, and treatment allocation (hematopoietic stem cell transplantation, enzyme replacement therapy with laronidase, both, or neither) over time for all disease phenotypes (Hurler, Hurler–Scheie, and Scheie syndromes). The interval between diagnosis and treatment has become shorter since laronidase became available in 2003 (gap during 2006–2009: Hurler—0.2 year, Hurler–Scheie—0.5 year, Scheie—1.4 years). However, the age at diagnosis has not decreased for any MPS I phenotype over time, and the interval between symptom onset and treatment initiation remains substantial for both Hurler–Scheie and Scheie patients (gap during 2006–2009, 2.42 and 6.71 years, respectively). Among transplanted patients, an increasing proportion received hematopoietic stem cells from cord blood (34 out of 64 patients by 2009) and was also treated with laronidase (42 out of 45 patients by 2009). *Conclusions*: Despite the availability of laronidase since 2003, the diagnosis of MPS I is still substantially delayed for patients with Hurler–Scheie and Scheie phenotypes, which can lead to a suboptimal treatment outcome. Increased awareness of MPS I signs and symptoms by primary care providers and pediatric subspecialists is crucial to initiate early treatment and to improve the quality of life of MPS I patients.

Keywords Mucopolysaccharidosis I · Hurler · Hurler–Scheie · Scheie · Laronidase · Enzyme replacement therapy · Hematopoietic stem cell transplant

Introduction

The Mucopolysaccharidosis I (MPS I) Registry was created in 2003 with the purpose of characterizing the natural history and long-term health and treatment outcomes of this rare, life-

K. D'Aco (✉) · P. Kaplan
Section of Metabolic Diseases,
Children's Hospital of Philadelphia,
3501 Civic Center Boulevard,
Philadelphia, PA 19104, USA
e-mail: dacok@email.chop.edu

L. Underhill · L. Rangachari · G. F. Cox
Genzyme, a Sanofi Company,
Cambridge, MA, USA

P. Arn
Division of Genetics, Nemours Children's Clinic,
Jacksonville, FL, USA

G. F. Cox
Division of Genetics, Children's Hospital Boston,
Department of Pediatrics, Harvard Medical School,
Boston, MA, USA

R. Giugliani
Department of Genetics/UFRGS,
Medical Genetics Service/HCPA and INAGEMP,
Porto Alegre, Brazil

T. Okuyama
Center for Lysosomal Storage Diseases,
National Center for Child Health and Development,
Tokyo 157-8535, Japan

F. Wijburg
Department of Pediatrics, Division of Metabolic Diseases
and Amsterdam Lysosome Center "Sphinx,"
Academic Medical Centre, University of Amsterdam,
Amsterdam, Netherlands

Published online: 11 January 2012

threatening genetic disorder [25]. This report looks at how diagnosis and disease-specific treatment of MPS I have changed over time by analyzing aggregate data from nearly 900 patients enrolled in the MPS I Registry.

MPS I (McKusick 607014) is an autosomal recessive lysosomal storage disorder that is caused by deficient enzyme activity of α-L-iduronidase (IDUA), leading to lysosomal accumulation of glycosaminoglycans (GAGs) in multiple tissues throughout the body. MPS I is a chronic, progressive disease affecting the heart, eyes, bones, joints, respiratory system, facial appearance, viscera, and often the central nervous system. It has historically been classified clinically into three syndromes based on age of onset, rapidity of progression, and presence and degree of cognitive involvement [23]. Hurler syndrome describes patients with the most severe form of MPS I, with signs and symptoms typically appearing in infancy and a median age of death of 6.8 years when untreated [20]. Patients with Scheie syndrome present later in childhood and demonstrate slower symptom progression with preservation of cognition and survival into adulthood [30]. Hurler–Scheie describes an intermediate form with no or mild cognitive impairment and death usually occurring in adolescence or early adulthood when untreated. Patients with Hurler–Scheie and Scheie syndromes also have been referred to as having attenuated MPS I. Collectively, these forms represent different degrees of severity along a disease spectrum without strict clinical, biochemical, or molecular diagnostic criteria in place to differentiate them.

With an overall prevalence of 1:100,000 live births [19, 20, 27] and significant variability in presentation, diagnosis of MPS I in all its forms poses a true challenge [8, 32]. Initial diagnosis is primarily based on physician recognition of signs and symptoms. Deficient IDUA activity and excess urinary GAG excretion are seen in all patients, but do not accurately predict disease severity or form. Although some genotype–phenotype correlations have been established [11, 29], most known disease-causing mutations (over 100 to date) are individually unique ("private") and uncharacterized. Treatment of MPS I has also proven challenging as disease-specific treatment options are limited, intensive, and not curative. Hematopoietic stem cell transplantation (HSCT) has been used to treat more than 500 patients with MPS I since 1981 [1, 18] and is typically recommended for patients with Hurler syndrome under 2 years of age with normal cognition (DQ>70), as it can prolong survival, preserve neurocognition, and ameliorate some somatic features [21]. However, due to its significant morbidity and mortality, HSCT is reserved for the most severe form of MPS I [5, 6, 28]. The allogenic stem cell infusion is given following conditioning with chemotherapeutic agents used to suppress the immune response; when successful, the transplant is a one-time procedure, though graft failure may necessitate subsequent transplants. Enzyme replacement therapy (ERT) with laronidase (recombinant human α-L-iduronidase; Aldurazyme®, BioMarin Pharmaceutical and Genzyme, a Sanofi Company) was approved in 2003 to treat the non-neurologic manifestations of MPS I and is the primary treatment option for patients with Hurler–Scheie and Scheie syndromes. Laronidase is also used to treat Hurler patients who are not candidates for HSCT because of age, health status, access to transplant, or parental choice. Laronidase must be given as a weekly peripheral or central intravenous infusion and is a lifelong therapy.

The timing of treatment initiation, and therefore of diagnosis, is thought to be an important factor for the success of both HSCT and laronidase. Developmental outcomes are better when transplant occurs before 24 months of age [26]. Laronidase may also be more beneficial when started early, as suggested by a case report of a sib pair with Hurler–Scheie syndrome [15]. Other than laronidase and HSCT, additional management of MPS I is symptom-based and largely supportive, such as surgical interventions (e.g., adenotonsillectomy, hernia repair, ventriculoperitoneal shunt, cardiac valve replacement, carpal tunnel release, spinal decompression); physical, occupational, and speech therapies; respiratory support (e.g., continuous positive pressure ventilation with oxygen supplementation); hearing aids; and medications for pain and gastrointestinal disturbances.

With the intent of improving the long-term health outcomes and quality of life of patients with MPS I worldwide, we report how the chronology of symptom onset, diagnosis, and treatment initiation with HSCT and laronidase have evolved over time among Registry patients. We also analyze treatment trends among patients in the MPS I Registry with regard to the use of HSCT and laronidase by reported phenotypes. Although there are some genotype–phenotype correlations in MPS I [29], genotype information was not included in the analysis since treatment decisions are usually based on clinical manifestations, as newborn screening is not yet available.

Methods

Variables analyzed and statistical methods

Data entered into the Registry as of March 2010 were analyzed. The 891 MPS I patients came from 179 sites in 33 countries (Table 1). Regionally, 46.6% of the patients came from Europe and the Middle East, 35.1% from North America, 14.9% from Latin America, and 3.4% from Asia Pacific. MPS I forms (Hurler, Hurler–Scheie, Scheie, and unknown phenotypes) were analyzed by year of treatment initiation and year of diagnosis with respect to the following treatment groups: HSCT, ERT with laronidase, both laronidase and HSCT, or neither treatment. A phenotype designation of "unknown" signifies that the reporting physician either checked

Table 1 Enrollment in the MPS I Registry by region and country

Region	Country	Number of patients
Europe and Middle East (47%, n=415)	Belgium	9
	Czech Republic	11
	Denmark	5
	France	63
	Germany	28
	Hungary	2
	Ireland	9
	Italy	26
	Netherlands	37
	Norway	1
	Poland	20
	Portugal	5
	Russia	1
	Saudi Arabia	2
	Slovakia	1
	Spain	25
	Sweden	4
	Turkey	3
	UK	163
North America (35%, n=313)	Canada	54
	USA	259
Latin America (15%, n=133)	Argentina	17
	Brazil	82
	Chile	7
	Colombia	6
	Mexico	20
	Venezuela	1
Asia Pacific (3%, n=30)	Australia	1
	Japan	8
	Korea	13
	New Zealand	1
	Singapore	1
	Taiwan	6
		Total 891

"undetermined" or left the field blank on the Patient Enrollment form. Treatment chronology was determined by analyzing age at symptom onset, diagnosis, and first disease-specific treatment (either HSCT or ERT) in relation to reported phenotype and year of diagnosis. These analyses excluded patients who reported symptom onset after diagnosis, as occurs in siblings of children already carrying an MPS I diagnosis, whose diagnosis and treatment chronology are not representative of the general MPS I population. Three time periods were examined: patients diagnosed before 2003 (prior to laronidase approval), patients diagnosed in 2003–2005, and patients diagnosed in 2006–2009. The latter two time periods are not event-specific but were chosen to allow for sufficient numbers of patients in each group for meaningful comparison. Among Hurler patients who underwent HSCT, age at first HSCT was analyzed by year of first HSCT.

Treatment initiation was defined as the initial laronidase infusion or the initial HSCT, whichever treatment modality was used first. Among transplanted patients, the stem cell source (bone marrow, umbilical cord blood, or peripheral blood) was determined. Among transplanted patients receiving laronidase, the timing of laronidase with respect to transplant was analyzed. Peri-transplant ERT was defined as laronidase given at any time during the interval 6 months prior and 3 months after HSCT. With respect to use of ERT in conjunction with transplantation, the distribution of patients by number and location (country) of treatment centers was also determined. Variables are summarized using descriptive statistics, including mean, median, standard deviation, designated percentiles, and minimum and maximum values. As data are not available for all variables in every patient, the number of observations is always designated.

Results

Patient demographics

The demographic profiles of the 891 Registry patients are shown in Table 2. Patients classified as having Hurler syndrome made up more than half of the study population, while patients with the Hurler–Scheie and Scheie forms made up one quarter and one tenth, respectively. Caucasian patients made up >80% of the Hurler and Scheie groups, but only 61% of the Hurler–Scheie group. Approximately 9% of patients had an unknown form. Males and females were equally distributed in this MPS I population, as expected for an autosomal recessive disorder. Consistent with clinical severity, the median ages of symptom onset, diagnosis, and treatment initiation (HSCT or laronidase) were earliest for Hurler patients (0.5 year, 0.8 year, and 1.4 years, respectively), intermediate for Hurler–Scheie patients (1.9, 3.8, and 8.6 years), and latest for Scheie patients (5.4, 9.4, and 17.1 years). The intervals between median age at onset of symptoms to diagnosis and from diagnosis to treatment initiation were on the order of several months for Hurler patients, a few years for Hurler–Scheie patients, and several years for Scheie patients.

Chronology of symptom onset, MPS I diagnosis, and treatment initiation

Figure 1 shows the median age at symptom onset, diagnosis, and initiation of treatment with either HSCT or laronidase for each disease form by year of diagnosis: <2003, 2003–2005, and 2006–2009. Several notable trends were observed. The

Table 2 Mucopolysaccharidosis I Registry: patient demographics

Baseline characteristics	Hurler	Hurler–Scheie	Scheie	Undetermined	Missing	Overall
Number of patients (%)	508 (57)	209 (23.5)	97 (10.9)	28 (3.1)	49 (5.5)	891
Male (%)	255 (50)	95 (46)	47 (49)	14	31	442 (50)
Caucasian (%)	405 (81.2)	127 (61.4)	81 (83.5)	21	3	637 (76.3)
Black (%)	16 (3.2)	13 (6.3)	2 (2.1)	0	0	31 (3.7)
Hispanic (%)	38 (7.6)	14 (6.8)	1 (1)	4	1	58 (6.9)
Asian (%)	13 (2.6)	37 (17.9)	7 (7.2)	1	1	59 (7.1)
Other ethnicity (%)	27 (5.4)	16 (7.7)	6 (6.2)	1	0	50 (6)
Median age at last data entry (range) [total number]	6.3 (0.4–35.9) [508]	13 (1.9–49.8) [209]	22.2 (5.4–64.3) [97]	6.9 (0.3–46.7) [28]	8.2 (0.9–40.1) [49]	8.6 (0.3–64.3) [891]
Median age of symptom onset (range) [total number]	0.5 (0–6.5) [485]	1.9 (0–12.4) [187]	5.4 (0–33.8) [87]	0.8 (0.1–7.2) [24]	1.6 (0.6–5.7) [4]	0.8 (0–33.8) [787]
Median age of diagnosis (range) [total number]	0.8 (0–23.8) [508]	3.8 (0–38.7) [209]	9.4 (0–54.1) [97]	1.3 (0–43.9) [28]	1.6 (0.3–33) [49]	1.3 (0–54.1) [891]
Median age of first treatment (range) [total number]	1.4 (0.1–31.2) [438]	8.6 (0.3–47.2) [197]	17.1 (3.1–62.9) [85]	2.9 (0.3–44) [23]	6.6 (0.5–34.8) [27]	2.8 (0.1–62.9) [770]
Median age at death (range) [total number]	3.8 (0.4–27.2) [156]	17.4 (7.5–30.3) [16]	29 (17.4–46.6) [4]	5.1 (1.8–9.7) [4]	0	5.1 (0.4–46.6) [180]

median age at symptom onset remained relatively stable for Hurler and Scheie patients over time, whereas it decreased by approximately 1.5 years for Hurler–Scheie patients after 2003. On the other hand, the age at diagnosis was stable over time for all forms. The interval between median age at diagnosis and initiation of treatment decreased for all groups after 2003, when laronidase was approved. The decrease was more notable for Hurler–Scheie and Scheie patients (several years) than for Hurler patients (several months). During 2006–2009, the median interval between age at diagnosis and initiation of treatment for individuals with Hurler–Scheie was 0.5 year, and for Scheie patients, 1.4 years. However, even after the 2003 approval of laronidase, the median interval between the age of symptom onset and the age of treatment initiation between 2006 and 2009 for the Hurler–Scheie and Scheie patients was 2.42 and 6.71 years, respectively.

Disease-specific treatment allocation by MPS I phenotype

The allocation of disease-specific treatments over time for the 770 treated patients is shown in Fig. 2. HSCT has been used primarily in Hurler patients, but especially so since 2003. Since laronidase became available, the proportion of patients treated with laronidase has increased in all disease forms. Among Hurler patients, almost half of those who began treatment in 2006–2009 received laronidase alone, and approximately two thirds of those who were transplanted also received laronidase.

Analysis of untreated MPS I patients

Of the 891 MPS I patients, 116 (13%) were listed as untreated with either laronidase or HSCT. Over time, the proportion of untreated patients has decreased. When analyzed by year of diagnosis, 83 of the 534 patients (16%) diagnosed before 2003 were untreated, as compared with 21 of the 197 patients (11%) diagnosed in 2003–2005, and 11 of the 159 patients (7%) diagnosed in 2006–2009. Among untreated patients, all three disease forms as well as patients with an undetermined/missing phenotype were represented. Of the 115 total untreated patients who had a date of diagnosis, 56 (49%) were Hurler patients diagnosed before 2003.

HSCT and laronidase treatment trends

Among all patients with Hurler syndrome receiving HSCT, the median age at first transplant has not changed over time (Fig. 3) though the proportion of patients receiving stem cells from cord blood or peripheral blood rather than bone marrow has increased from 26 out of 158 patients (16.5%) before 2003 to 33 out of 65 patients in 2003–2005 and 39 out of 64 patients in 2006–2009 (Fig. 4). Among patients receiving stem cells from bone marrow or peripheral blood, the majority of donors were unrelated and the proportion of

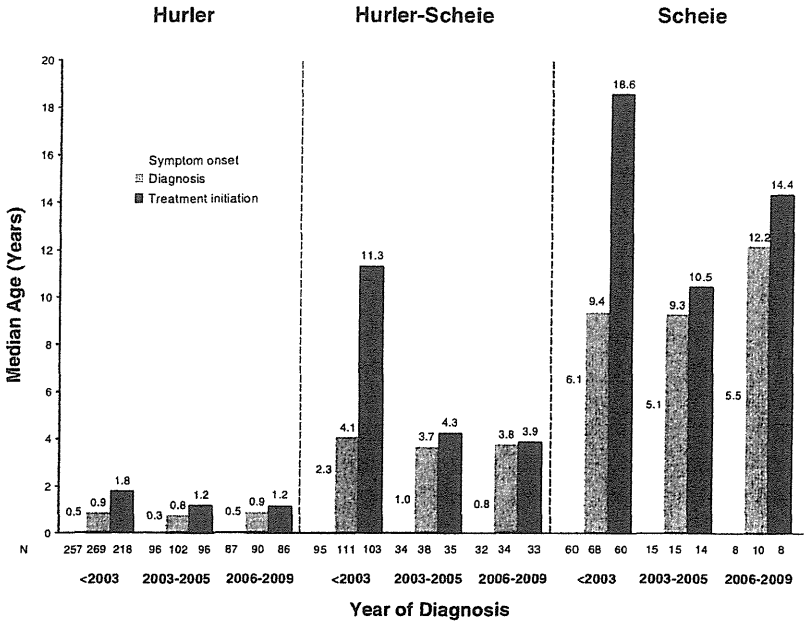

Fig. 1 Median age at symptom onset, diagnosis, and initiation of treatment. *Numbers* below each *bar* denote the number of Registry patients in each analysis and year of diagnosis. All data are as of March 2010. Median age is given in years

related versus unrelated did not change appreciably over time, although small sample sizes and missing donor information may have masked any trends.

The use of laronidase has greatly increased over time among patients receiving HSCT, the vast majority of whom have Hurler syndrome (Fig. 5). Since 2007, 42 out of 45

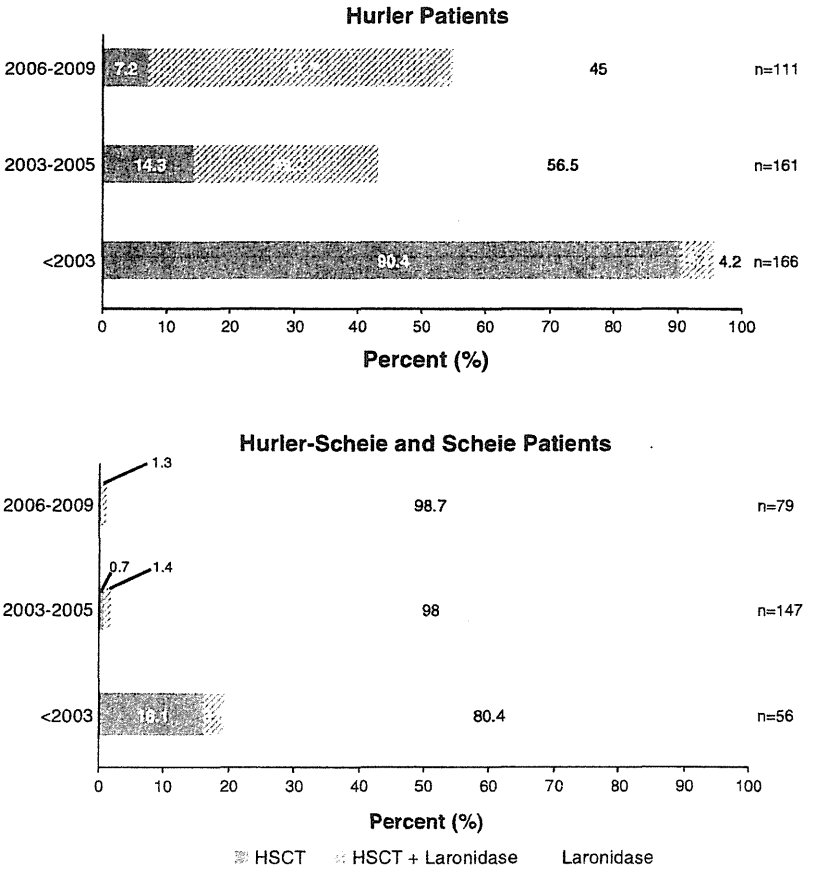

Fig. 2 Distribution of treatment modalities over time. *Data* represent all patients enrolled in the Registry as of March 2010 who report treatment with either HSCT, laronidase, or both. An additional 116 patients reported no treatment

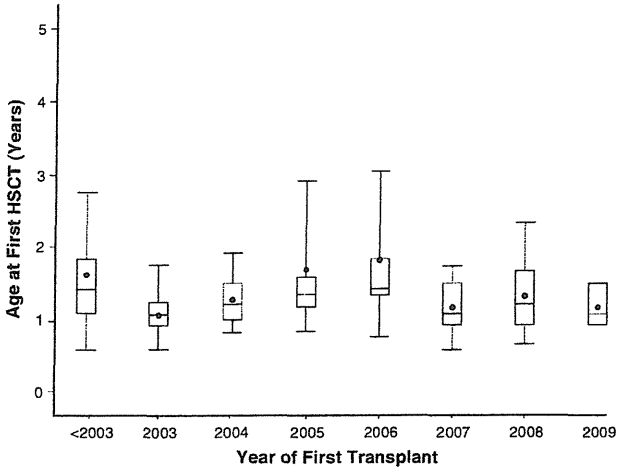

Fig. 3 Median age at first transplant by year of first transplant in Hurler patients. All data are as of March 2010. *Horizontal lines* within in each *box* represent the median age, and *dots* represent the mean age. *Lower and upper box edges* represent the 25th and 75th percentiles; *lower and upper whiskers* represent the 5th and 95th percentiles

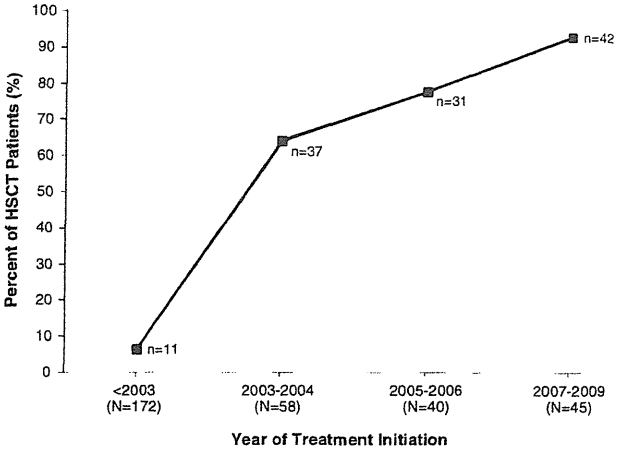

Fig. 5 Use of laronidase with HSCT by year of first treatment. First treatment is defined as either HSCT or laronidase, whichever occurred first. For all treatment periods, 92% of Registry patients who received both HSCT and laronidase received laronidase during the peri-transplant period, defined as any time during the interval 6 months before and 3 months after HSCT. All data are as of March 2010

transplanted patients also reported receiving laronidase. While this analysis included laronidase given to HSCT patients at any time in their disease course, nearly all patients (102 out of 111; 91.9%) who received both treatments after 2003 received laronidase in the peri-transplant period. The 42 patients who received laronidase and HSCT, with first treatment in 2007–2009, came from ten centers (18 patients) in the USA and seven centers (24 patients) in Europe (Spain, Belgium, Italy, UK, Netherlands, Czech Republic). Ten of the 17 centers reported only one patient, while the maximum number of patients treated at any one center was 11. The three patients who underwent their first transplant in 2007–2009 and did not receive laronidase came from different centers in Europe.

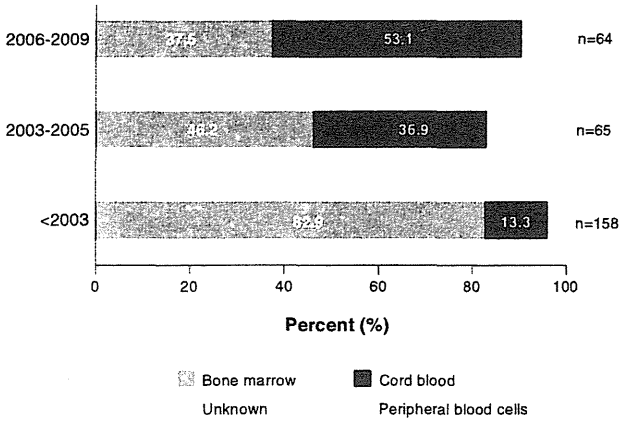

Fig. 4 Hematopoietic stem cell source by year of first HSCT in transplanted patients. Depicted are the relative proportions of various sources of stem cells used for HSCT. All data are as of March 2010

Discussion

Analysis of data from the MPS I Registry has allowed for improved understanding of the natural history of MPS I and will enable evaluation of the impact of therapeutic advances on morbidity and mortality [2, 3, 25, 30]. Limitations of observational registries such as the MPS I Registry, in which anonymized data are submitted voluntarily, include incomplete, missing, or inaccurate data, as well as losses to follow-up and lack of standardization of patient assessments. The voluntary nature of the Registry may also lead to biased data as not all physicians who manage MPS I patients use the MPS I Registry. Despite these potential biases, the data presented here offer insight into diagnosis and treatment trends in the largest cohort of MPS I patients ever evaluated.

Our data show that the median age at diagnosis has not decreased over time for any form of MPS I, despite available treatment options. Although Hurler patients tend to be diagnosed within a few months of symptom onset, Hurler–Scheie and Scheie patients still remain undiagnosed for years after symptom onset (Fig. 1). This gap not only represents years of reduced quality of life that would likely be ameliorated by treatment, but also a lost opportunity for preventing or delaying irreversible disease manifestations, as well as for genetic counseling about the risk of future siblings being affected. Sibling case studies and studies in the MPS I dog model suggest that starting enzyme replacement therapy with laronidase shortly after birth can significantly improve clinical outcome [13–15, 33]. Most patients who begin treatment at a very young age have an older sibling with a known diagnosis of MPS I. While studies of affected sib pairs will certainly provide more information

about the potential impact of early laronidase treatment, prompt clinical diagnosis will be imperative for the majority of MPS I patients to benefit from this information. Demonstration of substantial improvement in clinical outcomes with earlier diagnosis and treatment of MPS I along with an enhanced ability to predict phenotype at birth through genotype–phenotype correlations and/or disease biomarkers will underscore the need for broad implementation of a newborn screening program for this rare, life-threatening disorder and will aid in decisions of which patients to treat with what therapies, and how early to treat them.

As would be expected, the time interval from diagnosis to initiation of disease-specific treatment decreased following the availability of laronidase in 2003, especially for Hurler–Scheie and Scheie patients, who until then had generally been offered only palliative, symptom-based treatments. Prior to 2003, ERT with laronidase was available only through clinical trials, the largest of which enrolled mostly older children, adolescents, and young adults with a mean duration of 13 years since symptom onset [9]. In addition, the proportion of untreated patients has decreased over time. Despite the decrease in time from diagnosis to treatment, there still exists a delay of 0.5 year and 1.4 years between median ages at diagnosis and treatment for Hurler–Scheie and Scheie patients, respectively, during the most recent time interval of 2006–2009. In comparison, for patients with the Hurler form, there was almost no delay between diagnosis and treatment. This may be due to parents and/or diagnosing physicians perceiving the attenuated phenotypes as milder, or less urgent, thereby further delaying time to treatment. In addition, it may represent time needed for laronidase approval by reimbursement authorities [7]. There may also be regional differences in the delay between diagnosis and treatment, given that laronidase was not approved outside the European Union and the USA until 2005.

Interestingly, when looking at Fig. 1, the age of symptom onset appears to have decreased somewhat in both the Hurler–Scheie and Scheie populations. Rather than a true change in the natural history of MPS I disease, this finding is more likely to be secondary to small patient numbers and the retrospective awareness that certain common early symptoms (such as hernia and chronic otitis media) are often related to MPS I. Also, while the actual age of diagnosis has not greatly improved, parents and physicians may have improved in the retrospective recognition of the common early manifestations of the disease, which led to younger ages of symptom onset in the attenuated patient populations.

Of note, patients with an unknown disease form tended to present with MPS I symptoms between the ages of patients classified as Hurler and Hurler–Scheie. Similarly, they were typically diagnosed and treated at ages in between those of Hurler and Hurler–Scheie patients as well. This suggests that patients in the Registry with an unknown disease form are more likely to be on the more severe end of the MPS I spectrum.

This analysis identified several shifts in clinical practice, such as the increasing use of laronidase among transplanted patients, particularly in the peri-transplant period, and the increasing use of cord blood as a stem cell source. The proportion of unrelated versus related donors for HSCT has remained relatively stable as unrelated bone marrow donors have been replaced by unrelated cord blood donors. Use of laronidase in the peri-transplant period has been reported to be safe and well tolerated without interfering with engraftment or increasing the risk of graft-versus-host disease [6, 12, 16, 17, 31, 34]. Laronidase treatment may be particularly beneficial in patients in poor clinical condition prior to HSCT, to help improve their eligibility for transplant and tolerance of the full-intensity transplant conditioning [12, 16, 17, 24, 31].

In addition, an increasing proportion of Hurler patients in the Registry is receiving laronidase alone (Fig. 2), reflecting the fact that HSCT is not available in many parts of the world, while the majority of transplanted patients are from North America and Europe. A recent MPS I Registry analysis comparing patients from Latin America to those from the rest of the world found that less than 1% of patients in Latin America had been transplanted, compared to 27% of patients from the rest of the world [22]. Our MPS I Registry patients came from over 30 countries in Europe and the Middle East, North America, Latin America, and Japan and Asia Pacific (Table 1). Regional differences in treatment availability would also impact the ages at treatment initiation as well as the proportion of untreated patients. For example, in Brazil, which has a universal-access public health care system, neither laronidase nor HSCT is covered by government or specialized pharmaceutical programs [7].

Narrowing the gap between symptom onset and MPS I diagnosis is of the utmost importance and largely relies on increased recognition of clinical red flags by community physicians and pediatric specialists managing the many MPS I-related symptoms, such as otolaryngologists, orthopedists, rheumatologists, and ophthalmologists. While there is significant variability of first presenting symptom both within and between phenotypes, certain symptoms have been consistently noted to appear earlier than others in the MPS I population. Coarse facial appearance, abdominal distension, and corneal clouding are among the most common presenting symptoms in children with Hurler syndrome [4, 10]. In the patients with attenuated forms, joint stiffness/contractures, recurrent ENT symptoms, corneal clouding, and umbilical hernias are the most prevalent initial symptoms [4, 30, 32]. In addition, certain constellations of symptoms should raise a physician's suspicion of MPS I. In a 2009 study of surgical procedures in 544 MPS I Registry patients representing all clinical forms, 72% had had at least one surgical procedure, with a median of 3 to 4 surgeries per patient, and with nearly half of the patients reporting two or more surgeries by age 4 years [2]. Surgeries often appeared unrelated, such as combinations of ear tubes,

hernia repair, and tendon releases in the same patient, and often preceded the patient's MPS I diagnosis, particularly in the attenuated phenotypes. Many procedures were also performed at ages atypical for the general pediatric population, such as younger ages of tonsillectomy/adenoidectomy, and older ages of hernia repair in patients with MPS I.

Given the rarity of MPS I, increased efforts to educate community pediatricians and pediatric surgical subspecialists on clinical red flags that should prompt a genetics referral is of the utmost importance. An MPS I Registry analysis of early presenting symptoms for each MPS I form is underway to aid the front-line clinicians with earlier symptom recognition. Furthermore, future analysis of MPS I long-term therapeutic outcomes is merited, as large-scale evidence of improved outcomes with earlier diagnosis and treatment would reinforce the need for increased recognition of clinical red flags, allow better understanding of the therapeutic potential of current treatment modalities, and underscore the need for newborn screening for this disorder.

Acknowledgments The authors thank Jessica Kong, Gregory Fagan, and Ying Zhang of the MPS I Registry for expert biostatistical support and Sarah Kulke, MD (Genzyme Global Medical Affairs) for critical review of the manuscript.

Conflicts of interest KD receives grant funding from Genzyme for research in lysosomal storage diseases; LU, LR, and GC are employees of Genzyme, a Sanofi company, which distributes laronidase (Aldurazyme), a treatment for MPS I; PA receives honoraria and travel expenses to attend scientific meetings from Genzyme; RG receives research grants, travel expenses to attend scientific meetings, and speaker honoraria from Genzyme; TO receives honoraria and travel expenses to attend scientific meetings from Genzyme; FW receives research grants and reimbursement of expenses and honoraria for lectures on lysosomal storage diseases from Genzyme; PK receives honoraria for presentations and board meetings, travel expenses to meetings, and paid and unpaid consultancy work for Genzyme and has been a principal investigator in Genzyme-sponsored clinical trials.

References

1. Aldenhoven M, Boelens JJ, de Koning TJ (2008) The clinical outcome of Hurler syndrome after stem cell transplantation. Biol Blood Marrow Transplant 14:485–498
2. Arn P, Wraith J, Underhill L (2009) Characterization of surgical procedures in patients with mucopolysaccharidosis type I: findings from the MPS I Registry. J Pediatr 154(859–864):e853
3. Arn P, Whitley CB, Wraith JE, Underhill L, Rangachari L, Cox GF (2011) Postoperative mortality in patients with mucopolysaccharidosis I: findings from the MPS I Registry. J Pediatr Surg (in press)
4. Bodamer OA (2007) Clinical characteristics of MPS I patients in the MPS I Registry. The American Society of Human Genetics, San Diego
5. Boelens JJ, Wynn RF, O'Meara A, Veys P, Bertrand Y, Souillet G, Wraith JE, Fischer A, Cavazzana-Calvo M, Sykora KW, Sedlacek P, Rovelli A, Uiterwaal CS, Wulffraat N (2007) Outcomes of hematopoietic stem cell transplantation for Hurler's syndrome in Europe: a risk factor analysis for graft failure. Bone Marrow Transplant 40:225–233
6. Boelens JJ, Rocha V, Aldenhoven M, Wynn R, O'Meara A, Michel G, Ionescu I, Parikh S, Prasad VK, Szabolcs P, Escolar M, Gluckman E, Cavazzana-Calvo M, Kurtzberg J (2009) Risk factor analysis of outcomes after unrelated cord blood transplantation in patients with hurler syndrome. Biol Blood Marrow Transplant 15:618–625
7. Boy R, Schwartz IV, Krug BC, Santana-da-Silva LC, Steiner CE, Acosta AX, Ribeiro EM, Galera MF, Leivas PG, Braz M (2011) Ethical issues related to the access to orphan drugs in Brazil: the case of mucopolysaccharidosis type I. J Med Ethics 37:233–239
8. Cimaz R, Vijay S, Haase C, Coppa GV, Bruni S, Wraith E, Guffon N (2006) Attenuated type I mucopolysaccharidosis in the differential diagnosis of juvenile idiopathic arthritis: a series of 13 patients with Scheie syndrome. Clin Exp Rheumatol 24:196–202
9. Clarke L, Wraith JE, Beck M, Kolodny EH, Pastores G, Muenzer J, Rapoport DM, Berger KI, Sidman M, Cox GF (2009) Long-term efficacy and safety of laronidase in the treatment of mucopolysaccharidosis I. Pediatrics 123:229–240
10. Colville GA, Bax MA (1996) Early presentation in the mucopolysaccharide disorders. Child Care Health Dev 22:31–36
11. Cox GF, Wraith JE, Whitley CB, Wijburg FA, Guffon N (2009) Genotype frequencies in the MPS I Registry. Mol Genet Metab 96: S19 (abstract 31)
12. Cox-Brinkman J, Boelens JJ, Wraith JE, O'Meara A, Veys P, Wijburg FA, Wulffraat N, Wynn RF (2006) Haematopoietic cell transplantation (HCT) in combination with enzyme replacement therapy (ERT) in patients with Hurler syndrome. Bone Marrow Transplant 38:17–21
13. Dickson PI, Hanson S, McEntee MF, Vite CH, Vogler CA, Mlikotic A, Chen AH, Ponder KP, Haskins ME, Tippin BL, Le SQ, Passage MB, Guerra C, Dierenfeld A, Jens J, Snella E, Kan SH, Ellinwood NM (2010) Early versus late treatment of spinal cord compression with long-term intrathecal enzyme replacement therapy in canine mucopolysaccharidosis type I. Mol Genet Metab 101:115–122
14. Dierenfeld AD, McEntee MF, Vogler CA, Vite CH, Chen AH, Passage M, Le S, Shah S, Jens JK, Snella EM, Kline KL, Parkes JD, Ware WA, Moran LE, Fales-Williams AJ, Wengert JA, Whitley RD, Betts DM, Boal AM, Riedesel EA, Gross W, Ellinwood NM, Dickson PI (2010) Replacing the enzyme alpha-L-iduronidase at birth ameliorates symptoms in the brain and periphery of dogs with mucopolysaccharidosis type I. Sci Transl Med 2:60ra89
15. Gabrielli O, Clarke LA, Bruni S, Coppa GV (2010) Enzyme-replacement therapy in a 5-month-old boy with attenuated presymptomatic MPS I: 5-year follow-up. Pediatrics 125:e183–e187
16. Grewal SS, Wynn R, Abdenur JE, Burton BK, Gharib M, Haase C, Hayashi RJ, Shenoy S, Sillence D, Tiller GE, Dudek ME, van Royen-Kerkhof A, Wraith JE, Woodard P, Young GA, Wulffraat N, Whitley CB, Peters C (2005) Safety and efficacy of enzyme replacement therapy in combination with hematopoietic stem cell transplantation in Hurler syndrome. Genet Med 7:143–146
17. Hirth A, Berg A, Greve G (2007) Successful treatment of severe heart failure in an infant with Hurler syndrome. J Inherit Metab Dis 30:820
18. Hobbs JR, Hugh-Jones K, Barrett AJ, Byrom N, Chambers D, Henry K, James DC, Lucas CF, Rogers TR, Benson PF, Tansley LR, Patrick AD, Mossman J, Young EP (1981) Reversal of clinical features of Hurler's disease and biochemical improvement after treatment by bone-marrow transplantation. Lancet 2:709–712
19. Meikle PJ, Hopwood JJ, Clague AE, Carey WF (1999) Prevalence of lysosomal storage disorders. JAMA 281:249–254

20. Moore D, Connock MJ, Wraith E, Lavery C (2008) The prevalence of and survival in mucopolysaccharidosis I: Hurler, Hurler-Scheie and Scheie syndromes in the UK. Orphanet J Rare Dis 3:24
21. Muenzer J, Wraith JE, Clarke LA (2009) Mucopolysaccharidosis I: management and treatment guidelines. Pediatrics 123:19–29
22. Muñoz-Rojas M, Bay L, Sanchez L, van Kuijck M, Ospina S, Cabello J, Martins A (2011) Clinical manifestations and treatment of mucopolysaccharidosis type I patients in Latin America as compared with the rest of the world J Inher Metab Dis 34:1029–1037
23. Neufeld EF, Muenzer J (2001) The mucopolysaccharidoses. In: Scriver C, Beaudet A, Sly W, Valle D, Childs B, Kinzler K, Vogelstein B (eds) The metabolic and molecular bases of inherited disease. McGraw Hill, New York, pp 3421–3452
24. Orchard PJ, Milla C, Braunlin E, Defor T, Bjoraker K, Blazar BR, Peters C, Wagner J, Tolar J (2009) Pre-transplant risk factors affecting outcome in Hurler syndrome. Bone Marrow Transplant 45:1239–1246
25. Pastores G, Arn P, Beck M, Clarke J, Guffon N, Kaplan P, Muenzer J, Norato D, Shapiro E, Thomas J, Viskochil D, Wraith J (2007) The MPS I registry: design, methodology, and early findings of a global disease registry for monitoring patients with mucopolysaccharidosis type I. Mol Genet Metab 91:37–47
26. Peters C, Shapiro EG, Anderson J, Henslee-Downey PJ, Klemperer MR, Cowan MJ, Saunders EF, deAlarcon PA, Twist C, Nachman JB, Hale GA, Harris RE, Rozans MK, Kurtzberg J, Grayson GH, Williams TE, Lenarsky C, Wagner JE, Krivit W (1998) Hurler syndrome: II. Outcome of HLA-genotypically identical sibling and HLA-haploidentical related donor bone marrow transplantation in 54 children. The Storage Disease Collaborative Study Group. Blood 91:2601–2608
27. Poorthuis BJ, Wevers RA, Kleijer WJ, Groener JE, de Jong JG, van Weely S, Niezen-Koning KE, van Diggelen OP (1999) The frequency of lysosomal storage diseases in the Netherlands. Hum Genet 105:151–156
28. Prasad VK, Kurtzberg J (2010) Transplant outcomes in mucopolysaccharidoses. Semin Hematol 47:59–69
29. Terlato NJ, Cox GF (2003) Can mucopolysaccharidosis type I disease severity be predicted based on a patient's genotype? A comprehensive review of the literature. Genet Med 5:286–294
30. Thomas JA, Beck M, Clarke JT, Cox GF (2010) Childhood onset of Scheie syndrome, the attenuated form of mucopolysaccharidosis I. J Inherit Metab Dis 33:421–427
31. Tolar J, Grewal SS, Bjoraker KJ, Whitley CB, Shapiro EG, Charnas L, Orchard PJ (2008) Combination of enzyme replacement and hematopoietic stem cell transplantation as therapy for Hurler syndrome. Bone Marrow Transplant 41:531–535
32. Vijay S, Wraith JE (2005) Clinical presentation and follow-up of patients with the attenuated phenotype of mucopolysaccharidosis type I. Acta Paediatr 94:872–877
33. Wang RY, Cambray-Forker EJ, Ohanian K, Karlin DS, Covault KK, Schwartz PH, Abdenur JE (2009) Treatment reduces or stabilizes brain imaging abnormalities in patients with MPS I and II. Mol Genet Metab 98:406–411
34. Wynn R, Mercer J, Page J, Carr T, Jones S, Wraith J (2009) Use of enzyme replacement therapy (laronidase) before hematopoietic stem cell transplantation for mucopolysaccharidosis I: experience in 18 patients. J Pediatr 154:135–139

Administration of Anti-CD3 Antibodies Modulates the Immune Response to an Infusion of α-glucosidase in Mice

Toya Ohashi[1–3], Sayoko Iizuka[1], Yohta Shimada[1], Takashi Higuchi[2], Yoshikatsu Eto[2], Hiroyuki Ida[1–3] and Hiroshi Kobayashi[1–3]

[1]Department of Gene Therapy, Institute of DNA Medicine, Research Center for Medical Sciences, The Jikei University School of Medicine, Tokyo, Japan; [2]Department of Genetic Disease and Genomic Science, The Jikei University School of Medicine, Tokyo, Japan; [3]Department of Pediatrics, The Jikei University School of Medicine, Tokyo, Japan

Animal and human studies of enzyme replacement therapy (ERT) for Pompe disease (PD) have indicated that antibodies (Abs) generated against infused recombinant human α-glucosidase (rhGAA) can have a negative impact on the therapeutic outcome and cause hypersensitivity reactions. We showed that parenteral administration of anti-CD3 Abs into mice can reduce the titer of anti-human GAA Abs in wild-type mice administered the enzyme. Mice that had been treated with anti-CD3 Abs and then subjected to a secondary challenge with rhGAA showed a lower increase in Ab titers than control mice. Moreover, the administration of anti-CD3 Abs also reduced the levels of pre-existing Abs. Treatment with anti-CD3 Abs also prevented a lethal hypersensitivity reaction and reduced the Ab titers in a mouse model of PD. Mice treated with anti-CD3 Abs showed reduced numbers of $CD4^+$ and $CD8^+$ cells, and an increased ratio of $CD4^+CD25^+/CD4^+$ and $CD4^+CD25^+FoxP3^+/CD4^+$ cells. When the $CD4^+CD25^+$ cells were depleted using anti-CD25 Abs, the observed reduction in Abs against the enzyme by anti-CD3 Abs was abrogated. This suggests that $CD4^+CD25^+$ cells are important for the immune suppressive activity of anti-CD3 Abs. In summary, anti-CD3 Abs are useful for inducing immune tolerance to ERT for PD.

Received 30 January 2012; accepted 17 June 2012; advance online publication 7 August 2012. doi:10.1038/mt.2012.133

INTRODUCTION

Pompe disease (PD) (also known as glycogen storage disease II [MIN 232300]) is a lysosomal storage disease (LSD) characterized by a deficiency of acid α-glucosidase (GAA) activity. Because of this deficiency, glycogen accumulates progressively in the heart and skeletal muscles with the resultant presentation of cardiomyopathy and muscle weakness. PD can be divided into two clinical entities: infantile- and late-onset PD. Patients with infantile-onset PD present with hypertrophic cardiomyopathy, hypotonia, muscle weakness, respiratory failure, feeding problems, and failure to thrive within the first few months of life. The disease progresses rapidly, resulting in premature death typically in the first year of life if left untreated. Late-onset PD (child and adult type) has a variable clinical presentation. The onset of clinical signs can occur as early as the first year of life and as late as the seventh decade of life. Patients with late-onset PD present with muscle weakness and respiratory failure, but not cardiac symptoms. Until 2006, there were no therapies to target the underlying basis of PD. The only available treatment was supportive therapy for heart and respiratory failure. In 2006, enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA) (aglucosidase alfa) (Myozyme; Genzyme) was approved for treating this disease in many countries. Lumizyme (aglucosidase alfa; Genzyme) was also approved for late-onset PD in the United States in 2010. Both enzymes harbor the same protein sequence, but have a slightly different carbohydrate composition. In a clinical trial involving infants, patients who were not undergoing ventilation were treated with biweekly infusions of rhGAA at either 20 or 40 mg/kg.[1] A nontreated historical cohort was used as the control group.[2] The treated patients lived longer and the proportion of ventilation-free patients was larger compared with the historical cohort. These observations clearly indicated that rhGAA was effective in treating infantile-onset PD. On the basis of these results, rhGAA was approved; however, until recently, there has been no research that clearly shows the effectiveness of rhGAA for late-onset PD. Recently, a randomized control trial was carried out in late-onset PD patients, and ERT was associated with an improved walking distance and stabilization of pulmonary function over an 18-month period.[3] From these findings, ERT for PD would appear to be effective for both infantile- and late-onset types. Although ERT has been shown to be effective in treating PD patients, some challenges remain. One of these challenges is the immune response to the infused enzyme. Animal and human studies of ERT for PD have indicated that the formation of antibodies (Abs) against rhGAA can reduce the efficacy of treatment.[1,4–9] Kishnani *et al.* retrospectively studied infantile-onset PD in patients with an onset at <6 months of age who received ERT.[7] They divided the patients into two groups: cross-reactive immune material (CRIM) negative and CRIM positive. The baseline demographics

Correspondence: *Toya Ohashi, Department of Gene Therapy, Institute of DNA Medicine, Research Center for Medical Sciences/Department of Pediatrics, The Jikei University School of Medicine 3-25-8 Nishishinbashi, Minato-ku, Tokyo 105-8461, Japan. E-mail: tohashi@jikei.ac.jp*

and disease-related characteristic were comparable between the two groups. Immunoglobulin G (IgG) Abs to rhGAA developed earlier and titers were higher and more sustained in the CRIM-negative group. The CRIM-negative patients also exhibited poorer clinical outcomes. They concluded that the effect of CRIM status on outcome appears to be mediated by Ab responses to the infused rhGAA. More recently, some CRIM-positive infantile-onset PD patients also developed high titers of Abs against rhGAA and had poorer clinical outcomes, similar to the CRIM-negative infantile-onset PD patients.[9] From these observations, we argue that the induction of tolerance against rhGAA would be highly desirable to improve therapeutic outcome and prevent hypersensitivity reactions.

There are reports describing approaches to inducing immune tolerance to rhGAA. These include the administration of methotrexate,[10] anti-CD20 Abs,[11,12] anti-IgE Abs,[13] adeno-associated vector-expressing GAA into the thymus,[14] genetically modified GAA-expressing self bone marrow cells,[15,16] oral rhGAA,[17] and adeno-associated vector containing a liver-specific promoter at the beginning of therapy.[18–20] In addition, Lipinski *et al.* reported that immune tolerance could also be induced by a gradual escalation of the administered dose of rhGAA.[21] The ideal agents for the induction of tolerance should be antigen-specific, present with minimal side-effects, and have a long-lasting effect; however, methotrexate, anti-CD20 Abs, and anti-IgE Abs do not meet these requirements. Recently, Waters *et al.* reported that parenteral administration of anti-CD3 Abs could induce immune tolerance to coagulation factor supplementation treatment.[22] This induction of tolerance was long-lasting and, once it was established, tolerance was maintained for a sustained period; moreover, they suggested that this tolerance was probably antigen-specific. From this observation, we decided to test whether parenteral administration of anti-CD3 Abs could also induce tolerance to rhGAA for PD. Here, we investigated whether parenteral administration of anti-CD3 Abs can reduce the host immune response to infused rhGAA in mice.

RESULTS

Immune response to rhGAA in wild-type (Balb/c and C57BL/6) and PD model (B6;129-*Gaa*^{tm1Rabn}/J) mice

In order to determine whether there was a difference in the immune reaction between wild-type (7–8-weeks-old Balb/c and C57BL/6) and PD model (23-27-weeks-old B6;129-*Gaa*^{tm1Rabn}/J) mice, rhGAA (10 mg/kg) was administered to three groups of mice once a week for a total of 4 doses. One week after the final administration of rhGAA, the anti-rhGAA Ab titers were analyzed. As shown in **Supplementary Figure S1**, Ab titers against rhGAA in PD mice were significantly higher than in the control groups (Balb/c and C57BL/6) ($P \leq 0.05$, Kruskal–Wallis test followed by *post-hoc* Dunn's test), whereas the Ab titers in the Balb/c mice were not significantly different to those in the C57BL/6 mice. We started this experiment using seven Balb/c, six C57BL/6, and eight PD model mice. Following the repeated administration (four times) of rhGAA, three of the eight PD model mice died from anaphylactic shock, and the Ab titers of these mice could not be assayed; however, the Ab titers in these expired mice were probably very high. Thus, if we had been able to include the data of the Ab titers from these expired mice, the Ab titers of the PD model mice would probably be much higher than the presented data. In addition, significant levels of Abs against rhGAA also developed in both groups of wild-type mice following the repeated administration of rhGAA, but they rarely died from anaphylaxis. Therefore, to prevent loss of mice from anaphylaxis, we used wild-type mice in most of the following experiments, unless otherwise stated.

Prevention of Ab formation by anti-CD3 Abs in wild-type mice

After the intravenous administration of anti-CD3 Abs (10 μg per dose) for five consecutive days, rhGAA (10 mg/kg) was administered to the mice once a week for a total of four doses. One week after the final administration of rhGAA, the anti-rhGAA Ab titers were analyzed. Anti-CD3 Abs significantly reduced the formation of Abs in BALB/c ($P = 0.0034$; **Figure 1a**) and C57BL/6 mice ($P = 0.0002$; **Figure 1b**). Therefore, we performed the following experiments using BALB/c mice, unless otherwise stated. To assess the duration of the effects of the anti-CD3 Abs, rhGAA (10 mg/dose once a week for a total of four doses) was readministered at 11 weeks after the first rhGAA challenge. The anti-rhGAA Ab titers were analyzed at 1 week after the final infusion of rhGAA. As shown in **Figure 1c**, the Ab titers just before the second rhGAA challenge were reduced in both hamster IgG-treated groups. After the second challenge, the Ab titers in the anti-CD3 Ab-treated group were significantly lower than in the hamster IgG-treated control group ($P = 0.0061$; **Figure 1c**). This observation indicated that the immune-suppressive effect of the anti-CD3 Abs was long-lasting.

Immune-suppressive effect of anti-CD3 Abs against pre-existing anti-rhGAA Abs in wild-type mice

We also tested the effect of anti-CD3 Abs against pre-existing anti-rhGAA Abs. BALB/c mice were immunized once weekly for a total of four doses with rhGAA followed by the administration of anti-CD3 Abs or hamster IgG as a control for five consecutive days. Three days after the final administration of the anti-CD3 Abs, the Ab titers against rhGAA were measured. The study design is shown in **Figure 2a**. The Abs against rhGAA were elevated to the same level in both groups following immunization with rhGAA (indicated as post-immune *by rhGAA*, $P = 0.7132$), but the Ab titers in the anti-CD3 Ab group were significantly lower than in the hamster IgG-treated group (indicated as post-hamster IgG or post-anti-CD3 Abs, respectively, $P = 0.0373$; **Figure 2b**).

Prevention of lethal hypersensitivity reactions and Ab titers in PD model mice

PD mice repeatedly administered rhGAA frequently died because of hypersensitivity reactions. We tested whether the anti-CD3 Abs can prevent this lethal hypersensitivity reaction. As in the studies using the wild-type mice, anti-CD3 Abs or control hamster IgG were administered to PD mice. Then, rhGAA was administered intravenously at 20 mg/kg once every other week for up to 20 injections. The experimental schedule is shown in **Figure 3a** and the Kaplan-Meier survival curve is shown in **Figure 3b**. Only 1 of 11 mice survived in the cohort treated with hamster IgG. By contrast, 10 of 12 mice survived in the anti-CD3 Ab-treated

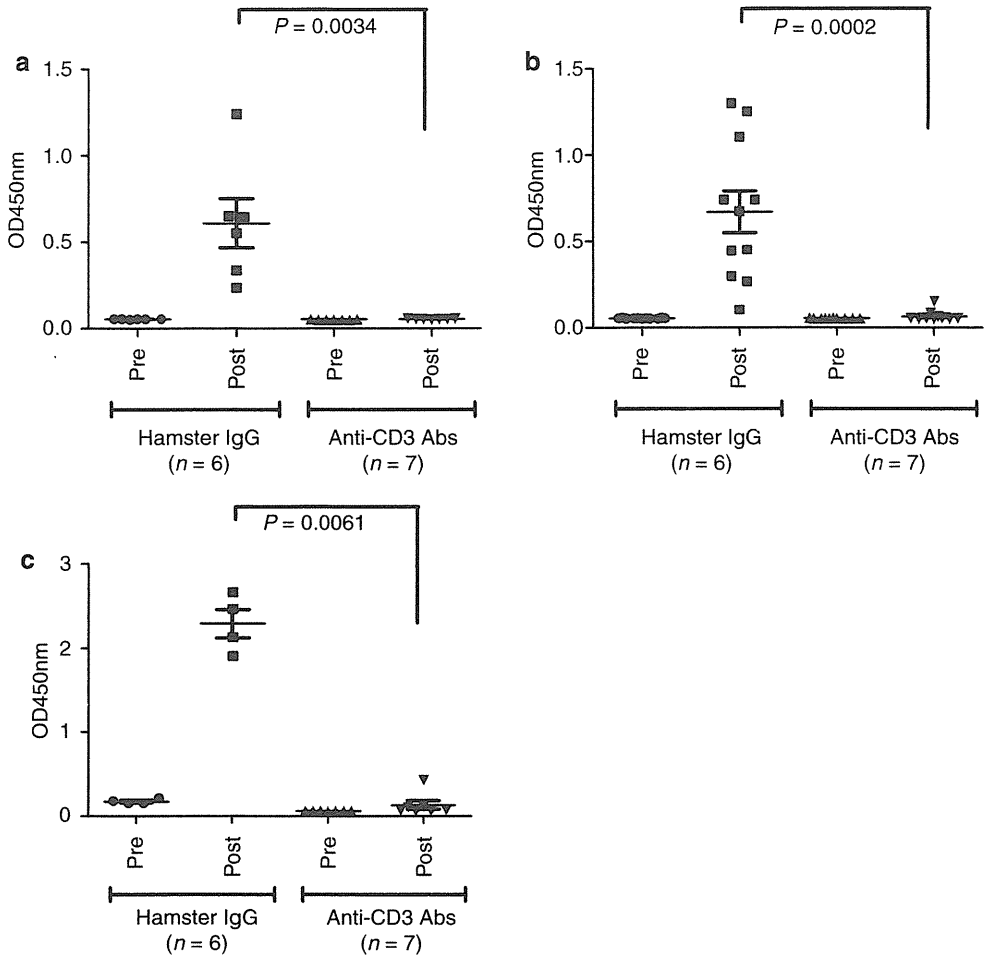

Figure 1 Prevention of antibody (Ab) formation by anti-CD3 Abs in wild-type mice. Anti-rhGAA Ab formation as a result of four doses of recombinant human α-glucosidase (rhGAA) was significantly reduced by anti-CD3 Abs in (**a**) wild-type BALB/c ($P = 0.0034$) and (**b**) C57BL/6 mice ($P = 0.0002$). In BALB/c mice, Ab formation due to a second challenge of rhGAA was also significantly lower in the anti-CD3 Ab-administered group ($P = 0.0061$). (**c**) "Pre" refers to the initial day of rhGAA administration. "Post" refers to 1 week after the final administration of rhGAA. The individual Ab values are shown as mean values and SEM.

group. The anti-CD3 Ab-treated mice survived for a significantly longer period than those injected with hamster IgG ($P = 0.0002$, log-rank test).

Anti-rhGAA IgG Ab levels were analyzed at various time points. As shown in **Figure 4a**, the Ab titers against rhGAA in most of the hamster IgG-treated group ($n = 11$) increased rapidly, and the animals typically died due to a hypersensitivity reaction. The Ab titers against rhGAA in 2 of the mice were only elevated slightly and these animals survived for a longer period. In one mouse (indicated by *), the Ab titer increased moderately during the early period, but this mouse died at 22 weeks after treatment (4 hours after the final rhGAA injection). Typically, the lethal hypersensitivity reactions occurred within 1 hour of the rhGAA injection, so it is likely that this mouse died from another cause. One mouse (indicated by **) survived for a longer period and its Ab titer declined. In contrast, in the anti-CD3 Ab-treated group ($n = 12$), the titers in most of the mice (10 of 12 mice) were elevated less than in the hamster IgG-treated group and these levels eventually declined (**Figure 4b**). It is interesting that four mice did not exhibit any increase in their Ab titer. After 8 weeks, only 2 mice were still alive in the hamster IgG-treated group, so we were unable to perform a statistical comparison of the Ab titers. Therefore, the Ab titers were compared at 2, 4, 6, and 8 weeks after ERT. The Ab titers were significantly lower in the anti-CD3 Ab group at all time points (**Table 1**). However, this immune-suppressive effect seemed to be less than in the wild-type mice.

The effect of anti-CD3 Abs on the lymphocyte population

Anti-CD3 Abs (10 μg) or the same dose of hamster IgG were administered to BALB/c mice for five consecutive days. Three days after the last administration of anti-CD3 Abs or hamster IgG, spleen cells were harvested and analyzed using flow cytometry for $CD4^+$, $CD8^+$, $CD4^+CD25^+$, and $CD4^+CD25^+FoxP3^+$ cells with fluorescently labeled Abs against cell surface and intracellular markers. The administration of anti-CD3 Abs significantly reduced the numbers of $CD4^+$ ($P = 0.0079$; **Figure 5a**) and $CD8^+$ ($P = 0.0079$; **Figure 5b**) cells and increased the ratio of $CD4^+CD25^+/CD4^+$ ($P = 0.0286$; **Figure 5c**) and $CD4^+CD25^+FoxP3^+/CD4^+$ ($P = 0.0286$; **Figure 5d**) cells. We analyzed the levels of $CD4^+$ and $CD8^+$ cells in

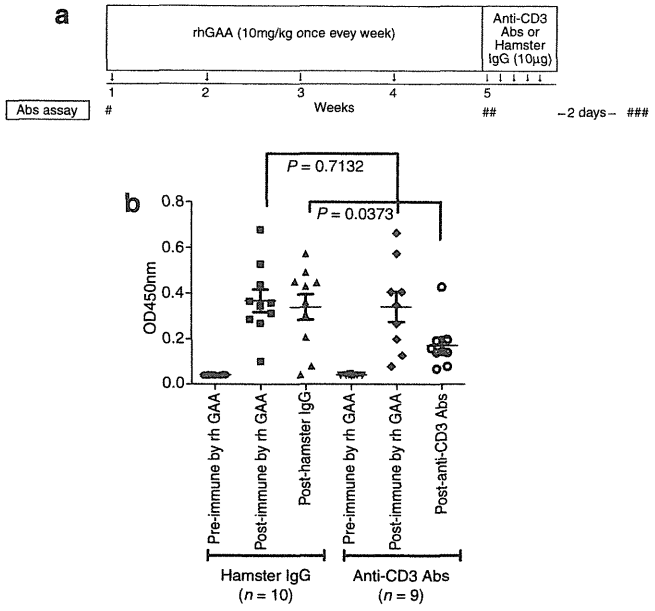

Figure 2 Suppressive effect of anti-CD3 antibodies (Abs) against pre-existing anti-recombinant human α-glucosidase (rhGAA) Abs in wild-type mice (BALB/c). The study design and immunization schedule are shown in **a**. (**b**)"Preimmune by rhGAA" refers to the initial day of rhGAA administration (# in **a**). "Postimmune by rhGAA" refers to 1 week after the final administration of rhGAA and the initial day of hamster immunoglobulin G (IgG) or anti-CD3 Ab administration (## in **a**). "Post-hamster IgG" or "Post-anti-CD3 Abs" refers to 3 days after the final administration of hamster IgG or anti-CD3 Abs, respectively (### in **a**). After rhGAA immunization, the Ab titers increased to the same extent in both the hamster- and anti-CD3 Ab-treated groups (P = 0.7132); however, after the administration of anti-CD3 Abs, the Ab titers were significantly reduced compared with the hamster IgG-treated group (P = 0.0373). The individual Ab values are shown as mean values and SEM.

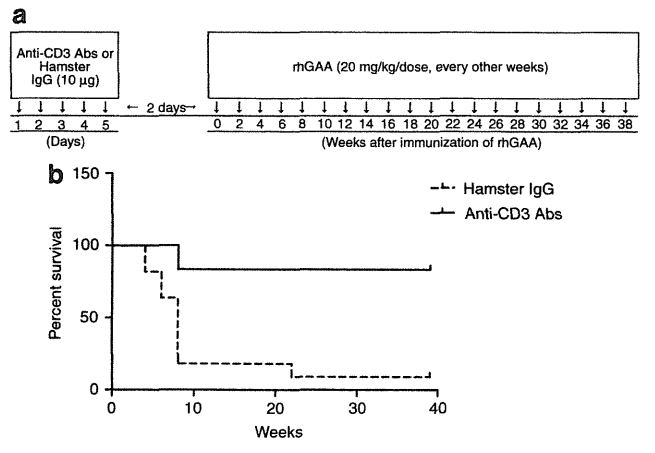

Figure 3 Prevention of the lethal hypersensitivity reaction and antibody (Ab) titers in Pompe disease (PD) mice. PD mice that were administered repeated doses of recombinant human α-glucosidase (rhGAA) usually died as a result of a lethal hypersensitivity reaction. The immunization schedule is shown in (**a**).The PD mice that received anti-CD3 Abs survived for a longer period than the hamster immunoglobulin G (IgG)-treated group (log-rank test, P = 0.0002) (**b**). "0" refers to the initial day of rhGAA administration.

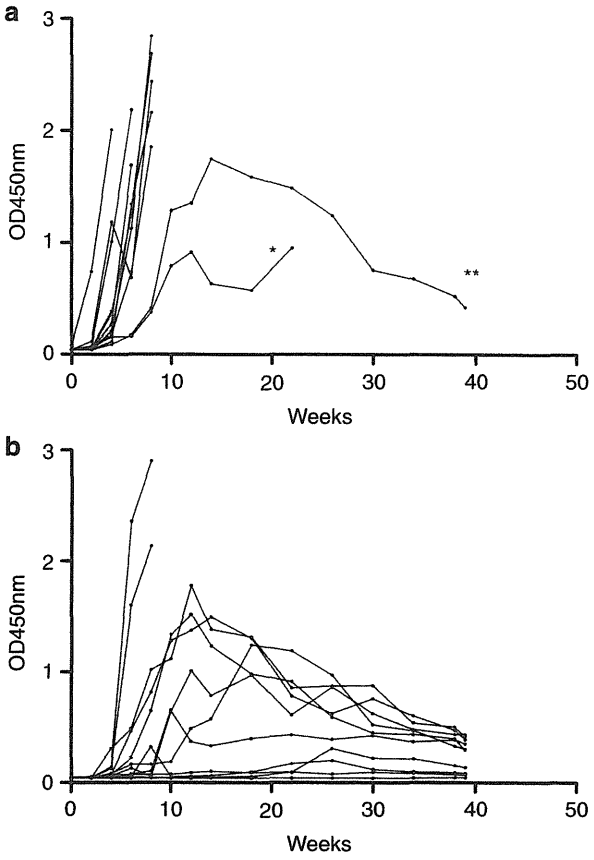

Figure 4 Titers of Abs against recombinant human α-glucosidase (rhGAA) following the repeated administration of rhGAA in individual Pompe disease (PD) mice. The antibody (Ab) titers of individual mice that received rhGAA repeatedly are shown. The Ab titers of the hamster group are shown in (**a**) and those of the anti-CD3 Ab group are shown in (**b**). "0" refers to the initial day of rhGAA administration.

peripheral blood up to 48 days after administration of Anti-CD3 Abs or hamster IgG, and found that level of $CD4^+$ cells did not recover to control levels for up to 36 days after the initiation of anti-CD3 Ab treatment (**Figure 6a,b**). Representative examples of flow cytometry plots for $CD4^+CD25^+FoxP3^+/CD4^+$ and $CD4^+CD25^+/CD4^+$ cells are shown in **Supplementary Figures S2** and **S3**.

Depletion of $CD4^+CD25^+$ cells

Because anti-CD3 Ab treatment increased the ratio of $CD4^+CD25^+/CD4^+$ and $CD4^+CD25^+FoxP3^+/CD4^+$T cells, we speculated that $CD4^+CD25^+$ cells may play an important role in the immune-suppressive activity of the anti-CD3 Abs. We depleted $CD4^+CD25^+$ cells by administering anti-CD25 Abs to BALB/c mice and tested whether the suppressive effect of anti-CD3 Abs on the formation of Abs against rhGAA was abrogated. Depletion of $CD4^+CD25^+$ cells was confirmed by flow cytometry using anti-CD4-FITC and anti-CD25-PE clone 7D4 (data not shown). When $CD4^+CD25^+$ cells were depleted by anti-CD25 Abs, the Ab titers were increased after rhGAA challenge (group 1, **Figure 7**). When the isotype control of the anti-CD25 Ab was administered, the increase in Abs against rhGAA was prevented (group 2, **Figure 7**). If neither anti-CD25 nor isotype control Abs were administered, anti-CD3 Abs prevented the increase in Abs, but hamster IgG did not (groups

3 and 4, respectively, **Figure 7**). This observation indicated that $CD4^+CD25^+$ cells play an important role in preventing the formation of Abs against rhGAA by anti-CD3 Abs during ERT for PD.

DISCUSSION

Clinical studies of ERT for LSDs have highlighted some challenges associated with this approach. One of these challenges is the immune response to the infused enzyme. There are several preclinical reports, including from our laboratory,[23,24] indicating that such Abs can have a negative impact on therapeutic efficacy in patients and in mouse models of LSDs. However, it is very difficult to make this conclusion in the clinic as LSDs are very rare and clinically heterogeneous disorders. Moreover, in some LSDs, the clinical manifestations are slow to develop, making correlations between Abs and clinical improvement due to ERT difficult. However, PD, especially the infantile-onset form, although rare and clinically heterogeneous, is a relatively rapidly progressing disease. Thus, among the various LSDs, it may be the most suitable to study the influence of Ab formation on clinical outcome following ERT. As mentioned above, several studies have demonstrated that Abs against rhGAA might have a negative impact on the efficacy of ERT for PD. To overcome this obstacle, several methods have been tested for the induction of immune tolerance against rhGAA in mouse models and human PD patients. Among them, we chose the oral administration of rhGAA[17] and anti-CD3 Abs because these methods might induce an antigen-specific and long-lasting tolerance. In this study, we focused on the administration of anti-CD3 Abs.

Table 1 Statistical comparison of the anti-rhGAA Ab titers following the repeated administration of rhGAA in PD mice at 2, 4, 6, and 8 weeks after rhGAA treatment

Weeks	Hamster IgG	*n*	Anti-CD3 Ab	*n*	*P* value
0	0.048 ± 0.004	11	0.044 ± 0.001	12	
2	0.123 ± 0.062	11	0.044 ± 0.001	12	0.0004
4	0.545 ± 0.182	11	0.095 ± 0.021	12	0.0006
6	1.042 ± 0.224	9	0.476 ± 0.212	12	0.0302
8	1.830 ± 0.389	7	0.695 ± 0.268	12	0.0312

Abbreviations: Ab, antibody; IgG, immunoglobulin G; PD, Pompe disease; rhGAA, recombinant human α-glucosidase
"0" refers to the initial day of rhGAA administration. The data are presented as mean values and SEM.

We showed that the parenteral administration of anti-CD3 Abs before ERT effectively reduced Ab formation against the infused enzyme in mice. Moreover, anti-CD3 Abs reduced the titers of pre-existing Abs against rhGAA. This is a very important observation as most CRIM-negative PD patients usually develop high Ab titers against rhGAA;[7,9] therefore, in the clinical setting, anti-CD3 Abs should be administered prior to the initiation of ERT. By contrast, it is very difficult to predict whether or not CRIM-positive patients will develop high Ab titers. It is not practical for all CRIM-positive PD patients to receive anti-CD3 Abs before ERT. Perhaps, once CRIM-positive patients develop high Ab titers, anti-CD3 Abs should be administered because they also reduce the levels of pre-existing Abs.

Anti-CD3 Abs appeared to be more effective in the wild-type mice than in the PD model mice. This may be due to the complete absence of GAA in this mouse model of PD, resulting in a robust immune reaction against rhGAA, compared with the wild-type mice. Therefore, the anti-CD3 Ab dose used for the wild-type mice may not be suitable for the PD mice, and dose adjustment of anti-CD3 Abs might be necessary for the PD mice. However, even in the PD mice, anti-CD3 Abs prevented the lethal hypersensitivity reaction and reduced the Ab titers against rhGAA. Only 1 mouse

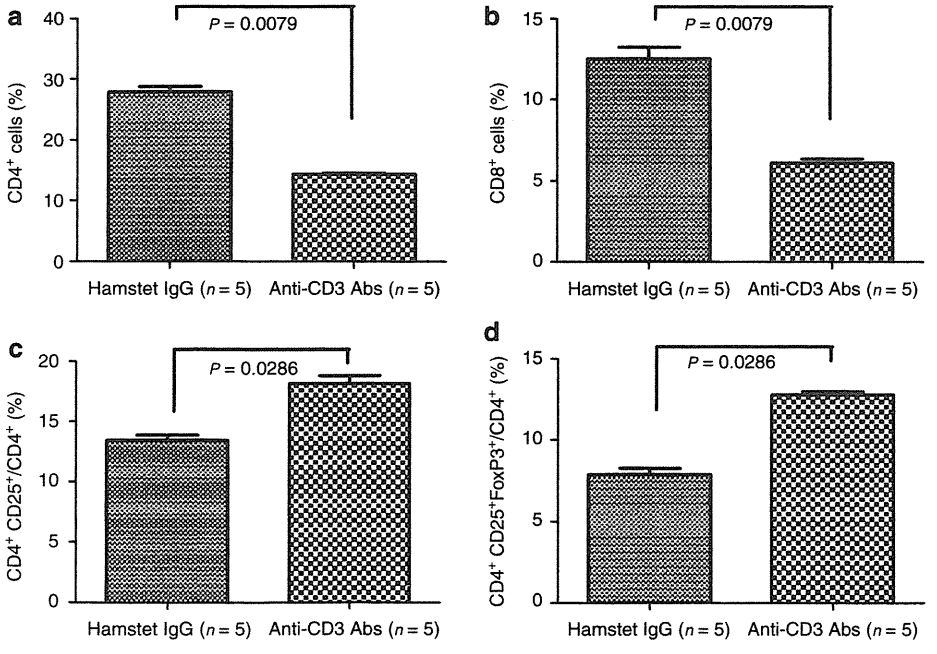

Figure 5 The effect of anti-CD3 antibodies (Abs) on effector T cells and regulatory T cells in the spleen. The lymphocyte population in the spleen from mice that received hamster immunoglobulin G (IgG) or anti-CD3 Abs is shown. The anti-CD3 Abs significantly reduced the number of (**a**) $CD4^+$ and (**b**) $CD8^+$ cells and increased the ratio of (**c**) $CD4^+CD25^+/CD4^+$ and (**d**) $CD4^+CD25^+FoxP3^+/CD4^+$ cells.

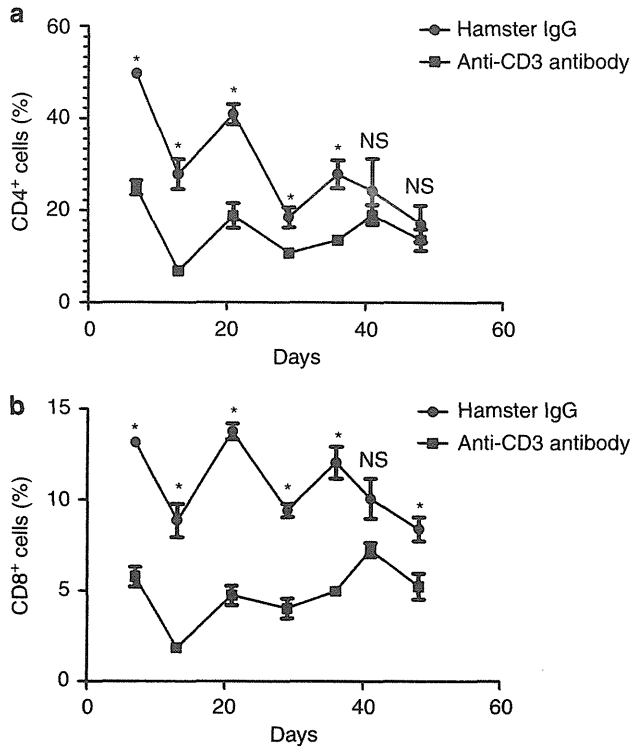

Figure 6 Effect of anti-CD3 Abs on the number of $CD4^+$ and $CD8^+$ cells in the peripheral blood. The levels of (**a**) $CD4^+$ and (**b**) $CD8^+$ cells in peripheral blood from mice that received hamster immunoglobulin G (IgG) or anti-CD3 Abs are shown. The administration of anti-CD3 Abs for five consecutive days reduced the levels of $CD4^+$ and $CD8^+$ cells in the peripheral blood at various time points for up to 36 days after the initiation of anti-CD3 antibody (Ab) or hamster IgG treatment. "0" refers to the initial day of anti-CD3 Abs or hamster IgG administration. *Significant difference between the two groups and "NS" indicates no significant difference between the two groups.

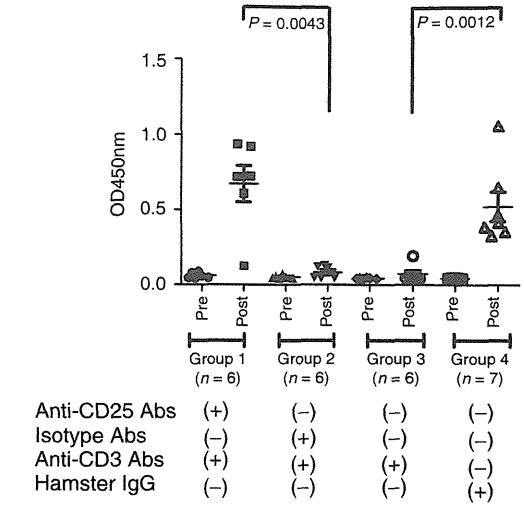

Figure 7 Depletion of $CD4^+CD25^+$ cells. Group 1: $CD4^+CD25^+$ cells were depleted by the administration of anti-CD25 Abs and tolerance was induced by the administration of anti-CD3 Abs. Group 2: As a control for group 1, isotype control anti-CD25 Abs were administered prior to tolerance induction by anti-CD3 Abs. Group 3: Tolerance was induced by anti-CD3 Abs without any pretreatment. Group 4: As a control for group 3, hamster immunoglobulin G (IgG) was administered instead of anti-CD3 Abs without any pretreatment. "Pre" refers to the initial day of recombinant human α-glucosidase (rhGAA) administration. "Post" refers to 1 week after the final administration of rhGAA. The individual antibody (Ab) values are shown as mean values and SEM.

out of 11 survived in the control hamster IgG-administered group. The Ab titer against rhGAA in the mouse that survived increased, but it then declined over time, even when ERT was continued without any immune-suppressive treatment (including anti-CD3 Ab administration). Even if the decrease in the number of $CD4^+$ and $CD8^+$ cells and the increase in the ratio of $CD4^+CD25^+$ /$CD4^+$ and $CD4^+CD25^+$ $FoxP3^+$/$CD4^+$ cells following the administration of anti-CD3 Abs are transient, once lethal hypersensitivity has been induced, which occurred rapidly following ERT and was prevented by the administration of anti-CD3 Abs, tolerance might be induced by repeated intravenous infusions of rhGAA.

An important issue to consider is whether the generation of Abs against rhGAA inhibits the effects of ERT. To clarify this issue, we infused PD mice with 10 mg/kg rhGAA once a week (for a total of five infusions) and with or without anti-CD3 Ab administration before ERT. The day after the final administration of rhGAA in both groups, the mice were killed and GAA activity in the liver was analyzed (**Supplementary Figure S4**). We examined GAA activity in the liver of two nontolerant hamster IgG-administered group mice exhibiting the highest Ab titers and three tolerant anti-CD3 Ab-administered group mice exhibiting no Abs. As a control, GAA activity in liver from untreated PD model mice was also examined (n = 3). GAA activity was not significantly different in the liver of the nontolerant hamster IgG-administered group mice exhibiting high Ab titer and the tolerant anti-CD3 Ab-administered group mice exhibiting no Ab. One reason for this observation is that the mice that exhibited very high Ab titers died after the third or fourth infusion of rhGAA. Consequently, we were unable to measure the GAA activity in these mice. We only assayed GAA activity in the mice that survived, and these animals had relatively low Ab titers. Therefore, in this study, we were not able to show the effect of anti-CD3 Abs on clinical outcome through the induction of immune tolerance. However, many reports have shown that the induction of tolerance improves the therapeutic effect of ERT in PD patients. So there is no doubt that the induction of tolerance is essential to maximize the effect of ERT in PD patients.

Here, we did not study the mechanism of tolerance induction by the anti-CD3 Abs in detail. The mechanism by which anti-CD3 Abs induce tolerance in protein-supplement therapy was previously studied extensively using mouse models of hemophilia.[22] The authors concluded that the induction of immune tolerance occurred through the generation of distinct regulatory T cells (Tregs), including $CD4^+CD25^+$ cells, and by T cells polarized toward a Th1 immune response. Regarding the induction of Tregs by anti-CD3 Abs, their results were consistent with ours. Although the mechanism of Treg induction by the anti-CD3 Abs is not clear, it has recently become clear that anti-CD3 Abs increase the levels of CD25 by inducing its expression on peripheral effector $CD4^+CD25^-$ cells, rather than by expanding thymically derived $CD4^+CD25^+$ Tregs.[25,26] In addition to the expansion of $CD4^+CD25^+$ Tregs, the levels of $CD4^+$ cells were low in anti-CD3 Ab-administered mice for a long period, even though their levels gradually increased. Taking this observation into account,

both the expansion of Tregs and the reduction of $CD4^+$ effector cells are probably necessary to induce tolerance in anti-CD3-administered mice. In other words, the ratio of regulatory to effector cells is probably an important component of tolerance in this setting. Recently, the immune responses following ERT for PD were reported to be caused by the activation of T cells.[27] In this regard, it is logical to use anti-CD3 Abs to induce immune tolerance.

The clinical efficacy and safety of anti-CD3 Abs have been studied in various autoimmune diseases including autoimmune diabetes,[28] ulcerative colitis,[29] and Crohn's disease.[30] The results in some diseases are very encouraging, but not all. In general, the induction of immune tolerance was difficult in situations in which there is an existing immune response, such as autoimmune diseases. In the case of ERT for PD, we can induce tolerance prior to the establishment of an immune reaction using anti-CD3 Abs. Thus, it is probably easier to induce tolerance in PD using anti-CD3 Ab treatment than in autoimmune diseases in which the immune reaction is already established. As such, we believe that the administration of anti-CD3 Abs prior to ERT is a very promising strategy to maximize the efficacy of ERT for PD.

The Abs used in this study were anti-murine CD3 antigens. We do not know whether anti-human CD3 Abs, which have been used in clinical trials, will be as effective as the anti-murine CD3 Abs. In this regard, Kuhn *et al.* developed a good animal model that expresses human CD3 antigens and was used to demonstrate the efficacy of antihuman CD3 Abs against autoimmune insulin-dependent diabetes.[31] This animal model can be used as a preclinical study tool to assess the use of anti-CD3 Abs for immune tolerance induction therapy in ERT for PD.

In conclusion, the administration of anti-CD3 Abs is a very promising strategy to induce immune tolerance to ERT for PD to maximize its efficacy and prevent hypersensitivity reactions.

MATERIALS AND METHODS

Animals. Wild-type BALB/C and C57BL/6 mice and PD mice (B6;129-*Gaa*^{tm1Rabn}/J) were used in this study. Both types of wild-type mice were purchased from Sankyo Labo Service (Tokyo, Japan). The PD mice were a generous gift from Dr N. Raben (NIH, Bethesda, MD). In this study, we generally used wild-type mice because most of the PD mice that received rhGAA repeatedly died due to a hypersensitivity reaction at an early stage if there was no immune-suppressive treatment. Therefore, it was difficult to compare the Ab titers in treated and untreated mice. All animal experiments were reviewed and approved by the Animal Care Committee of The Jikei University School of Medicine.

Anti-CD3 Abs. Anti-CD3 Abs (clone 145-2C11) were obtained from Bioexpress (Lebanon, NH). We used whole IgG anti-CD3ε or non-FcR-binding anti-CD3ε $F(ab')_2$ Abs. Both Abs reduced the Ab titers against rhGAA to the same extent in a preliminary experiment (data not shown). However, the latter Ab had fewer side-effects than the former. As a control, we used hamster whole IgG or $F(ab')_2$ (Bioexpress).

Assay for rhGAA-specific IgG. IgG Abs against rhGAA were assayed using an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 1 μg of rhGAA in phosphate-buffered saline (PBS) overnight at 4 °C. The plates were blocked by adding 100 μl PBS/1% bovine serum albumin and incubating for 5 h at room temperature. After this step, the wells were washed with PBS/0.05% Tween 20. The serum samples from mice were diluted 20,000-fold with PBS/1% bovine serum albumin, and 100 μl diluted serum was added to each well and incubated for 1 h at room temperature. The plate was then washed with the same buffer and reacted with 100 μl 5,000-fold diluted peroxidase-conjugated anti-mouse IgG Ab (Kirkegaard & Perry Labs., Gaithersburg, MA). After incubation for 30 minutes at room temperature, the plates were washed again and color was generated by the addition of 3,3′,5′,5′-tetramethylbenzidine substrate reagent (Promega, Madison, WI) for 10 minutes. The reaction was stopped by adding 100 μl 0.6 N H_2SO_4, and the optical density was measured at 450 nm using an ARVOMX/Light plate reader (PerkinElmer, Waltham, MA). The Ab titer was reported as the value of the optical density at 450 nm. To compare the Ab titers between the two groups, all of the samples were assayed in one 96-well plate to avoid variability among the experiments.

Flow cytometry. Abs specific for CD4-FITC (clone H129.19), CD8-FITC (53-6.7), CD25-PE (clone PC61), CD25-PE (clone 7D4), and FoxP3-Alexa Fluor 647 (MF23) were used (BD Biosciences, San Jose, CA). Single-cell suspensions were pooled from the spleens or peripheral blood of the mice. The cells were incubated with an Fc blocker (CD16/32); BD Biosciences) for 15 minutes and then stained for the expression of surface markers for 30 minutes at 4 °C. To detect FoxP3 expression, the cells were permeabilized using a Cytofix/Cytoperm Kit (BD Biosciences), incubated with the Fc blocker and surface maker stain as described earlier, and stained with anti-FoxP3-Alexa Fluor 647 for 30 minutes at room temperature. The data were acquired on a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using MACSQuantify Software (Miltenyi Biotec).

Induction of tolerance by anti-CD3 Abs in wild-type mice. We administered 10 μg of anti-CD3 Abs intravenously for five consecutive days to each 10-week-old Balb/c or C57BL/6 mouse. As a control, the same dose of hamster IgG was administered. Three days after the final administration of the anti-CD3 Abs or hamster IgG, the mice received 10 mg/kg rhGAA intravenously once every week for a total of four doses. One week after the final administration of rhGAA, blood was collected from the retro-orbital plexus. Eleven weeks after the first administration of the anti-CD3 Abs, Balb/c mice were challenged with 10 mg/kg rhGAA intravenously once every week for a total of four doses. One week after the final administration of rhGAA, Abs against rhGAA were assayed using ELISA.

Effect of anti-CD3 Abs against pre-existing Abs in wild-type mice. To immunize 10-week-old BALB/c mice, 10 mg/kg rhGAA was administered intravenously once every week for a total of four doses. One week after the final administration of rhGAA, 10 μg anti-CD3 Abs were administered for five consecutive days. As a control, the same amount of hamster IgG was administered. Three days after the final administration of the anti-CD3 Abs or hamster IgG, anti-rhGAA Abs were assayed using ELISA.

Prevention of the hypersensitivity reaction to ERT in PD mice. PD mice develop a lethal hypersensitivity against rhGAA when it was infused repeatedly; therefore, we tested whether anti-CD3 Abs inhibit this reaction. We administered 10 μg anti-CD3 Abs for 5 consecutive days to PD mice (9–18 weeks old). As a control, the same dose of hamster IgG was administered. Three days after the final administration of the anti-CD3 Abs or hamster IgG, the mice received 20 mg/kg rhGAA intravenously once every other week for a total of 20 doses. We defined lethal hypersensitivity as death within 30 minutes after the intravenous administration of rhGAA. The occurrence of a lethal hypersensitivity reaction was monitored and Ab titers were also assayed at various time points.

Depletion of $CD4^+CD25^+$ cells. Wild-type BALB/c mice were injected intraperitoneally with 1 mg anti-CD25 Abs (clone PC61; Bioexpress) or isotype control Abs (rat IgG1; Bioexpress) on days 0 and 5; on days 3–7, they received 10 μg/day anti-CD3 Abs or control hamster IgG. Three days after the final injection of the anti-CD3 Abs, the mice were killed and splenocytes were analyzed for the expression of CD4 and CD25 using anti-CD4-FITC and anti-CD25-PE clone 7D4. An identical treatment regimen was

performed, but at 3 days after the anti-CD3 or hamster IgG injections (day 10), the mice received the first of 4 weekly rhGAA immunizations (10 $mg \cdot kg^{-1} \cdot day^{-1}$). The subsequent immune response to rhGAA was determined at 1 week after the final rhGAA immunization using ELISA.

Statistical analysis. The results are expressed as the mean ± SEM. Statistical differences between two groups were determined using the Mann–Whitney test, and differences between three groups were determined by analysis of variance with the Kruskal–Wallis and Dunn's multiple comparison tests. In addition, we calculated the risk of lethal hypersensitivity to rhGAA infusion using the Kaplan–Meier method, while the log-rank test was used to assess the differences between the study groups. P values <0.05 were considered to be significant.

SUPPLEMENTARY MATERIAL

Figure S1. Immune response to rhGAA in wild-type (Balb/c and C57BL/6) and PD model mice.

Figure S2. Representative flow cytometry plot demonstrating that anti-CD3 Abs increased the ratio of $CD4^+CD25^+/CD4^+$ cells.

Figure S3. Representative flow cytometry plot demonstrating that anti-CD3 Abs increased the ratio of $CD4^+CD25^+FoxP3^+/CD4^+$ cells.

Figure S4. GAA activity in the liver from nontolerant or tolerant PD mice.

ACKNOWLEDGMENTS

The authors thank Nina Raben (NIH, Bethesda, MD) for providing the PD mouse. We also wish to thank Seng H. Cheng (Genzyme) for critically reviewing the manuscript. This research was partially supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports, and Culture of Japan. T.O., H.I., and Y.E. have active research support from the Genzyme. These activities have been fully disclosed and are managed under a Memorandum of Understanding with the Conflict of Interest Resolution Board of The Jikei University School of Medicine. This work was performed at the Department of Gene Therapy, Institute of DNA Medicine, Research Center for Medical Sciences/Department of Pediatrics, The Jikei University School of Medicine, 3-25-8 Nishishinbashi, Minato-ku, Tokyo 105-8461, Japan.

REFERENCES

1. Kishnani, PS, Corzo, D, Nicolino, M, Byrne, B, Mandel, H, Hwu, WL *et al.* (2007). Recombinant human acid [alpha]-glucosidase: major clinical benefits in infantile-onset Pompe disease. *Neurology* **68**: 99–109.
2. Kishnani, PS, Hwu, WL, Mandel, H, Nicolino, M, Yong, F and Corzo, D; Infantile-Onset Pompe Disease Natural History Study Group (2006). A retrospective, multinational, multicenter study on the natural history of infantile-onset Pompe disease. *J Pediatr* **148**: 671–676.
3. van der Ploeg, AT, Clemens, PR, Corzo, D, Escolar, DM, Florence, J, Groeneveld, GJ *et al.* (2010). A randomized study of alglucosidase alfa in late-onset Pompe's disease. *N Engl J Med* **362**: 1396–1406.
4. Raben, N, Danon, M, Gilbert, AL, Dwivedi, S, Collins, B, Thurberg, BL *et al.* (2003). Enzyme replacement therapy in the mouse model of Pompe disease. *Mol Genet Metab* **80**: 159–169.
5. Amalfitano, A, Bengur, AR, Morse, RP, Majure, JM, Case, LE, Veerling, DL *et al.* (2001). Recombinant human acid alpha-glucosidase enzyme therapy for infantile glycogen storage disease type II: results of a phase I/II clinical trial. *Genet Med* **3**: 132–138.
6. Kishnani, PS, Nicolino, M, Voit, T, Rogers, RC, Tsai, AC, Waterson, J *et al.* (2006). Chinese hamster ovary cell-derived recombinant human acid alpha-glucosidase in infantile-onset Pompe disease. *J Pediatr* **149**: 89–97.
7. Kishnani, PS, Goldenberg, PC, DeArmey, SL, Heller, J, Benjamin, D, Young, S *et al.* (2010). Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab* **99**: 26–33.
8. de Vries, JM, van der Beek, NA, Kroos, MA, Ozkan, L, van Doorn, PA, Richards, SM *et al.* (2010). High antibody titer in an adult with Pompe disease affects treatment with alglucosidase alfa. *Mol Genet Metab* **101**: 338–345.
9. Banugaria, SG, Prater, SN, Ng, YK, Kobori, JA, Finkel, RS, Ladda, RL *et al.* (2011). The impact of antibodies on clinical outcomes in diseases treated with therapeutic protein: lessons learned from infantile Pompe disease. *Genet Med* **13**: 729–736.
10. Joseph, A, Munroe, K, Housman, M, Garman, R and Richards, S (2008). Immune tolerance induction to enzyme-replacement therapy by co-administration of short-term, low-dose methotrexate in a murine Pompe disease model. *Clin Exp Immunol* **152**: 138–146.
11. Mendelsohn, NJ, Messinger, YH, Rosenberg, AS and Kishnani, PS (2009). Elimination of antibodies to recombinant enzyme in Pompe's disease. *N Engl J Med* **360**: 194–195.
12. Messinger, YH, Mendelsohn, NJ, Rhead, W, Dimmock, D, Hershkovitz, E, Champion, M *et al.* (2012). Successful immune tolerance induction to enzyme replacement therapy in CRIM-negative infantile Pompe disease. *Genet Med* **14**: 135–142.
13. Rohrbach, M, Klein, A, Köhli-Wiesner, A, Veraguth, D, Scheer, I, Balmer, C *et al.* (2010). CRIM-negative infantile Pompe disease: 42-month treatment outcome. *J Inherit Metab Dis* **33**: 751–757.
14. Chu, Q, Moreland, RJ, Gao, L, Taylor, KM, Meyers, E, Cheng, SH *et al.* (2010). Induction of immune tolerance to a therapeutic protein by intrathymic gene delivery. *Mol Ther* **18**: 2146–2154.
15. Douillard-Guilloux, G, Richard, E, Batista, L and Caillaud, C (2009). Partial phenotypic correction and immune tolerance induction to enzyme replacement therapy after hematopoietic stem cell gene transfer of alpha-glucosidase in Pompe disease. *J Gene Med* **11**: 279–287.
16. van Til, NP, Stok, M, Aerts Kaya, FS, de Waard, MC, Farahbakhshian, E, Visser, TP *et al.* (2010). Lentiviral gene therapy of murine hematopoietic stem cells ameliorates the Pompe disease phenotype. *Blood* **115**: 5329–5337.
17. Ohashi, T, Iizuka, S, Shimada, Y, Eto, Y, Ida, H, Hachimura, S *et al.* (2011). Oral administration of recombinant human acid a-glucosidase reduces specific antibody formation against enzyme in mouse. *Mol Genet Metab* **103**: 98–100.
18. Sun, B, Bird, A, Young, SP, Kishnani, PS, Chen, YT and Koeberl, DD (2007). Enhanced response to enzyme replacement therapy in Pompe disease after the induction of immune tolerance. *Am J Hum Genet* **81**: 1042–1049.
19. Sun, B, Kulis, MD, Young, SP, Hobeika, AC, Li, S, Bird, A *et al.* (2010). Immunomodulatory gene therapy prevents antibody formation and lethal hypersensitivity reactions in murine pompe disease. *Mol Ther* **18**: 353–360.
20. Sun, B, Li, S, Bird, A, Yi, H, Kemper, A, Thurberg, BL *et al.* (2010). Antibody formation and mannose-6-phosphate receptor expression impact the efficacy of muscle-specific transgene expression in murine Pompe disease. *J Gene Med* **12**: 881–891.
21. Lipinski, SE, Lipinski, MJ, Burnette, A, Platts-Mills, TA and Wilson, WG (2009). Desensitization of an adult patient with Pompe disease and a history of anaphylaxis to alglucosidase alfa. *Mol Genet Metab* **98**: 319–321.
22. Waters, B, Qadura, M, Burnett, E, Chegeni, R, Labelle, A, Thompson, P *et al.* (2009). Anti-CD3 prevents factor VIII inhibitor development in hemophilia A mice by a regulatory $CD4^+CD25^+$-dependent mechanism and by shifting cytokine production to favor a Th1 response. *Blood* **113**: 193–203.
23. Ohashi, T, Iizuka, S, Ida, H and Eto, Y (2008). Reduced alpha-Gal A enzyme activity in Fabry fibroblast cells and Fabry mice tissues induced by serum from antibody positive patients with Fabry disease. *Mol Genet Metab* **94**: 313–318.
24. Ohashi, T, Sakuma, M, Kitagawa, T, Suzuki, K, Ishige, N and Eto, Y (2007). Influence of antibody formation on reduction of globotriaosylceramide (GL-3) in urine from Fabry patients during agalsidase beta therapy. *Mol Genet Metab* **92**: 271–273.
25. Belghith, M, Bluestone, JA, Barriot, S, Mégret, J, Bach, JF and Chatenoud, L (2003). TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med* **9**: 1202–1208.
26. You, S, Leforban, B, Garcia, C, Bach, JF, Bluestone, JA and Chatenoud, L (2007). Adaptive TGF-beta-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment. *Proc Natl Acad Sci USA* **104**: 6335–6340.
27. Banati, M, Hosszu, Z, Trauninger, A, Szereday, L and Illes, Z (2011). Enzyme replacement therapy induces T-cell responses in late-onset Pompe disease. *Muscle Nerve* **44**: 720–726.
28. Sherry, N, Hagopian, W, Ludvigsson, J, Jain, SM, Wahlen, J, Ferry, RJ Jr *et al.*; Protégé Trial Investigators. (2011). Teplizumab for treatment of type 1 diabetes (Protégé study): 1-year results from a randomised, placebo-controlled trial. *Lancet* **378**: 487–497.
29. Baumgart, DC, Targan, SR, Dignass, AU, Mayer, L, van Assche, G, Hommes, DW *et al.* (2010). Prospective randomized open-label multicenter phase I/II dose escalation trial of visilizumab (HuM291) in severe steroid-refractory ulcerative colitis. *Inflamm Bowel Dis* **16**: 620–629.
30. van der Woude, CJ, Stokkers, P, van Bodegraven, AA, Van Assche, G, Hebzda, Z, Paradowski, L *et al.*; Initiative on Crohn's and Colitis, The Netherlands. (2010). Phase I, double-blind, randomized, placebo-controlled, dose-escalation study of NI-0401 (a fully human anti-CD3 monoclonal antibody) in patients with moderate to severe active Crohn's disease. *Inflamm Bowel Dis* **16**: 1708–1716.
31. Kuhn, C, You, S, Valette, F, Hale, G, van Endert, P, Bach, JF *et al.* (2011). Human CD3 transgenic mice: preclinical testing of antibodies promoting immune tolerance. *Sci Transl Med* **3**: 68ra10.
32. Kyosen, SO, Iizuka, S, Kobayashi, H, Kimura, T, Fukuda, T, Shen, J *et al.* (2010). Neonatal gene transfer using lentiviral vector for murine Pompe disease: long-term expression and glycogen reduction. *Gene Ther* **17**: 521–530.

Molecular Genetics and Metabolism 107 (2012) 122–128

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Enzyme replacement therapy (ERT) procedure for mucopolysaccharidosis type II (MPS II) by intraventricular administration (IVA) in murine MPS II

Takashi Higuchi [a], Hiromi Shimizu [a,b], Takahiro Fukuda [c], Shiho Kawagoe [a], Juri Matsumoto [a], Yohta Shimada [e], Hiroshi Kobayashi [a,d,e], Hiroyuki Ida [a,d,e], Toya Ohashi [a,d,e], Hideto Morimoto [f], Tohru Hirato [f], Katsuya Nishino [f], Yoshikatsu Eto [a,*]

[a] Department of Genetic Diseases & Genomic Science, The Jikei University School of Medicine, Tokyo, Japan
[b] Department of Physics, Faculty of Science and Engineering, Waseda University, Tokyo, Japan
[c] Neuropathology, The Jikei University School of Medicine, Tokyo, Japan
[d] Department of Pediatrics, The Jikei University School of Medicine, Tokyo, Japan
[e] Department of Gene Therapy, Institute of DNA Medical, The Jikei University School of Medicine, Tokyo, Japan
[f] JCR Pharmaceuticals Co., Ltd. Hyogo, Japan

ARTICLE INFO

Article history:
Received 8 March 2012
Received in revised form 1 May 2012
Accepted 2 May 2012
Available online 18 May 2012

Keywords:
Hunter syndrome
Mucopolysaccharidosis
Lysosomal storage disease
Iduronate-2-sulfatase
Intraventricular
Enzyme replacement therapy

ABSTRACT

Mucopolysaccharidosis type II (MPS II), or Hunter syndrome, is a lysosomal storage disorder caused by a deficiency of iduronate-2-sulfatase (IDS) and is characterized by the accumulation of glycosaminoglycans (GAGs). MPS II has been treated by hematopoietic stem cell therapy (HSCT)/enzyme replacement therapy (ERT), but its effectiveness in the central nervous system (CNS) is limited because of poor enzyme uptake across the blood–brain barrier (BBB). To increase the efficacy of ERT in the brain, we tested an intraventricular ERT procedure consisting of repeated administrations of IDS (20 μg/mouse/3 weeks) in IDS-knockout, MPS II model mice. The IDS enzyme activity and the accumulation of total GAGs were measured in mouse brains. The IDS activity was significantly increased, and the accumulation of total GAGs was decreased in the MPS II mouse brains treated with multiple administrations of IDS via intraventricular ERT. Additionally, a high level of IDS enzyme activity was appreciated in other MPS II mouse tissues, such as the liver, spleen, testis and others. A Y-maze was used to test learning and memory after repeated intraventricular ERT with IDS. The IDS-treated mouse groups recovered the capacity for short-term memory and activity. Although large and small vacuoles were found at the margin of the cerebellar Purkinje cells in the disease-control mice, these vacuoles disappeared upon treated with IDS. Loss of vacuoles was also observed in other tissues (liver, kidney and testis). These results demonstrate the possible efficacy of an ERT procedure with intraventricular administration of IDS for the treatment of MPS II.

1. Introduction

Mucopolysaccharidosis type II (MPS II), also known as Hunter syndrome, is an X-linked, recessive inborn error of metabolism caused by mutations in the gene coding for the lysosomal enzyme, iduronate-2-sulfatase (IDS) [1]. The enzyme IDS removes the sulfate group from heparin sulfate and dermatan sulfate. In the absence of IDS, these sulfated glycosaminoglycans (GAGs) accumulate in the lysosomes. In Japan, MPS II is the most common type of mucopolysaccharidosis, accounting for 60% of documented cases [2]. MPS II may occur in mild or severe forms, and there is wide variability in symptoms for individual patients. The severe form is characterized by progressive somatic and neurological disease. Early treatment of MPS II patients with enzyme replacement therapy (ERT)/hematopoietic stem cell therapy (HSCT) is very important [3].

Treatment with ERT is one of the therapeutic approaches for patients who cannot be treated with HSCT with at least 6 different lysosomal storage diseases (LSDs) such as Gaucher disease, Pompe disease, Fabry disease, and MPS I, II, and VI [4–9]. Usually, ERT treatments are performed by intravenous (IV) infusion of the enzyme. ERT has been successful in treating hepatosplenomegaly, cardiomegaly, and skin, etc., ERT has not been proven to be ineffective in CNS disease, but may have limited effects on the CNS and skeletal system. The neuropathic forms of LSD, those that affect the CNS, are generally difficult to treat with ERT because of limited enzyme uptake

Abbreviations: MPS II, mucopolysaccharidosis type II; ERT, enzyme replacement therapy; IDS, iduronate-2-sulfatase; BBB, blood–brain barrier; CNS, central nervous system; HSCT, hematopoietic stem cell therapy; LSD, lysosomal storage disease; IV, intravenous infusion; GAG, glycosaminoglycan; CT, computed tomography; H&E, hematoxylin and eosin.

* Corresponding author at: Department of Genetic Diseases and Genomic Science, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-0003, Japan. Fax: +81 3 5400 1293.
E-mail address: eto.y@jikei.ac.jp (Y. Eto).

1096-7192/$ – see front matter.
doi:10.1016/j.ymgme.2012.05.005

across the blood–brain barrier (BBB) [10]. In response to this challenge, many trials have been established to evaluate potential treatments for the neuropathic forms of LSDs [11–13].

Here, we treated the *Ids* gene-knockout, MPS II mouse model with direct, repeated intraventricular administrations of IDS. This procedure bypasses the BBB, allowing for direct uptake of the IDS enzyme by mouse neuronal cells. The IDS enzyme activity, accumulation of total GAGs, and histopathology were examined in the MPS II mouse tissues. This examination was conducted after repeat administrations of IDS via intraventricular ERT, and it demonstrated that IDS enzyme activity was maintained highly and total GAGs were decreased in the MPS II mouse brain. Furthermore, the mice that underwent treatment showed improved short-term memory and activity during the Y-maze test. In addition, histological findings demonstrated a loss of vacuoles at the margin of Purkinje cells and other cell types (liver cells, tubule cells, and Leydig cells). These results strongly suggest the potential of intraventricular ERT as an effective treatment for MPS II.

2. Materials and methods

2.1. Animals

Male MPS II model mice, iduronate-2-sulfatase knockout (IDS-KO) mice, were produced by JCR Pharmaceuticals Co., Ltd. (Hyogo, Japan). In these mice, the *Ids* gene was deleted from exon 2 to exon 5. The IDS-KO mouse was bred from a C57BL/6 background strain and has a null mutation in the *Ids* gene. The normal control, wild-type mice were bred from a C57BL/6 strain. Mice were housed in groups of 3 per cage in a colony room under a 12-hour light–dark cycle. Rodent food and water were available ad libitum. All animal studies were approved by the Animal Experiments Committee at The Jikei University.

2.2. Computed tomography (CT) scan experiment

The skulls of the mice were scanned using a Latheta LCT-200 experimental animal CT system (Hitachi-Aloka Medical, LTD, Tokyo, Japan).

2.3. Foot print pattern analysis

The treated MPS II mice and the control, wild-type mice were tested during the same sessions to minimize variability. Analysis of the walking footprint pattern was performed as previously described [14]. Gait abnormalities were identified by painting the mouse paws with non-toxic, washable paint and then by placing the mice in a corridor lined with white paper.

2.4. Intraventricular ERT administration

Mice were anesthetized with pentobarbital sodium (Abott Japan Co., Ltd., Tokyo, Japan) and mounted in a stereotaxic apparatus (Narishige Co., Ltd., Tokyo, Japan). A total of 20 μg of recombinant human iduronate-2-surfatase (JCR Pharmaceuticals Co., Ltd., Hyogo, Japan) or saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was injected into the lateral ventricle which was repeated 4, 6 and 8 times every 3 weeks with a 30-gauge needle [coordinates relative to bregma: anteroposterior −3.0 mm; lateral +2.0 mm (midline); dorsoventral −3.0 mm from skull surface]. The IDS dose was determined by reference to the previous report [15].

2.5. Tissue collection

Tissue samples were collected from the treated and untreated IDS-KO and wild-type mice at the end of each treatment session. The mice were anesthetized and sacrificed by cardiac perfusion using 40 ml of phosphate-buffered saline (PBS (−)). The tissue samples were collected, and half of each sample was fixed for histopathological analysis (H&E staining, Alcian blue staining, and immunostaining), while the other half was frozen in dry ice prior to processing with the IDS activity assay and total GAG accumulation analysis. The brains were divided into three regions: front section cerebral, posterior section cerebral, and cerebellum.

2.6. IDS activity analysis

IDS activity was determined with homogenized tissues in water, as described using the artificial substrate 4-methylumbelliferyl-alpha-iduronide-2-sulfate (MU-αIdu-2S) [Moscerdam Substrates, Oegstgeest, Netherlands]. Tissue protein concentrations were determined using the BCA protein assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The measurement of IDS activity was conducted as previously described [15]. A total of 15 μg of protein was incubated with 20 μl of the *substrate solution* (1.25 mM MU-αIdu-2S-Na_2, 0.1 M Na-acetate/0.1 M acetic acid buffer (pH 5.0), 10 mM Pb-acetate) for 4 h at 37 °C in a dark room. Next, 40 μl of *Pi/Ci buffer* (0.4 M Na-phosphate/0.2 M citrate buffer (pH 4.5), 0.02% Na-azide) and 10 μl *LEBT* (purified Lysosomal Enzymes from Bovine Testis) were added to the incubation samples, which were then incubated for an additional 24 h at 37 °C. Finally, the reaction was stopped by adding 200 μl of *stop buffer* (0.5 M $NaHCO_3$/0.5 M Na_2CO_3 buffer (pH 10.7), 0.025% Triton X-100) to the mixture. The liberated fluorescence of the 4-methylumbelliferone was then measured with a RF-5300PC spectrofluorophotometer at 365 nm excitation and 460 nm emission (SIMADZU Co., Kyoto, Japan). The enzyme activity was expressed as nmol/4 h/μg protein, as calculated with the standard curve of the fluorogenic substrate, 4-methylumbelliferyl-α-iduronide (Sigma-Aldrich Japan Co., LLC. Tokyo, Japan). We took the average of 3–4 experiments or more.

2.7. Tissue total GAG accumulation assay

Treated and untreated MPS II and wild-type mouse tissues were homogenized in an NaCl-T buffer (0.9% NaCl, 0.2% Triton X-100). The samples were shaken overnight at 4 °C and microcentrifuged for 5 min at 3000 rpm, and the supernatants were collected. Total GAG levels were measured using the Wieslad™ sGAG quantitative Alcian blue-binding assay kit (Euro-Diagnostica, Malmö, Sweden) [15]. First, 50 μl of homogenized samples was incubated with 50 μl of 8 M Guanidine-HCl at room temperature (RT) for 15 min. Then, 50 μl of STA solution (0.3% H_2SO_4, 0.75% Triton X-100) was added for 15 min at RT, with a solution of Alcian blue working in the solution for 15 min. The samples were then centrifuged for 15 min at 12,000 rpm and rinsed with DMSO solution (40% DMSO, 0.05 M $MgCl_2$). Finally, 500 μl of Gu-prop solution (4 M Guanidine-HCl, 33% 1-propanol, 0.25% Triton X-100) were added to the pellets, and the mix was allowed to completely dissolve. Alternatively, absorbance was read in the SH-9000 spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan) at 600 nm. GAG was normalized to the protein concentration. Protein concentration was measured with BCA Protein Assay kits. The GAG ratio was expressed as mg of GAG/mg of protein, as calculated through the standard curve of the GAG substrate, chondroitin sulfate-6 (Euro-Diagnostica Co., Malmö Sweden). We took the average of 3–4 experiments or more.

2.8. Spontaneous alternation behavior

Spontaneous alternation behavior requiring attention and working memory was assessed using a Y-maze [16,17]. Each arm of the Y-maze was 40-cm long, ranging from 3 to 10 cm wide from the bottom to the top and 12.5 cm high. The arms were labeled A, B, or C, and both arms were positioned at equal angles (diverging at a 120°). The