J. Inherit. Metab. Dis. 28 (2005) 575–583

Enzyme replacement therapy in Japanese Fabry disease patients: The results of a phase 2 bridging study

Y. ETO[1*], T. OHASHI[1], Y. UTSUNOMIYA[1], M. FUJIWARA[1], A. MIZUNO[2], K. INUI[3], N. SAKAI[3], T. KITAGAWA[4], Y. SUZUKI[5], S. MOCHIZUKI[1], M. KAWAKAMI[1], T. HOSOYA[1], M. OWADA[4], H. SAKURABA[6], and H. SAITO[7]

[1]*Tokyo Jikei University School of Medicine, Tokyo;* [2]*Chubu National Hospital, Chubu;* [3]*Osaka University School of Medicine, Osaka;* [4]*Nihon University, Nihon;* [5]*International University of Health and Welfare;* [6]*Tokyo Metropolitan Institute of Medical Science, Tokyo;* [7]*National Children's Hospital, Japan*

**Correspondence: Nishishimbashi 3-25-8, Minato-ku, Tokyo, Japan.*
E-mail: yosh@sepia.ocn.ne.jp

MS received 07.06.03 Accepted 28.11.04

Summary: Fabry Disease (α-galactosidase A deficiency) is an X-linked hereditary disorder leading to the pathological accumulation of globotriaosylceramide (GL-3) in lysosomes, particularly in the vascular endothelium of the kidney, heart and brain. We report the results of an open-label phase 2 study that was undertaken to evaluate whether ethnic differences exist that would affect agalsidase beta (Fabrazyme) treatment of Fabry patients in the Japanese population, relative to safety and efficacy. The study design mirrored the design of the completed phase 3 clinical trial that led to approval of the product agalsidase beta. The 13 Japanese, male Fabry patients enrolled in the study received the enzyme replacement therapy over a period of 20 weeks as biweekly infusions. All selected efficacy end points showed improvements that were comparable with findings from the phase 3 study. These improvements included reductions of GL-3 accumulation in both kidney and skin capillary endothelial cells to (near) normal levels (92% of patients). Kidney and plasma GL-3 levels decreased by 51.9% and 100%, respectively, by ELISA. Renal function remained normal. Fabry-associated pain, and quality of life, showed improvement over baseline in multiple categories. Related adverse events were mild or moderate in intensity and mostly infusion-associated (fever and rigors). As expected, IgG antibody formation was observed in 85% of the patients, but had no effect on treatment response. These results suggest that treatment with agalsidase beta is safe and effective in Japanese patients with Fabry disease. With regard to safety and efficacy, no differences were observed as compared to the caucasian population.

Fabry disease (McKusick 301500) is an X-linked inborn error of metabolism characterized by deficient activity of the lysosomal hydrolase α-galactosidase A (α-GalA; EC 3.2.1.22). The prevalence in males is estimated as 1:40 000 to 1:60 000 (Desnick et al 2001; Meikle et al 1999). In classically affected male individuals, residual activity of α-galactosidase A is (nearly) absent, which results in the pathological accumulation of α-galactosyl-terminated neutral glycosphingolipids, predominantly globotriaosylceramide (GL-3), in cellular lysosomes. Accumulation of GL-3 occurs in virtually all tissues of the body, but particularly in the endothelial, perithelial and smooth-muscle cells of blood vessels, ganglion cells of the autonomic nervous system, glomeruli and tubules of the kidney and the cardiomyocytes of the heart (Desnick et al 2001). In the classical phenotype, deterioration of renal function will ultimately progress to end-stage renal disease in the third to fifth decades of life (Desnick et al 2001). Cardiac manifestations causing significant morbidity may include cardiomyopathy, angina pectoris, congestive heart failure, myocardial ischaemia and arrythmias (Linhart et al 2002). Cerebrovascular involvement can lead to transient ischaemic attacks, stroke and other neurological disorders (Kolodny and Pastores 2002). The clinical spectrum of Fabry disease also includes a 'renal variant' and a 'cardiac variant' phenotype in patients without classic symptoms who predominantly develop end-stage renal disease (Nakao et al 2003) and cardiac manifestations (Elleder et al 1990; von Scheidt et al 1991), respectively. Heterozygotes are also prone to manifest disease symptoms owing to the phenomenon of random X-chromosome inactivation (lyonization) (Lyon 2002). The disease spectrum in female carriers may range from asymptomatic disease to the classic phenotype.

Treatment of Fabry disease used to be limited to supportive care such as management of pain and hypertension (Desnick and Wasserstein 2001). Clinical trials with two enzyme replacement therapies, agalsidase alfa (Replagal; Transkaryotic Therapies, Inc., Cambridge, MA, USA) (Schiffmann et al 2000, 2001) and agalsidase beta (Fabrazyme; Genzyme Corporation, Cambridge, MA, USA) (Eng et al 2001a,b) have led to the commercial availability of these two products: both agalsidase beta and agalsidase alfa in the European Union and only agalsidase beta in the United States.

A phase 2 open-label trial was undertaken in patients of Japanese descent to evaluate whether ethnic differences exist that would affect treatment of Fabry disease with agalsidase beta in this population, in relation to safety and efficacy. The study design, including the safety and efficacy end points, mirrored the design of the completed phase 3 double-blind study with agalsidase beta (Eng et al 2001b). This choice of design was based on the concern that the relatively small number of patients with Fabry disease in Japan would not adequately power a primary end point in an open-label, single treatment group study design. The results of this phase 2 bridging study are reported here.

PATIENTS AND METHODS

Study design, patients, treatment regimen: This multicentre, phase 2, open-label trial was designed to evaluate the efficacy and safety of agalsidase beta treatment

and enrolled 13 male patients with confirmed Fabry disease. The patients received 1 mg/kg of agalsidase beta administered as 11 biweekly infusions over 20 weeks. Participating study sites were The Jikei University School of Medicine, Chubu National Hospital, Osaka University School of Medicine, Nagoya University School of Medicine, and Kyusyu University School of Medicine. The protocol was approved by the relevant institutional review boards and the trial was conducted in accordance with the Good Clinical Practice guidelines.

Patient eligibility criteria, as well as the clinical, biochemical, biopsy tissue (kidney, heart, skin) and safety assessments, were identical to those applicable for the phase 3 double-blind study. Refer to the publication by Eng et al (2001b) for details. For the management of potential infusion-associated reactions, the patients were pretreated with acetaminophen ($\leqslant$500 mg) or ibuprofen ($\leqslant$200 mg) and hydroxyzine (up to 30 mg) administered 1 h prior to each infusion. The infusion rate was less than 0.25 mg/min.

Evaluation of efficacy: The renal efficacy end point was the proportion of patients with a zero score for GL-3 deposits at week 20 (11 infusions). Additional tissue efficacy end points included microvascular endothelial deposits of GL-3 in the heart, skin and other kidney cell types. Biopsy tissues were scored (0–1–2–3 scoring system) by three blinded, independent pathologists. Specimens with no microvascular endothelial deposits or only trace amounts (normal or nearly normal) were given a score of 0; specimens in which the majority of vessels had evidence of a single endothelial inclusion (mild GL-3 accumulation) were given a score of 1; those with multiple aggregates of granules in the majority of capillaries were given a score of 2 (moderate GL-3 accumulation); and those with numerous aggregates of granules within the endothelium (often bulging into the lumen) in the majority of vessels were given a score of 3 (severe GL-3 accumulation). Majority scores were calculated per organ as well as summed for all organs. Change from baseline to week 20 was also assessed for GL-3 concentrations (ELISA assay) in kidney, urine sediment and plasma. Quality of life measurements included the Short Form McGill Pain Questionnaire and SF-36 Health Status Survey.

Statistical analysis: An exact binomial matched pair procedure was used primarily for the analysis of GL-3 accumulation in the capillary endothelium of the kidney to analyse the proportions of patients with a score of 0 at baseline compared to week 20. This test was also used to analyse GL-3 accumulation in the other cell types in the kidney and skin. A one-sample Wilcoxon signed-rank test was used to determine whether there was a significant difference from zero in the median change score from baseline to week 20. Descriptive statistics (n, mean, standard deviation, minimum, median, maximum) were displayed at baseline, at week 20, and for changes from baseline to week 20 for multiple parameters, such as the kidney, urinary and plasma GL-3 (ELISA), GL-3 accumulation in additional cell types, and the quality of life results as measured by the Short Form McGill Pain Questionnaire and SF-36 Health Status Survey. Change in SF-36 parameters was analysed by the Wilcoxon signed-rank test.

RESULTS

Patients: Baseline characteristics and demographic data for the 13 patients enrolled in the study are presented in Table 1.

Kidney GL-3 clearance

Kidney capillary endothelial cells. Reduction in kidney capillary endothelial cell GL-3 accumulation from non-0 scores to a 0 score (clearance) by week 20 was achieved by 12/13 (92%) patients ($p < 0.001$). At baseline, GL-3 accumulation was mild (score = 1) for 10 of 13 (77%) patients and moderate (score = 2) for 3/13 (23%) patients. All patients (10/10) with mild accumulation showed clearance at week 20. The same observation was made for 2 of 3 (67%) patients who had moderate accumulation at baseline. The third patient exhibited a reduction from moderate to mild. The overall change in median histology score for all 13 patients was −1.0 ($p < 0.001$).

Other kidney cell types. All 11 patients with kidney glomerular endothelial cell GL-3 accumulation (non-0 score) at baseline achieved clearance (0 score) at week 20. Of these patients, 7/11 (64%) had severe GL-3 accumulation (score = 3) at baseline, and 4/11 (36%) had moderate accumulation (score = 2).

All 12 patients with kidney noncapillary (arteriolar) interstitial endothelial cell GL-3 accumulation (non-0 score) at baseline achieved a 0 score at week 20. Of these 12 patients, 10 (83%) had severe GL-3 accumulation and 2 (17%) had moderate GL-3 accumulation in noncapillary (arteriolar) interstitial endothelial cells at baseline.

Skin GL-3 clearance

Skin capillary endothelial cells. Twelve of 13 (92%) patients achieved a reduction of skin capillary endothelial cell GL-3 from non-0 scores to a 0 score (clearance) at week 20 ($p < 0.001$). For these 12 patients, at baseline GL-3 accumulation was severe

Table 1 Patient details

Age (years)	
Mean ± SD	26.6 ± 5.5
Range	16–34
Weight (kg) (mean ± SD)	59.1 ± 8
Sex (*n*)	
Male	13
Female	0
Plasma α-GAL activity	BDL[a]
Leukocyte α-GAL activity	BDL[a]
Serum creatinine (mg/dl) (mean ± SD)	1.1 ± 0.28
Completed study *n* (%)	13 (100)

[a] BDL, below detectable level (<0.78 nmol/h per ml for plasma α-GAL and <0.78 nmol/h per mg for leukocyte α-GAL)

(score = 3) for 5 patients, moderate (score = 2) for 6, and mild (score = 1) for 1 patient. One patient with moderate GL-3 accumulation at baseline achieved a reduction to mild at week 20. The overall change in median histology score for all patients was −2.0 ($p < 0.001$).

Other skin cell types. Of patients with deep-vessel endothelial cell GL-3 accumulation in the skin (non-0 score) at baseline, 10/12 (83%) patients achieved reduction to a 0 score at week 20, and 2/12 (17%) patients achieved a decrease in GL-3 accumulation from moderate (score = 2) to mild (score = 1) at week 20.

Heart GL-3 clearance—heart capillary endothelial cells: Only one patient met the criteria for baseline cardiac biopsy as defined in the protocol. The cardiac capillary endothelial cells from this patient showed reduction of GL-3 from 'mild' (score = 1) GL-3 accumulation in cardiac capillary endothelial cells at baseline, to complete clearance at week 20.

GL-3 clearance—kidney, urine, plasma (by ELISA): The results of GL-3 clearance in the kidney, urinary sediment and plasma are shown in Table 2. In the 13 patients, the median kidney, urinary, and plasma GL-3 levels decreased by 51.9% ($p = 0.003$), 0.003), 55.4% ($p = 0.244$), and 100% ($p < 0.001$), respectively, between baseline and week 20.

Creatinine clearance: Creatinine clearance was used as a measure of the change in renal filtration function from baseline to week 20. The median creatinine clearance was 125.9 ml/min (mean ± SD = 126.6 ± 41.8) at baseline and 120.2 ml/min (mean ± SD = 115.3 ± 30.4) at week 20. This difference was not statistically significant ($p = 0.216$; Wilcoxon signed rank test). Median serum creatinine levels also

Table 2 Mean change from baseline to week 20 (11 infusions) for GL-3 levels in kidney, urine, and plasma (ELISA)

Organ		*Baseline*	*Week 20*	*% Change from baseline (week 20)*	*p-Value*[a]
Kidney (ng/mg)	Mean ± SD	2972 ± 1529	1667 ± 1760	−46.2	0.003
	Median	3149	1182	−51.9	
	Range	341–5098	171–6122		
Urine (nmol/filter)	Mean ± SD	4085 ± 2077	2687 ± 2514	65.1[b]	0.244
	Median	3680	1278	−55.4[b]	
	Range	62–7340	313–8080		
Plasma (ng/μl)	Mean ± SD	3.9 ± 2.7	0.2 ± 0.8	−89.4	<0.001
	Median	3.6	0	−100	
	Range	0–9	0–3		

[a] *p*-Value derived from a Wilcoxon signed rank test on change from baseline to week 20
[b] Values are correct. The upper end of the range (min–max) consisted of only a few patients; therefore, the median fell on a negative value

remained relatively stable at week 20 (not shown), suggesting maintenance of renal function.

Clinical assessments: Results from laboratory tests indicate that treatment with r-hαGAL appears to have no toxic effect. Ophthalmic, ECG, and echocardiogram findings further support this observation.

Quality of life assessments: Overall, median pain scores were at the low end at baseline and showed slight improvement at week 20 in all parameters. The median change score for present pain intensity (PPI) approached statistical significance ($p=0.063$). Likewise, there was improvement in all categories for the SF-36 Health Status Survey. Statistically significant improvement was observed in median values for the General Health ($p=0.023$) and the Mental Component Scale (MCS) scores ($p=0.048$). The median values from baseline to week 20 for the category Role – Emotional approached statistical significance ($p=0.063$). p-Values presented in this section refer to the Wilcoxon signed rank test.

Safety: All patients completed the study and each received all 11 infusions of agalsidase beta. All patients reported at least one adverse event (AE). Relation of AEs to the study drug were defined as possible, probable, definite or unknown. The most frequently reported related AEs were rigors (chills) and fever. These were infusion-associated reactions (related events that occurred on the same day as the infusion) and were mild or moderate in intensity (Table 3). These events were often managed with antihistamines and antipyretics or with a reduction in the infusion rate.

One patient experienced a serious adverse event considered to be related to the infusion. The patient was hospitalized overnight for observation owing to persistent malaise (related to fever) and limb pain after the infusion (probably related). The patient recovered without sequelae.

IgG seroconversion occurred in 11/13 (85%) patients. The mean time to seroconversion was 63.3 days. Seroconversion did not affect the patient's response to treatment. No IgE antibody formation was detected in any of the patients.

Table 3 Related adverse experiences occurring in >10% of patients

	Severity of adverse experience (% of patients)	
WHOART preferred term	*Mild*	*Moderate*
Rigors	3 (23)	2 (15)
Fever	2 (15)	2 (15)
Malaise	2 (15)	0
Dyspnoea	2 (15)	0
Rhinitis	2 (15)	0
Hypertension	1 (8)	1 (8)

AE counted once (most severe occurrence) if reported more frequently. There were no adverse experiences of severe intensity

DISCUSSION

This phase 2 open-label trial in patients of Japanese descent was conducted to evaluate whether ethnic differences exist in relation to safety and efficacy that could affect treatment of Fabry disease with agalsidase beta in this population. The study design, including the safety and efficacy end points, paralleled the design of the completed phase 3 double-blind study with agalsidase beta (Eng et al 2001b). The renal efficacy end point was the proportion of patients with clearance of GL-3 deposits from capillary endothelial cells at week 20 (11 infusions). Additional tissue efficacy end points included microvascular endothelial deposits of GL-3 in the heart, skin and other kidney cell types.

In the current study, the percentages of patients who achieved GL-3 clearance (zero score) from capillary endothelial cells after 20 weeks of agalsidase beta treatment were 92% ($p < 0.001$) for the kidney and 92% ($p < 0.001$) for skin. For those patients who received active drug during the double-blind portion of the completed phase 3 study, these percentages were 69% and 100%, respectively. After the 6-month open label extension portion of the phase 3 study, zero scores were reached in 98% and 96% of the patients, respectively. Clearance of GL-3 deposits (0 score) was observed for other cell types of the kidney as well, i.e. for all patients with glomerular endothelial cell GL-3 accumulation or noncapillary interstitial cell GL-3 accumulation. Although not assessed during the current study, variable responses to enzyme therapy may be observed for cell types such as podocytes and interstitial smooth-muscle cells. This may be due to either greater total GL-3 accumulation or/and relatively lower accessibility of enzyme therapy. Prolonged treatment may be necessary to remove a lifetime of accumulated GL-3. Clearance of GL-3 in heart tissue was not directly comparable to patients from the phase 3 study because a biopsy specimen was only obtained from one of the patients in the current study. This patient achieved clearance at 20 weeks.

Other efficacy end points, i.e. kidney, urine and plasma GL-3 measurements, creatinine clearance and quality of life measurements, also showed improvement and were comparable with findings from the phase 3 double-blind study. Median kidney, urinary and plasma GL-3 levels decreased by 51.9% ($p = 0.003$), 55.4% (not statistically significant) and 100% ($p < 0.001$), respectively, between baseline and week 20. For urinary GL-3, some outlier patients in our study may have shifted mean values for this category. Creatinine clearance and median serum creatinine levels also remained normal, suggesting maintenance of renal function. However, a longer duration of follow-up is needed to assess the change in renal function over time.

Patients had low baseline values for measurement of pain as determined by the McGill Pain Questionnaire but did show an overall improvement in all categories evaluated by the questionnaires. However, this study was not designed as a pain study and patients were not chosen on the basis of the presence of pain. In addition, there was no restriction on the use of pain medications. The statistically significant improvement in the General Health and Mental Component Scale scores of the SF-36 Health Status Survey are particularly encouraging since Fabry disease is a chronic disease and can have a significant impact on patients' quality of life. Long-term treatment is

needed to establish this particular effect more conclusively, as patients were allowed to continue on prophylactic pain medications while participating in the study.

The profile of adverse events considered in relation to treatment in the current phase 2 study is consistent when compared to the agalsidase beta treatment group in the phase 3 study and its extension. Infusion-associated events such as rigors, fever, dyspnoea and rhinitis coincided with the development of IgG antibodies, an expected response with the infusion of recombinant protein therapy. The proportion of patients who developed IgG antibodies was almost identical compared with patients in the phase 3 study, i.e. 85% and 83%, respectively. Seroconversion did not affect the patient's response to treatment. No patients developed IgE antibodies.

We conclude that the results of this phase 2 trial demonstrate that agalsidase beta is effective in the treatment of Fabry disease in Japanese patients and is well tolerated, as there were no safety issues. Because classical Fabry disease is progressive in nature, it often culminates in renal failure, cardiac failure and/or stroke resulting in death in the third to fifth decades of life. The long-term administration of agalsidase beta may significantly reduce the physical effects of Fabry disease and improve quality of life and possibly life expectancy. Results from the current study and the completed phase 3 study suggest that there are no ethnic differences between the Japanese and caucasian Fabry patient populations with regard to safety and efficacy of agalsidase beta treatment.

FUNDING

This study was supported by a grant from Genzyme Corporation, Cambridge, MA, USA.

REFERENCES

Desnick RJ, Wasserstein MP (2001) Fabry disease: clinical features and recent advances in enzyme replacement therapy. *Adv Nephrol Necker Hosp* **31**: 317–339.

Desnick RJ, Ioannou YA, Eng CM (2001) α-Galactosidase A deficiency: Fabry disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds; Childs B, Kinzler KW, Vogelstein B, assoc. eds. *The Metabolic and Molecular Bases of Inherited Disease*, 8th edn. New York: McGraw-Hill, 3733–3774.

Elleder M, Bradova V, Smid F, et al (1990) Cardiocyte storage and hypertrophy as a sole manifestation of Fabry's disease. Report on a case simulating hypertrophic non-obstructive cardiomyopathy. *Virchows Arch A Pathol Anat Histopathol* **417**(5): 449–455.

Eng CM, Banikazemi M, Gordon RE, et al (2001a) A phase 1/2 clinical trial of enzyme replacement in Fabry disease: pharmacokinetic, substrate clearance, and safety studies. *Am J Hum Genet* **68**(3): 711–722.

Eng CM, Guffon N, Wilcox WR, et al (2001b) Safety and efficacy of recombinant human α-galactosidase A replacement therapy in Fabry's disease. *N Engl J Med* **345**(1): 9–16.

Kolodny EH, Pastores GM (2002) Anderson–Fabry disease: extrarenal, neurologic manifestations. *J Am Soc Nephrol* **13**(supplement 2): S150–153.

Linhart A, Magage S, Palecek T, et al (2002) Cardiac involvement in Fabry disease. *Acta Paediatr Suppl* **91**(439): 15–20.

Lyon MF (2002) X-chromosome inactivation and human genetic disease. *Acta Paediatr Suppl* **91**(439): 107–112.

Meikle PJ, Hopwood JJ, Clague AE, et al (1999) Prevalence of lysosomal storage disorders. *JAMA* 281(3): 249–254.

Nakao S, Kodama C, Takenaka T, et al (2003) Fabry disease: detection of undiagnosed hemodialysis patients and identification of a 'renal variant' phenotype. *Kidney Int* 64(3): 801–807.

von Scheidt W, Eng CM, Fitzmaurice TF, et al (1991) An atypical variant of Fabry's disease with manifestations confined to the myocardium. *N Engl J Med* 324(6): 395–399.

Schiffmann R, Murray GJ, Treco D, et al (2000) Infusion of alpha-galactosidase A reduces tissue globotriaosylceramide storage in patients with Fabry disease. *Proc Natl Acad Sci USA* 97(1): 365–370.

Schiffmann R, Kopp JB, Austin HA 3rd, et al (2001) Enzyme replacement therapy in Fabry disease: a randomized controlled trial. *JAMA* 285(21): 2743–2749.

MOLECULAR REPRODUCTION AND DEVELOPMENT 70:464–470 (2005)

Birth of Offspring After Transfer of Mongolian Gerbil (*Meriones Unguiculatus*) Embryos Cryopreserved by Vitrification

KEIJI MOCHIDA,[1] TERUHIKO WAKAYAMA,[2] KAORU TAKANO,[3] YOKO NOGUCHI,[3] YOSHIE YAMAMOTO,[3] OSAMU SUZUKI,[3] JUNICHIRO MATSUDA,[3] AND ATSUO OGURA[1]*

[1]*Bioresource Center, RIKEN, Tsukuba, Japan*
[2]*Center for Developmental Biology, RIKEN, Kobe, Japan*
[3]*Department of Veterinary Science, National Institute of Infectious Diseases, Tokyo, Japan*

ABSTRACT The Mongolian gerbil (*Meriones unguiculatus*) has been used as a laboratory species in many fields of research, including neurology, oncology, and parasitology. Although the cryopreservation of embryos has become a useful means to protect valuable genetic resources, its application to the Mongolian gerbil has not yet been reported. In this study, we investigated the in vitro and in vivo developmental competence of Mongolian gerbil embryos cryopreserved by vitrification. In vivo-fertilized embryos were vitrified on the day of collection using the ethylene glycol (EG)-based solutions EFS20 and EFS40, which contained 20% and 40% EG, respectively, in PB1 containing 30% (w/v) Ficoll 70 and 0.5M sucrose. First, we compared one-step and two-step vitrification protocols. In the one-step method, the embryos were directly transferred into the vitrification solution (EFS40), whereas in the two-step method, the embryos were exposed serially to EFS20 and EFS40 and then vitrified. After liquefying (thawing), late two-cell embryos (collected on day 3) vitrified by the two-step method showed significantly better rates of in vitro development to the morula stage compared to those vitrified by the one-step method (65% vs. 5%, $P < 0.0001$). We then examined whether the same two-step method could be applied to early two-cell embryos (collected on day 2), four-cell embryos (day 4), morulae (day 5), and blastocysts (day 6). After liquefying, 87%–100% of the embryos were morphologically normal in all groups, and 23% and 96% developed to the compacted morula stage from early two- and four-cell embryos, respectively. After transfer into recipient females, 3% (4/123), 1% (1/102), 5% (4/73), and 10% (15/155) developed to full-term offspring from vitrified and liquefied early two-cell embryos, late two-cell embryos, morulae, and blastocysts, respectively. This demonstrates that Mongolian gerbil embryos can be safely cryopreserved using EG-based vitrification solutions. *Mol. Reprod. Dev. 70: 464–470, 2005.* © 2005 Wiley-Liss, Inc.

Key Words: cryopreservation; ethylene glycol; embryo transfer

INTRODUCTION

The Mongolian gerbil (*Meriones unguiculatus*), also called the "laboratory gerbil", is a myomorph rodent that is native to China and Mongolia. It has been widely used as a laboratory animal in biomedical research, including the study of epilepsy (Jobe et al., 1991), tumor (Meckley and Zwicker, 1979), hypercholesterolemia (Dictenberg et al., 1995), and cerebral ischemia (Levine and Payan, 1966). This species has also been used to develop good animal models for a variety of infectious diseases caused by bacteria, viruses, and parasites; for example, *Helicobacter pylori* (Yokota et al., 1991; Sugiyama et al., 1998), Borna disease virus (Nakamura et al., 1999), *Echinococcus multilocularis* (Williams and Oriol, 1976), *Cryptospordium muris* (Koudela et al., 1998), *Brugia-pahangi* (Klei et al., 1981), *Giardia duodenalis* (Buret et al., 1991), and *Entamoeba histolytica* (Chadee and Meerovitch, 1984). Although gerbils were randomly bred in closed laboratory colonies for the first decades of their use, selective breeding has recently been conducted to establish laboratory strains that are suited for each research purpose. The best-characterized strains include seizure-sensitive and -resistant strains (Loskota et al., 1974; Robbins, 1976; Seto-Ohshima et al., 1997) and mutant strains with different coat colors (Robinson, 1973; Shimizu et al., 1990).

Grant sponsor: MEXT, Japan; Grant sponsor: MHLW, Japan; Grant sponsor: CREST, Japan; Grant sponsor: Human Science Foundation, Japan.

*Correspondence to: Atsuo Ogura, Bioresource Center, RIKEN, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan.
E-mail: ogura@rtc.riken.jp

Received 16 July 2004; Accepted 11 October 2004
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20226

For many domestic and laboratory species, assisted reproductive technologies have been developed to enhance live animal production and safe cryopreservation of genetic materials. However, the major assisted reproductive technologies—including in vitro fertilization, embryo culture, embryo transfer, and embryo cryopreservation—have not yet been established for Mongolian gerbils. This is a major drawback of working with Mongolian gerbils and it impedes their broad exploitation in biomedical research. The present study was undertaken to develop a reliable experimental protocol for cryopreservation of gerbil embryos. For this purpose, we employed embryo vitrification methods, which have been successfully used for mice (Kasai et al., 1990), rabbits (Kasai et al., 1992), cattle (Tachikawa et al., 1993), horses (Hochi et al., 1994), humans (Mukaida et al., 1998), mastomys (Mochida et al., 2001), and rats (Han et al., 2003). As little information is available concerning techniques related to embryo manipulation in gerbils, we also examined whether the protocols for superovulation and embryo transfer conventionally used for mice and rats could be applied to gerbils.

MATERIALS AND METHODS

Animals

Mongolian gerbils (*Meriones unguiculatus*) from inbred strains MGS/Sea (agouti coat color, Seac Yoshitomi, Ltd., Fukuoka, Japan) and MGB (black coat color, from the Nippon Medical School, Tokyo, Japan; Shimizu et al., 1990) were maintained under specific-pathogen-free conditions at the National Institute of Infectious Diseases, Japan. They were kept under controlled lighting conditions (light: 05:00–19:00) and provided with water and commercial laboratory mouse chow *ad libitum*. All animals were maintained and handled in accordance with the guidelines of the National Institute of Infectious Diseases, Japan. As the results obtained from different strains of gerbil were not significantly different, they were combined in this study.

Collection of Embryos

Mature females (7–18 weeks of age) were induced to superovulate by intraperitoneal injection of 10 IU pregnant mare's serum gonadotrophin (PMSG) between 3 and 5 pm, followed by injection of 10 IU human chorionic gonadotrophin (hCG) 44–46 hr later. The superovulated females were mated with mature males in cages with wire net floors. The next morning (designated day 1 of pregnancy), the presence of a copulation plug was confirmed. The early two-cell embryos, late two-cell embryos, four-cell embryos, and morulae were collected by flushing the oviducts (at 48, 72, 96, 120 hr post-hCG) with modified phosphate buffered saline (PB1, Whittingham, 1971a); blastocysts were also collected by flushing the uteri (144 hr post-hCG). The collected embryos were placed in culture dishes containing droplets of M16 medium (Whittingham, 1971b) covered with paraffin oil and cultured at 37°C under 5% CO_2 in air until cryopreservation or embryo transfer.

Cryopreservation of Embryos

In the first series of experiments, we assessed the toxicity of different cryoprotectants to optimize the cryopreservation solution suitable for gerbil embryos. Freshly collected late two-cell (day 3) embryos were immediately suspended in 2M solutions of ethylene glycol (EG), glycerol, dimethyl sulfoxide (DMSO), propylene glycol (PG), or acetamide in PB1 at room temperature (22°C) for 10 min. After washing by serial transfers into three drops of PB1 at room temperature, the rate of in vitro development to the compacted morula stage was assessed using the culture conditions described above. Preliminary experiments showed that the morula stage was the most advanced stage to which fresh two-cell embryos developed under our in vitro culture conditions.

Vitrification was performed according to the method developed for mouse embryos by Kasai et al. (1990), with slight modifications. We prepared two vitrification solutions, EFS20 and EFS40, which consisted of 20% and 40% (v/v) EG, respectively, in PB1 solution containing 30% (w/v) Ficoll (average molecular weight 70,000), and 0.5M sucrose. In this study, we employed both one- and two-step vitrification protocols. For the one-step method, 13–20 embryos, together with a minimal amount of culture medium, were directly introduced, using a fine glass pipette, into approximately 40 μl EFS40 solution in a 0.25-ml plastic straw held horizontally. The plug ends were sealed with polyvinyl alcohol powder. After exposure of embryos to the EFS solution for 2 min at room temperature, the straw was immersed in liquid nitrogen. For the two-step method, 13–20 embryos were suspended in EFS20 solution for 2 min at room temperature. They were then directly transferred to approximately 40 μl EFS40 solution in a straw, as described above. After exposure of the embryos to EFS40 solution at room temperature for 30 sec, the sealed straw was immersed in liquid nitrogen.

To liquefy, (thaw; for terminology, see Shaw and Jones, 2003) the embryos for further evaluation, after storage in liquid nitrogen for at least 2 days, a straw was warmed rapidly in 22°C water for about 8 sec. Immediately after warming, the EFS solution containing the embryos was expelled from the straw onto a watch glass using a metal rod. The solution was diluted by addition of 0.8 ml PB1 medium containing 0.25M or 0.5M sucrose (S-PB1) and the embryos were retrieved into fresh S-PB1 medium. At 5 min after liquefying, the embryos were transferred to PB1 medium.

Embryo Transfer

Two types of pseudopregnant recipient females were prepared for embryo transfer: hormone-treated recipients and nontreated recipients. For the former, females were pre-treated with hormones for superovulation, as described above. Only virgin females were used for both groups. The vasectomized males for induction of pseudopregnancy were prepared at least 4 weeks before the experiments and infertility was confirmed by the

absence of sperm in the vagina of females after mating. Each recipient female was mated with a mature vasectomized male in a cage with a wire net floor. On the following morning (designated day 1 of pseudopregnancy), the females were examined for the presence of a vaginal plug and used for embryo transfer. Shortly before embryo transfer, the recipient females were anesthetized with an intraperitoneal injection of sodium pentobarbital (57.5 mg/kg).

Statistical Analysis

The results were evaluated using Fisher's exact probability test. Values of P less than 0.05 were considered statistically significant.

RESULTS

Effects of Cryoprotectants on the Development of Gerbil Embryos

Gerbil embryos were exposed to one of five different cryoprotectants and their subsequent development was assessed. As shown in Figure 1, embryos exposed to EG and PG showed rates of development to the compacted morula stage similar to those of nonexposed control embryos ($P > 0.05$). In contrast, those exposed to glycerol or acetamide had significantly decreased developmental competence (Fig. 1). Exposure to DMSO had an intermediate effect on embryo development. Therefore, we selected EG-based solutions for use in our cryopreservation experiments for gerbil embryos, as has been done for mouse embryos (Kasai et al., 1990).

Comparison of One- and Two-step Methods

Late two-cell (day 3) gerbil embryos that had been vitrified using the one- or two-step methods were liquefied and examined for subsequent development in vitro. More embryos developed to the compacted morula stage in vitro when they had been vitrified by the two-step method using EFS20 and EFS40, and liquefied in 0.25M sucrose solution (36/55, 69%) as compared to the other vitrification and liquefying procedures (Fig. 2). Only 5% of embryos (2/43) developed to the morula stage after the one-step vitrification method using EFS40 alone (Fig. 2).

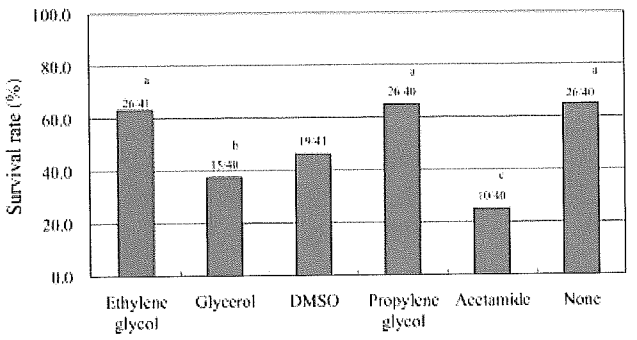

Fig. 1. Survival of late two-cell (day 3) gerbil embryos after exposure to cryoprotectants in PB1 for 10 min at 22°C. The numbers on the bars indicate surviving embryos per exposed embryos. The values with different letters (a, b, and c) are statistically different ($P < 0.05$).

In light of these results, we employed the two-step method for the subsequent vitrification experiments.

Effect of the Stage of Development Upon Cryopreservation on Subsequent In Vitro Embryo Development

Using the two-step method described above, we vitrified embryos at different stages of development and later liquefied and examined them to determine their survival and subsequent development in vitro and in vivo. When recovered into normal culture medium, most embryos (>87%) appeared to be morphologically normal, regardless of the stage at which the embryos were vitrified (Table 1). When cultured in vitro, embryos vitrified at the late two-cell (day 3) and four-cell (day 4) stages showed in vitro development comparable to that of nonvitrified control embryos at the same stage (Table 1). When embryos were vitrified at the early two-cell stage (day 2), in vitro developmental competence was significantly lower than that of controls (Table 1).

Development of Vitrified Gerbil Embryos After Embryo Transfer

We found that hormonal treatment of the recipient females made the efficiency of mating with males more consistent compared with natural mating, which occurs at random. However, as hormonal treatment may compromise the oviductal and uterine environments for transferred embryos, we first assessed whether hormonally treated recipient females conceived after embryo transfer. Early two-cell embryos (day 2) and blastocysts (day 6) transferred into the recipient oviducts and uteri, respectively, developed to term, regardless of whether the recipient females had been treated with hormones (Table 2). For further embryo transfer experiments, therefore, we used recipient females prepared by natural mating without hormone treatment, to optimize the conditions for efficient embryo transfer.

Early two-cell embryos (day 2), late two-cell embryos (day 3), morulae (day 5), and blastocysts (day 6) were vitrified using the two-step method, liquefied, and assessed for their developmental competence after transfer into recipient females. Four-cell embryos (day 4) were not transferred because day 3 oviduct (ampullar) is not an appropriate transfer site in gerbils owing to the distance from the position of the native oocytes, which at this stage have descended to a point near the uterine–oviductal junction.

Although the rates of development to full-term offspring were not high, normal pups were born from embryos vitrified at all stages, indicating that at least a portion of the embryos were completely viable and competent after vitrification and liquefying (Table 3; Fig. 3). The implantation rates were also low (Table 3), indicating that most embryos died before implantation, or simply failed to attach to the uterine epithelium. The weaning rates varied according to the experiment, but all of weaned pups developed into adults with normal appearance, to the extent examined.

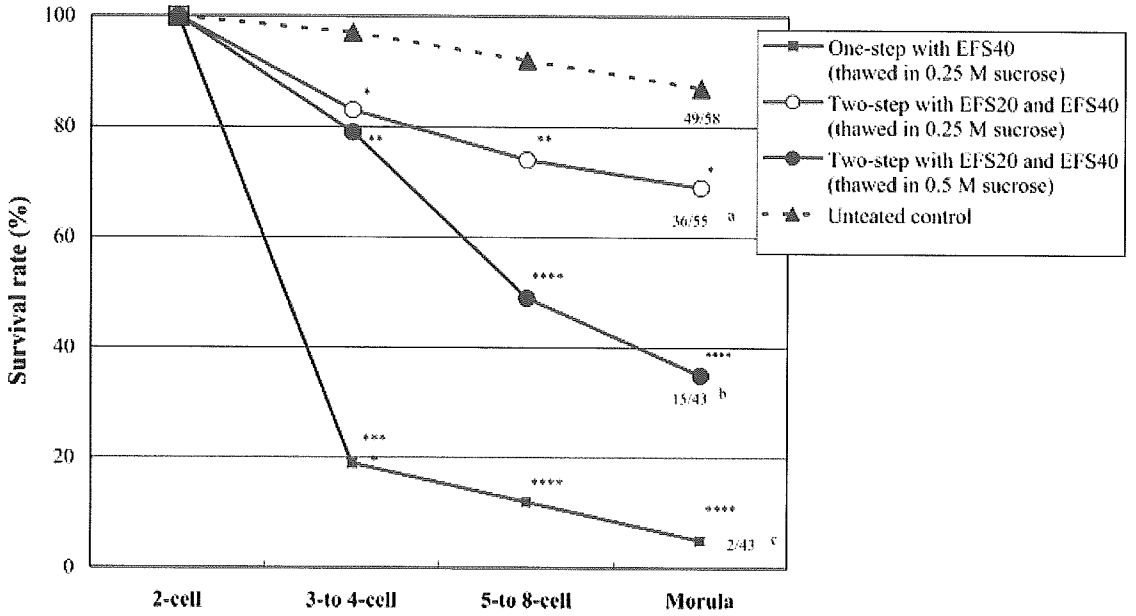

Fig. 2. In vitro development of late two-cell (day 3) gerbil embryos vitrified in EFS solution by the one-step or two-step method. Numbers indicate surviving embryos per cultured embryos. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.001$, as compared with the nonvitrified control at the same stage. $^{a,b}P < 0.001$, $^{b,c}P < 0.005$, and $^{a,c}P < 0.0001$.

DISCUSSION

The present study clearly demonstrated that Mongolian gerbil embryos could survive freezing and liquefying procedures at high rates and that some of them develop into normal full-term offspring. We employed a vitrification method using EG-based cryoprotectant solutions. Embryo cryopreservation by vitrification was first developed by Rall and Fahy (1985) for mouse embryos. It has potential advantages over conventional slow-freezing methods because of its very rapid cooling time and minimal cell injury caused by extracellular crystallization (Rall, 1987). However, the original vitrification solution consisted of four cryoprotectants, including acetamide, which is known to be very toxic to embryos; therefore, its application eventually was limited to embryos of certain strains of mice. Later, this complication was overcome by development of less toxic vitrification solutions using EG or glycerol (Kasai et al., 1990; Zhu et al., 1994; Rall et al., 2000). The use of EG also increased the flexibility of the conditions for vitrification protocols (e.g., exposure time to cryoprotectant) and thus increased the reproducibility of the vitrification experiments (Kuleshova et al., 1999; Nowshari and Brem, 2001). In this study, we confirmed the low toxicity of EG for gerbil embryos, but glycerol showed moderate toxic effects for gerbil embryos. To date, successful vitrification using EG has been reported for

TABLE 1. In Vitro Development of Gerbil Embryos After Vitrification by the Two-Step Method With EFS20 and EFS40

		No. (%) of embryos				
Stage of embryos (day)	Treatment	Vitrified	Recovered	Morphologically normal	Cultured	Developed to morula
Early two-cell (2)	Vitrified	40	39 (98)	39 (100)	39	9 (23)*
	Control	—	—	—	36	31 (86)*
Late two-cell (3)	Vitrified	176	169 (96)	147 (87)	55	38 (69)
	Control	—	—	—	49	39 (80)
Four-cell (4)	Vitrified	54	50 (93)	50 (100)	50	48 (96)
	Control	—	—	—	60	51 (85)
Morula (5)	Vitrified	94	92 (98)	87 (95)	—	—
Blastocyst (6)	Vitrified	50	46 (92)	46 (100)	—	—

$^{*}P < 0.001$.

TABLE 2. In Vivo Development of Gerbil Embryos Transferred Into Pseudopregnant Recipients With or Without Treatment for Induction of Sterile Mating

Stage of embryos (day)	Treatment before sterile mating	No. (%) of recipients that became pregnant	No. of embryos transferred	No. (%)		
				Implanted	Live offspring delivered	Offspring weaned
Early two-cell (2)	No treatment	3/7 (43)	80	12 (15)	9 (11)	4 (5)
	Hormone treatment	4/8 (50)	124	23 (19)	10 (8)	7 (6)
Blastocyst (6)	No treatment	5/5 (100)	77	40 (52)	37 (48)*	31 (40)*
	Hormone treatment	5/7 (71)	111	34 (31)	20 (18)*	15 (14)*

Early two-cell and blastocyst were transferred into the oviducts (day 1) and uteri (day 5) of recipient females, respectively.
*$P < 0.001$.

many mammalian species (see Introduction). In mice, its applicability for embryos at each developmental stage has been assessed in detail (Miyake et al., 1993).

As vitrification solutions contain very high concentrations of cryoprotectants and sucrose to circumvent intracellular ice formation, the embryos are exposed to extremely high osmolality before they are vitrified. It had been reported that this osmotic shock may compromise the viability of the embryos, but can be reduced by the step-wise exposure of embryos to solutions with increasing osmolalities. We found that this was also the case with gerbil embryos. When late two-cell gerbil embryos were vitrified using the two-step method with EFS20 and EFS40, their developmental competence was significantly improved; as many as 65% of the frozen and liquefied embryos reached the morula stage in vitro. The osmolalities of EFS20 and EFS40 were about 4.5 Osmol/kg and 9 Osmol/kg, respectively, as measured by an automated osmometer. In mice, we found that better survival rates could be achieved for embryos from certain strains (e.g., DBA/2, ddY) using the two-step method, as compared to a one-step method with EFS30. Thus far, mouse embryos from 248 strains, including genetically modified mice, have been safely cryopreserved using the two-step method and their viability confirmed by full-term development after liquefying and embryo transfer (unpublished).

In the last series of experiments, we assessed the viability and competence of vitrified gerbil embryos by transfer into recipient females. Because there have been very few studies on embryo transfer in gerbils, the best combinations of the embryo stage and the day of pseudopregnancy of the recipient females are not known. In preliminary experiments, late two-cell (day 3) embryos did not survive when transferred into 1-day pseudopregnant oviducts. In this study, therefore, the recipient females were implanted with embryos 1 day older than the female's pseudopregnancy. The oviducts and uteri of gerbils accepted embryos at different stages of development as long as a 1-day difference existed between the recipients and embryos. Embryos at every developmental stage developed into normal offspring, regardless of whether the embryos had been vitrified. However, the rates of normal birth per transfer were very low, being less than 10% in most cases. We also found that the implantation rates of both vitrified and fresh embryos were low, except in the case of fresh 6-day embryos. This indicates that embryo transfer techniques for gerbils can still be improved, probably by optimizing the transfer timing. The in vitro culture medium for gerbil embryos should also be improved, because no two-cell or four-cell embryos reached the blastocyst stage in M16 medium, which was originally developed for mouse embryos. We have previously reported that embryos of

TABLE 3. In Vivo Development of Vitrified Gerbil Embryos After Transferred Into Pseudopregnant Females

Stage of embryos (day)	Recipient female			No. of embryos transferred	No. (%) of		
	Stage (day)	Transfer site	No. (%) that became pregnant		Implanted	Live offspring delivered	Offspring weaned
Early two-cell (2)	1	Oviduct	2/8 (25)	123	6 (5)*	4 (3)**	2 (2)**
Late two-cell (3)	2	Oviduct	1/6 (17)	102	6 (6)*	1 (1)*	1 (1)**
Morula (5)	4	Uterus	2/6 (33)	73	9 (12)	4 (5)	2 (3)
Blastocyst (6)	5	Uterus	3/10 (33)	155	30 (19)*	15 (10)*,**	13 (8)**
Blastocyst (6)	5[a]	Uterus	3/7 (43)	110	25 (23)	17 (15)	15 (14)

Embryos were transferred into recipient females on the day of thawing.
[a]Without hormone treatment (natural cycle).
*$P < 0.005$.
**$P < 0.05$.

Fig. 3. Gerbil pups born after transfer of vitrified blastocysts (black). They looked normal and showed active movement.

mastomys, a laboratory rodent native to Africa, developed into blastocysts in glutamine-containing medium, but not in a medium lacking glutamine (Ogura et al., 1997). Future experiments should examine whether this is also the case with gerbil embryos.

No conventional methods yet exist for embryo transfer in the Mongolian gerbil, in part because, unlike other rodents such as mice and rats, induction of pseudopregnancy in female gerbils is difficult due to the unique character of the reproductive biology of this species. First, females and males caged together after reaching sexual maturity often show very aggressive behavior because of their monogamous nature. It has been reported that the incidence of fighting and mortality can be decreased to some extent by combining an elder male and a virgin female (Norris and Adams, 1972). We employed this combination for our embryo transfer experiments. Second, unlike in mice and rats, it is difficult to identify the estrous cycle in gerbils by vaginal smears due to their irregular patterns. The estrous cycle of the Mongolian gerbil generally lasts for 4 to 7 days, varying by individual animal (Marston and Chang, 1965). In a preliminary experiment, we examined whether the cycle could be synchronized by the hormone treatment used for superovulation. On the day following hCG administration and mating, 61% (n = 41) of females had a copulation plug. As these hormone-treated females were proven to conceive after embryo transfer, we conventionally employed this method to conduct transfer experiments using vitrified embryos. Successful embryo transfer in Mongolian gerbils was first reported by Norris and Rall (1983), who used lactating pregnant females as recipients after ligating their single oviduct during early pregnancy. This method gave excellent results by exploiting lactation-induced delayed implantation for embryo transfer.

In conclusion, Mongolian gerbil embryos can be cryopreserved safely using a two-step vitrification method with EG-based cryoprotectant solutions. The offspring derived from vitrified embryos appeared normal and grew into fertile adults. This strategy will enable efficient maintenance of gerbil breeding colonies and avoid microbiological and genetic contamination that may occur during natural breeding.

ACKNOWLEDGMENTS

The authors thank Dr. M. Kasai, Kochi University, for helpful discussion, and Dr. K. Shichinohe and Dr. M. Shimizu-Suganuma, Nippon Medical School, for generous donation of MGB gerbils.

REFERENCES

Buret A, Gall DG, Olson ME. 1991. Growth, activities of enzymes in the small intestine, and ultrastructure of microvillus border in gerbils infected with *Giardia duodenalis*. Parasitol Res 77:109–114.

Chadee K, Meerovitch E. 1984. The Mongolian gerbil (*Meriones unguiculatus*) as an experimental host for *Entamoeba histolytica*. Am J Trop Med Hyg 33:47–54.

Dictenberg JB, Pronczuk A, Hayes KC. 1995. Hyperlipidemic effects of trans fatty acids are accentuated by dietary cholesterol in gerbils. J Nutr Biochem 6:353–361.

Han MS, Niwa K, Kasai M. 2003. Vitrification of rat embryos at various developmental stages. Theriogenology 59:1851–1863.

Hochi S, Fujimoto T, Braun J, Oguri N. 1994. Pregnancies following transfer of equine embryos cryopreserved by vitrification. Theriogenology 42:483–488.

Jobe PC, Mishra PK, Ludvig N, Dailey JW. 1991. Scope and contribution of genetic models to an understanding of the epilepsies. Crit Rev Neurobiol 6:183–220.

Kasai M, Komi JH, Takakamo A, Tsudera H, Sakurai T, Machida T. 1990. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J Reprod Fertil 89:91–97.

Kasai M, Hamaguchi Y, Zhu SE, Miyake T, Sakurai T, Machida T. 1992. High survival of rabbit morulae after vitrification in an ethylene glycol-based solution by a simple method. Biol Reprod 46: 1042–1046.

Klei TR, Enright FM, Blanchard DP, Uhl SA. 1981. Specific hyporesponsive granulomatous tissue reactions in *Brugia pahangi*-infected birds. Acta Tropica 38:267–276.

Koudela B, Modry D, Vitovec J. 1998. Infectivity of *Cryptsporidium muris* isolated from cattle. Vet Parasitol 76:181–188.

Kuleshova LL, MacFarlane DR, Trounson AO, Shaw JM. 1999. Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. Cryobiology 38:119–130.

Levine S, Payan H. 1966. Effects of ischemia and other procedures on the brain and retina of the gerbil (*Meriones unguiculatus*). Exp Neurol 16:255–262.

Loskota WJ, Lomax P, Rich ST. 1974. The gerbil as a model for the study of the epilepsies. Seizure patterns and ontogenesis. Epilepsia 15: 109–119.

Marston JH, Chang MC. 1965. The breeding, management and reproductive physiology of the Mongolian gerbil (*Meriones unguiculatus*). Lab Anim Care 15:34–48.

Meckley PE, Zwicker GM. 1979. Naturally-occurring neoplasms in the Mongolian gerbil. Lab Anim 13:203–206.

Miyake T, Kasai M, Zhu SE, Sakurai T, Machida T. 1993. Vitrification of mouse oocytes and embryos at various stages of development in an ethylene glycol-based solution by a simple method. Theriogenology 40:121–134.

Mochida K, Matsuda J, Suzuki O, Nakahira M, Noguchi A, Nakayama K, Kurosawa S, Takano K, Noguchi Y, Yamamoto Y, Ogura A. 2001. Development of reproductive biotechniques in mastomys. In: Miyamoto H, Manabe N, editors. Reproductive biotechnology. Kyoto: Hokuto Shobo, pp 279–284.

Mukaida T, Wada S, Takahashi K, Pedro PB, An TZ, Kasai M. 1998. Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. Hum Reprod 13:2874–2879.

Nakamura Y, Nakaya T, Hagiwara K, Momiyama N, Kagawa Y, Taniyama H, Ishihara C, Sata T, Kurata T, Ikuta K. 1999. High susceptibility of Mongolian gerbil (*Meriones unguiculatus*) to Borna disease virus. Vaccine 17:480–489.

Norris ML, Adams CE. 1972. Aggressive behaviour and reproduction in the Mongolian gerbil, *Meriones unguiculatus*, relative to age and sexual experience at pairing. J Reprod Fertil 31:447–450.

Norris ML, Rall WF. 1983. Egg transfer in the Mongolian gerbil (*Meriones unguiculatus*) during lactational delay of implantation. J Reprod Fertil 68:123–128.

Nowshari MA, Brem G. 2001. Effect of freezing rate and exposure time to cryoprotectant on the development of mouse pronuclear stage embryos. Hum Reprod 16:2368–2373.

Ogura A, Matsuda J, Mochida K, Suzuki O, Takimoto K, Nakayama K, Naiki M. 1997. Preimplantation embryo development in mastomys (*Praomys coucha*) in vivo and in vitro. J Reprod Dev 43:65–71.

Rall WF. 1987. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology 24:387–402.

Rall WF, Fahy GM. 1985. Ice-free cryopreservation of mouse embryos at −196°C by vitrification. Nature 313:573–575.

Rall WF, Schmidt PM, Lin X, Brown SS, Ward AC, Hansen CT. 2000. Factors affecting the efficiency of embryo cryopreservation and rederivation of rat and mouse models. ILAR J 41:221–227.

Robbins ME. 1976. Seizure resistance in albino gerbils. Lab Anim 10: 233–235.

Robinson R. 1973. Acromilanic albinism in mammals. Genetica 44: 454–458.

Seto-Ohshima A, Murashima Y, Kawamura N, Aoi T, Ito M. 1997. Facial nerve innervating pinnae muscles of the gerbil: Three-dimensional construction with respect to neighboring structures. Acta Histochem Cytochem 30:653–660.

Shaw JM, Jones GM. 2003. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. Hum Reprod Update 9:583–605.

Shimizu M, Shichinohe K, Tsukidate S, Fujita K. 1990. Basic studies on the Mongolian gerbil as a susceptible host to filarial infection comparative studies on growth and reproduction among coat color mutants and genetic analysis of coat colors. Jpn J Trop Med Hyg 18: 301–310.

Sugiyama A, Maruta F, Ikeno T, Ishida K, Kawasaki S, Katsuyama T, Shimizu N, Tatematsu M. 1998. *Helicobacter pylori* infection enhances N-methyl-N-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. Cancer Res 58:2067–2069.

Tachikawa S, Otoi T, Kondo S, Machida T, Kasai M. 1993. Successful vitrification of bovine blastocysts derived by in vitro maturation and fertilization. Mol Reprod Dev 34:266–271.

Whittingham DG. 1971a. Survival of mouse embryos after freezing and thawing. Nature 233:125–126.

Whittingham DG. 1971b. Culture of mouse ova. J Reprod Fertil 14:7–12.

Williams JF, Oriol R. 1976. Immunological response to experimental echinococcosis in *Meriones unguiculatus*. J Parasitol 62:563–568.

Yokota K, Kurebayashi Y, Takayama Y, Hayashi S, Isogai H, Isogai E, Imai K, Yabana T, Yachi A, Oguma K. 1991. Colonization of *Helicobacter pylori* in the gastric mucosa of Mongolian gerbils. Microbiol Immun 35:475–480.

Zhu SE, Sakurai T, Edashige K, Machida T. Kasai M. 1994. Optimization of the procedures for the vitrification of expanded mouse blastocysts in glycerol-based solutions. J Reprod Dev 40:293–300.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Genetics and Metabolism 84 (2005) 332–343
Molecular Genetics and Metabolism
www.elsevier.com/locate/ymgme

GALC transduction leads to morphological improvement of the twitcher oligodendrocytes in vivo

Xing-Li Meng[a], Jin-Song Shen[a], Kazuhiko Watabe[a,c], Toya Ohashi[a,b,*], Yoshikatsu Eto[a,b]

[a] *Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine, Tokyo, Japan*
[b] *Department of Pediatrics, The Jikei University School of Medicine, Tokyo, Japan*
[c] *Department of Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan*

Received 6 November 2004; received in revised form 13 December 2004; accepted 13 December 2004
Available online 24 January 2005

Abstract

Globoid cell leukodystrophy (GLD, Krabbe disease) is a severe demyelinating disease caused by a genetic defect of β-galactocerebrosidase (GALC). To date treatment to GLD is limited to hematopoietic stem cell transplantation. Experimental approaches by means of gene therapy in twitcher mouse, an authentic murine model of human GLD, showed significant but only marginal improvements of the disease. To clarify whether the introduction of GALC could provide beneficial effects on the oligodendrocytes in GLD, we transduced twitcher oligodendrocytes by stereotactically injecting recombinant retrovirus encoding GALC-myc-tag fusion gene into the forebrain subventricular zone of neonatal twitcher mouse. In vivo effects of exogenous GALC on twitcher oligodendrocytes were studied histologically by combined immunostaining for the myc-epitope and the oligodendroglial specific marker, π form of glutathione-*S*-transferase, at around 40 days of age. We show here that GALC transduction led to dramatic morphological improvement of the twitcher oligodendrocytes comparing with those in untreated twitcher controls. This study provided direct in vivo evidence that GALC transduction could prevent or correct aberrant morphology of oligodendrocytes in GLD which may be closely related to the dysfunction and/or degeneration of oligodendrocytes and the demyelination in this disease.

Keywords: Krabbe disease; Twitcher; Galactocerebrosidase; Oligodendrocyte; Gene therapy; Retrovirus vector

Introduction

Globoid cell leukodystrophy (GLD, Krabbe disease) is a demyelinating disease caused by a genetic deficiency in the activity of a lysosomal enzyme, β-galactocerebrosidase (GALC, EC 3.2.1.46) [1]. In infantile form of this disease, the clinical symptoms occur soon after birth, progress rapidly and most patients die before 2 years of age. The rapid deterioration of the nervous system is postulated to be the result of the dysfunction and/or degeneration of myelin-forming cells (oligodendrocytes and Schwann cells) caused by an accumulation of galactosylsphingosine (psychosine), a cytotoxic metabolite and one of the substrates of GALC [2,3]. To date, there is no effective treatment to this disease except hematopoietic stem cell transplantation [4].

Twitcher mouse is a naturally occurring genetically authentic murine model of human GLD [5]. Deficiency in the activity of GALC is caused by a nonsense mutation at the coding region of GALC gene [6]. The clinical symptoms of the twitcher mouse include failure to gain weight, tremor, and progressive paralysis of hind limbs and early death around postnatal day 40 (P40). Similar to human GLD, the characteristic pathology in the central and peripheral nervous system (CNS and PNS) of the twitcher mouse is severe demyelination, infiltration

* Corresponding author. Fax: +81 3 3433-1230.
E-mail address: tohashi@jikei.ac.jp (T. Ohashi).

1096-7192/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2004.12.007

of periodic acid-Schiff (PAS)-positive macrophages, and gliosis. In the brain, oligodendrocytes show morphological alterations and are gradually depleted by apoptotic death [7,8].

The treatments to GLD, like those for most of the other lysosomal storage diseases, are to deliver the deficient enzyme to the cells affected by the storage. Bone marrow transplantation [9–11], neural stem cell transplantation [12], and vector-mediated gene therapy [13,14] in the twitcher mouse showed various degrees of correction of the biochemical, pathological, and clinical phenotypes of the disease. The level of GALC activity appeared responsible for the therapeutic effects. So far, little direct evidence showing that exogenous GALC enzyme was delivered to myelin-forming cells is available. Recent study [15] suggested that down-regulated immune-related molecules in twitcher mouse following bone marrow transplantation may also contribute to clinicopathological improvements. Whether transduction of GALC into myelin-forming cells alone has therapeutic benefit will be crucial in treating GLD, especially by means of gene transfer. In vitro studies showed retrovirus-mediated transduction of GALC led to morphological normalization in cultured oligodendrocytes derived from twitcher mouse [16,17], however taken that the culture system may be different from the in vivo environments such as the absence of immunological environments which is considered to play important roles in the destruction of oligodendrocytes and demyelination in twitcher [18], it is important to clarify whether GALC transduction improves the morphology of the oligodendrocytes in GLD in vivo.

In this study, we introduced human GALC cDNA tagged with myc-epitope into a small number of oligodendrocytes in the twitcher brain by stereotactic injection of retrovirus into the subventricular zone (SVZ) at birth. By combined immunostaining for the myc-tag and the oligodendroglial specific marker, π form of glutathione-*S*-transferase (GST-π), we clearly show that GALC transduction led to dramatic morphological improvement of the twitcher oligodendrocytes comparing with those in untreated twitcher controls. The results provided direct evidence that GALC transduction could prevent or correct aberrant morphology of oligodendrocytes in GLD in vivo which may be closely related to the degeneration of oligodendrocytes and the demyelination process in this disease.

Materials and methods

Plasmids

The full-length coding region of human GALC cDNA was cloned [13] and inserted into *Cla*I site of pBluescript II KS (Stratagene, La Jolla, CA). To assess the influence of ATG surrounding sequence to GALC expression level, the region around the initiation codon was removed using *Not*I (in +7 position of GALC) and *Sac*I (in the vector), and replaced by three different fragments generated from synthetic complementary oligonucleotides containing an additional *Cla*I site. The first fragment contains an ATG surrounding sequence initially reported [19,20] and named as "original ATG" in this study. The second fragment contains Kozak consensus sequence [21] named as "Kozak ATG," and the third fragment contains another in-frame ATG at 48 bp upstream to original ATG [22] and named as "1st ATG." The sequences of these fragments were shown in Fig. 1. These three GALC cDNA with different ATG surrounding sequence were excised from pBluescript II KS using *Cla*I and cloned into *Cla*I site of retrovirus

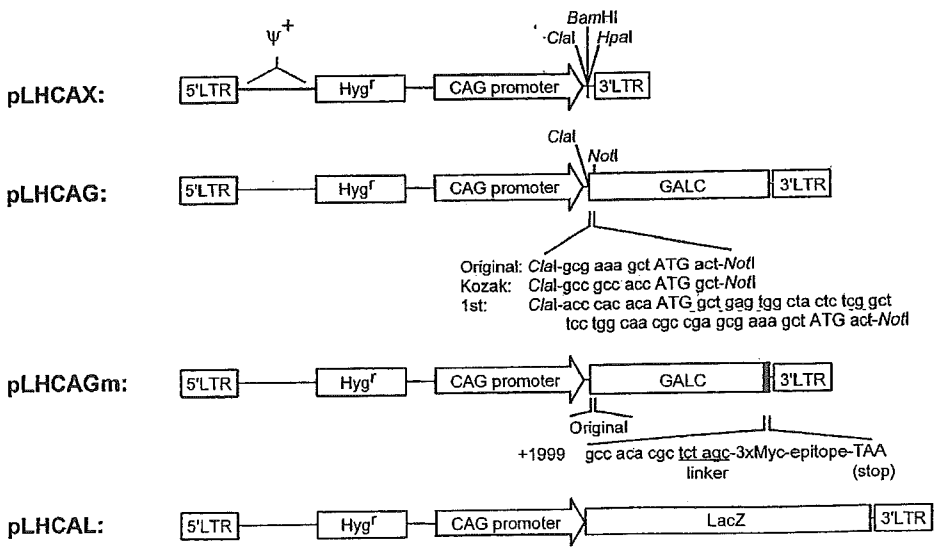

Fig. 1. Schematic representations of the retroviral constructs. See Materials and methods for details.

vector pLHCAX [23] to generate a series of retroviral vectors named pLHCAG (Fig. 1). In which, a hygromycin phosphotransferase gene was driven by the long terminal repeat (LTR) and GALC gene was driven by CAG promoter. To introduce myc-epitope, the stop codon (TAA, in +2008 position) of GALC (with "original ATG") was replaced with a unique *Xba*I site by polymerase chain reaction (PCR)-based mutagenesis. Complementary oligonucleotides encoding three tandem c-myc-epitope (EQKLISEEDL) followed by a stop codon (TAA) was cloned in-frame into *Xba*I site to create pLHCAGm (Fig. 1). In all ligated plasmids, the orientation and sequence of each insert was confirmed by DNA sequencing. A retrovirus vector expressing *Escherichia coli* β-galactosidase (β-gal), pLHCAL [23], generated by the insertion of LacZ gene between the *Bam*HI and *Hpa*I sites of pLHCAX was also used in this study (Fig. 1).

Transient expression study

Bosc23 and NIH/3T3 cells were grown in 12-well plates (NunC, Roskilde, Denmark) at 37 °C with 5% CO_2in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). When approximately 95% confluent, the cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation (1.6 μg DNA:4 μl lipofectamine 2000 per well). Two days after transfection, the cells were washed with phosphate-buffered saline (PBS), harvested and assayed for GALC activity (as described below).

Virus preparation

To produce recombinant retrovirus, ecotropic packaging cell line ψMP34 [24] was transfected by calcium phosphate method (Cellphect transfection kit, Amersham, Piscataway, NJ) with pLHCAGm. After selection with 0.5 mg/ml hygromycin B (Wako, Osaka, Japan), drug-resistant colonies were isolated and a subclone (ψMP34/pLHCAGm#92) producing the highest titer of LHCAGm was used in this study. Retrovirus was concentrated according to the protocol reported by Bowles et al. [25] as described previously [23].

Viral titer assay and in vitro infection

For the assessment of infectious viral titers, NIH/3T3 target cells were grown in 24-well plates (for LHCAGm) or 6 cm dishes (for LHCAL) in DMEM supplemented with 10% FBS. When approximately 60% confluent, cells were infected with serially diluted viral stocks overnight at 37 °C in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO). Forty-eight hours after infection, cells were fixed and stained to detect transgene expressing cells. The cells infected with LHCAGm were fixed with 4% paraformaldehyde in PBS for 10 min on ice, and stained for myc-epitope using rabbit polyclonal antibody to myc-tag (MBL, Nagoya, Japan, 1:1500) as described in histochemical analysis section below. The cells infected with LHCAL were fixed with 0.25% glutaraldehyde in PBS and stained with X-gal solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS) overnight at 37 °C. The number of positively stained colonies was counted under a microscope and the titer was calculated as follow: cfu/ml = number of positive colonies/virus volume (ml). The titers of concentrated LHCAGm and LHCAL were 1.2×10^7 and 7.3×10^7 cfu/ml, respectively.

To evaluate intracellular activity of transduced GALC gene, spontaneously immortalized fibroblasts derived from twitcher mouse designated as Tw2 were infected with LHCAGm in the presence of 8 μg/ml polybrene and selected with 0.5 mg/ml hygromycin B. A drug-resistant subclone (Tw2/LHCAGm#11) with the highest GALC activity was expanded and used for the galactocerebroside (GalCer)-loading study.

Animals and virus injection

Breeding pairs of twitcher heterozygotes (C57BL/6J, twi/+) were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in our laboratory under standard housing condition. To determine transduction pattern of SVZ injection in the mouse, nine normal C57BL/6J mice received LHCAL injection at P0 were analyzed for gene transfer to the neural cells by X-gal staining (at P4) or immunohistochemistry for β-gal (at P30). Twenty twitcher mice were used in this study: 10 mice received LHCAGm injection and seven out of eight surviving recipients were used for histochemical ($n=4$) or biochemical ($n=3$) analysis at P35–40; three received LHCAL injection and used for histochemical analysis at P38–39 and seven untreated twitcher mice were taken as controls for histochemical ($n=4$) or biochemical ($n=3$) analysis at P39–40. In addition, six untreated normal mice were used as controls for histochemical ($n=3$) or biochemical ($n=3$) analysis at P40.

The procedure of stereotactic injection of retrovirus into SVZ was modified from that reported by previous study in neonatal rats [26]. Briefly, the injection was performed on the day of birth after DNA diagnosis of the twitcher mutation by PCR [6]. The newborn mice were anesthetized by placing on ice for a few minutes, placed in a stereotactic apparatus (Narishige, Tokyo, Japan) and kept cold with ice packs. Approximately 1.0 μl HBSS containing retrovirus with 0.01% Fast Green (Sigma) was injected unilaterally through a 30 G needle with a micro syringe (Ito, Shizuoka, Japan). Stereotactic

coordinates (related to the intersection of transverse and longitudinal cerebral fissure) were anterior 1.5 mm and lateral 1.5 mm, at a depth of 2 mm. All pups used in this study were recovered from cryoanesthetization by warming prior to being returned to their mother.

The Fast Green dye was used to verify that the virus was not injected into the lateral ventricle. In pilot studies using LHCAL in normal mice showed that when retrovirus was injected into the cerebral ventricles few cells could be transduced in any location of the brain. In one twitcher mouse received LHCAGm injection in this study, virus entered the lateral ventricle and few myc$^+$ cell was found in the brain and no further analysis was carried out on this animal.

GALC activity assay

GALC activity was assayed as previously described [27] using ^{3}H-labeled GalCer as substrate. The cells were washed with PBS, harvested, and sonicated for 30 s on ice. For tissues, the forebrain was homogenized in distilled water with Potter–Elvehjem homogenizer and sonicated briefly. The homogenate corresponding to about 100 μg of total proteins was used for the assay. Protein concentrations were determined with BCA protein assay reagent (Pierce, Rockford, IL) with bovine albumin as standard.

Galactocerebroside-loading study

Galactocerebroside (GalCer)-loading study was performed essentially as described by Kobayashi et al. [28,29]. Briefly, ^{3}H-labeled GalCer in chloroform:methanol (2:1, v/v) and phosphatidylserine in chloroform:methanol (95:5, Sigma) were dried in a sterilized tube and mixed with DMEM supplemented with 10% FBS by sonication for 30 min. Confluent Tw2, Tw2/LHCAGm#11, and NIH/3T3 cells grown in 3.5 cm dishes were incubated with 1.5 ml the medium containing 0.33 μg [^{3}H]GalCer (12,000 dpm)/ml and 5 μg phosphatidylserine/ml. After 4 days, the medium was removed and cells were harvested by trypsin digestion. Intracellular [^{3}H]GalCer and released [^{3}H]galactose in medium was extracted as described [29], dissolved in ACSII scintillation cocktail (Amersham) and the radioactivities were counted in a scintillation counter (LS6500, Beckman Instruments, Fullerton, CA). Hydrolysis of GalCer was expressed as a percentage of the released galactose, on the basis of the total incorporated GalCer (the sum of galactose in the medium and intracellularly accumulated GalCer).

Western blot

The confluent cells grown in 6 cm dishes were lysed with 300 μl sample buffer. After heating at 95 °C for 5 min, 5 μl of total cell lysates were electrophoresed on a 10% sodium dodecylsulfate (SDS)–polyacrylamide gel and blotted to a PVDF membrane (Novex, San Diego, CA). The membrane was reacted with rabbit polyclonal antibody to myc-tag (MBL, 1:1000) at 4 °C overnight and the signals of reactive proteins were detected by ECL plus reagents (Amersham).

PCR and Southern blot

Total cellular RNA was extracted from the brain homogenate of the recipients and untreated control mice using RNeasy lipid tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions including DNase I treatment. The reverse-transcription reaction was performed using the SuperScript first-strand synthesis system (Invitrogen) with oligo(dT) primer. Genomic DNA was extracted from brain homogenate from treated twitcher mice and untreated control mice by DNeasy tissue kit (Qiagen). The primers specific for human GALC cDNA were designed as 5′-TGGAA CCCATTCAGCAAAAG-3′ (forward primer) and 5′-CTGCTTAAAAAGAAATCTTTCGGAT-3′ (reverse primer, amplicon length of 559 bp). The forward and reverse primer located in exons 8 and 12, respectively, spanning more than 17 kb of introns in genome, and the last 5–6 nucleotides (underlined) in 3′-termini are completely different from the sequences of the mouse GALC cDNA. Genomic DNA (100 ng) or cDNA corresponding to 0.6 μg total RNA were added to AmpliTaq Gold Master Mix (ABI, Tokyo, Japan) containing 0.5 μM of each primer in a final volume of 50 μl. The PCR was carried out as denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s and extension at 72 °C for 1 min. Under these conditions, no amplification occurs in mouse genomic DNA or cDNA.

Ten microliters of PCR products were electrophoresed on a 1.5% agarose gel, photographed after ethidium bromide staining and transferred to a nylon membrane. The membrane was hybridized with a [^{32}P]dCTP labeled human GALC probe (1.1 kb *Hin*dIII–*Sph*I fragment of GALC cDNA) and exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

Histochemical analysis

The mice were anesthetized and fixed with transcardial perfusion of chilled 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at the time of analysis. Brains were removed and postfixed in the same fixative overnight at 4 °C and cryoprotected in 15% sucrose in 0.1 M PB. Consecutive coronal sections of the forebrain were made at a thickness of 20 μm using a cryostat.

For immunohistochemistry, the sections were incubated with 0.3% Triton X-100 in 0.1 M PBS (PBST) for overnight at 4 °C, then treated with 0.5% H_2O_2 in PBST