Gb_3) in lysosomes. Lysosomal α-galactosidase is a glycoprotein, and is synthesized in rough-surfaced endoplasmic reticulum followed by the addition of *N*-linked high-mannose-type oligosaccharides. The enzyme is then transferred to the Golgi apparatus, where further modification, including addition of mannose-6-phosphate (M6P) residues and binding to M6P receptor, occurs. Subsequently, the enzyme is transported to endosomes via M6P receptors. The enzyme then moves to lysosomes, where it exerts its function. In some type of cells, including cultured fibroblasts, α-galactosidase can be incorporated into the cells from the extracellular milieu via M6P receptors on the plasma membrane and transported to lysosomes (Kornfeld and Sly 2001).

A deficiency of α-galactosidase results in widespread cellular deposition of CTH, thereby causing Fabry disease (MIM 301500) (Desnick et al. 2001). Fabry disease is an X-linked genetic disease exhibiting a wide clinical spectrum. Male patients with classic Fabry disease usually have no α-galactosidase activity and, in childhood or adolescence, there is pain in the peripheral extremities, angiokeratoma, hypohidosis and corneal opacity, followed by renal, cardiac and cerebrovascular involvement with increasing age (Desnick et al. 2003). The incidence of classic Fabry disease has been estimated to be 1 in 40,000 male newborns (Desnick et al. 2001). Patients with variant form Fabry disease have residual α-galactosidase activity and milder clinical manifestations with late onset (Sakuraba et al. 1990; Nakao et al. 1995). Females heterozygous for Fabry disease can be affected to a moderate or severe degree due to random X-chromosomal inactivation (Sakuraba et al. 1986; Fukushima et al. 1995; Itoh et al. 1993, 1996; Lyon 1962). However, a recent survey has revealed that many Fabry females can be affected similarly to Fabry males and thus should be considered as patients rather than carriers of the disease (Mehta et al. 2004). Fabry disease has been under-recognized, and the number of Fabry patients requiring treatment is thought to be much larger than previously assumed.

Recently, two different recombinant α-galactosidases were developed for enzyme replacement therapy for Fabry disease: agalsidase alfa (Replagal; Transkaryotic Therapies, Cambridge, MA) generated in human cultured fibroblasts (Schiffmann et al. 2000), and agalsidase beta (Fabrazyme; Genzyme Therapeutics, Cambridge, MA) produced in Chinese hamster ovary (CHO) cells (Eng et al. 2001a, b). The former has been approved in Europe, and the latter in Europe, the United States, and Japan, and many Fabry disease patients have been successfully treated with these drugs. However, these recombinant enzymes are produced in cultured mammalian cells and thus their production is very expensive. Furthermore, careful monitoring for infection by pathogens is essential because fetal calf serum is usually required for the culture of mammalian cells.

We have constructed a yeast cell line producing a recombinant human α-galactosidase with *N*-linked high-mannose-type sugar chains (yeast recombinant human α-galactosidase, yr-haGal), as described previously (Chiba et al. 2002). Effective incorporation of the enzyme into affected organs is very important for enzyme replacement therapy, and in Fabry disease successful targeting of α-galactosidase is strongly dependent on the presence of M6P residues on the sugar chains of the enzyme preparations. In this study, we improved the procedures for purification of α-galactosidase from the culture medium of yeast cells to obtain a large amount of highly purified enzyme protein with M6P residues that facilitate incorporation of the enzyme into affected organs, and analyzed the effect of the purified enzyme on cleavage of CTH accumulated in cultured Fabry fibroblasts and organs of Fabry mice.

Materials and methods

Purification of yr-haGal secreted into the culture medium of yeast cells

Here we used a yeast strain, HPY21G, constructed by introducing the human α-galactosidase cDNA into a *Saccharomyces cerevisiae* strain, HPY21, as described previously (Chiba et al. 2002). A large-scale culture (100 l) was performed to examine the effect of yr-haGal on Fabry mice. We had previously used Blue-Sepharose and ConA-Sepharose columns to purify yr-haGal (Chiba et al. 2002). However, these columns are very expensive and had only weak binding ability because of the characteristics of the affinity chromatography so we improved the purification procedure. All column materials used in the experiments reported here were purchased from Amersham Biosciences Japan (Tokyo, Japan). The culture medium of the HPY21G strain was collected and concentrated, and ammonium sulfate was added slowly to the supernatant to a final concentration of 55%. The precipitate was recovered by centrifugation, re-dissolved in 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, and then dialyzed against the same buffer. A sample was then applied to a HiLoad Q 16/10 Sepharose HP column equilibrated with the same buffer. After washing the column, α-galactosidase was eluted with a 0–1 M NaCl gradient in the same buffer. Fractions containing enzyme activity were pooled, and then a one-tenth volume of 3 M ammonium sulfate was added. A sample was then applied to a HiLoad 26/10 Phenyl HP column equilibrated with 25 mM MES buffer, pH 6.0, containing 0.3 M ammonium sulfate. After washing the column, α-galactosidase was eluted with a 0.3–0 M ammonium sulfate gradient in the same buffer. Fractions containing enzyme activity were dialyzed against 20 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl, and then were concentrated with an Amicon Ultra-4 (13,000 MWCO; Millipore, Bedford, MA). A sample was then applied to a HiLoad 16/60 Superdex 200pg column. Fractions containing enzyme activity then were pooled and subjected to α-mannosidase treatment to expose M6P residues at

the non-reducing ends of the sugar chains. Treatment of the recombinant α-galactosidase with the culture supernatant of SO-5, a new bacterium producing an α-mannosidase, was performed as described previously (Chiba et al. 2002). After the α-mannosidase treatment, the α-galactosidase protein was re-purified on HiLoad Q and HiLoad 16/60 Superdex 200pg columns under the conditions described above.

Biochemical analyses of the enzymatic properties of yr-haGal

The purity and molecular mass of yr-haGal produced in yeast cells were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Chiba et al. 2002). Reversed-phase high-performance liquid chromatography (HPLC) analysis of the purified yr-haGal was performed on a Cosmosyl $5C_4$-AR-300 (4.6×150 mm) column (Nacalai Tesque, Kyoto, Japan). The protein was eluted with a linear trifluoroacetic acid/acetonitrile gradient at a flow rate of 1 ml/min with ultraviolet detection at 215 nm. Deglycosylation of yr-haGal with *N*-glycanase F (Takara Bio, Shiga, Japan) was performed according to the method recommended by the manufacturer, and *N*-terminal amino acid sequence analysis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis were performed by Shimazu Corporation (Kyoto, Japan).

α-Galactosidase activity was measured fluorometrically with 4-methylumbelliferyl-α-galactopyranoside (Calbiochem, San Diego, CA) as a substrate in the presence of *N*-acetylgalactosamine (Sigma, St. Louis, MO), a specific inhibitor of α-*N*-acetylgalactosaminidase (Mayes et al. 1981). The protein concentration was determined with a *DC* assay kit (Bio-Rad, Richmond, CA), using bovine serum albumin (BSA) as a standard.

Sugar chain analysis of yr-haGal was performed according to the method reported previously (Takashiba et al. 2004). Briefly, the enzyme was hydrolyzed with 2 M trifluoroacetic acid and L-rhamnose, as an internal standard, at 100°C for 2 h, and monosaccharides derived from the sugar chains were then quantitated by means of capillary electrophoresis using a P/ACE MDQ equipped with a laser-induced fluorescence detector (Beckman Coulter, Fullerton, CA); authentic monosaccharides were used as standards for quantitation.

Examination of the effect of yr-haGal on cultured human Fabry fibroblasts

Cultured fibroblasts from a patient with Fabry disease and a normal control subject were established and maintained in our laboratory. The cells were cultured in Ham's F-10 medium containing 10% fetal calf serum and antibiotics at 37°C in an incubator containing 5% CO_2. The study involving the cultured human fibroblasts was approved by the Ethical Committee of our institute.

To examine uptake of yr-haGal by Fabry fibroblasts, yr-haGal produced in yeast cells was added to the culture medium of Fabry fibroblasts to give concentrations of 0, 0.25, 0.5, 1.0, 3.0 and 6.0 μg/ml. For examination of the inhibitory effect of M6P on the cellular uptake of yr-haGal, Fabry fibroblasts were cultured in medium containing 5 mM M6P and 1.0μg/ml yr-haGal. After 18 h culture, the cells were harvested mechanically, washed three times with phosphate-buffered saline (PBS), pH 7.4, and then collected as a pellet by centrifugation. An appropriate amount of water was then added to the pellet and the cells were sonicated; the resulting homogenate was used for α-galactosidase assay and protein determination.

To examine degradation of accumulated CTH by the incorporated recombinant α-galactosidase, Fabry fibroblasts were cultured with culture medium containing the recombinant α-galactosidase at concentrations of 0, 0.5, 1.0, 2.0 and 3.0μg/ml for 3 days. Alternatively, Fabry fibroblasts were cultured in medium containing 3.0μg/ml recombinant α-galactosidase for 0, 1, 3, 5, and 7 days. Cells grown on a Lab-Tek chamber slide (Nunc, Naperville, IL) were fixed with 2% paraformaldehyde in PBS, pH 7.4, for 10 min, followed by blocking with 5% BSA in PBS for 1 h. The cells were then incubated with a mouse monoclonal anti-CTH antibody (culture supernatant; IgG isotype) (Kotani et al. 1994) and rabbit polyclonal anti-α-galactosidase antibodies (1:100 diluted; IgG isotype) (Ishii et al. 1994) for 1 h. After washing, they were reacted for 1 h with a fluorescent isothiocyanate-conjugated goat anti-mouse IgG $F(ab')_2$ (diluted 1:200; Jackson Immuno Research, West Grove, PA) and a rhodamine-conjugated goat anti-rabbit IgG $F(ab')_2$ (diluted 1:400; Jackson Immuno Research). To determine the localization of the accumulated CTH, double staining with the anti-CTH antibody and a mouse monoclonal antibody to lysosome-associated membrane protein-1 (LAMP-1; Southern Biotechnology, Birmingham, AL), a marker for lysosome, was also performed according to a modified method described elsewhere (Kotani et al. 2004). The stained cells were observed under a microscope (Axiovert 100M; Zeiss, Oberkochen, Germany) equipped with a confocal laser scanning imaging system (LSM510; Zeiss).

Examination of the effect of yr-haGal on Fabry mice

Fabry mice (α-galactosidase knock-out mice, donated by Ashok B. Kulkarini and Toshio Oshima) and wild type C57BL/6 mice were used in this experiment according to the rules drawn up by the Animal Care Committee of our institute.

To examine the pharmacokinetics and biodistribution of the recombinant α-galactosidase, a single dose, 3.0 mg/kg body weight, of recombinant α-galactosidase was injected into the tail veins of Fabry mice. As a control, a single dose, 2.0 mg/kg body weight of agalsidase beta (purchased from Genzyme Japan, Tokyo, Japan)

was injected into litter-matched Fabry mice so that the injected enzyme activity was almost the same (6.0–6.4 mmol h^{-1} kg^{-1} body weight). Each group consisted of two mice. Blood samples were collected at 0, 1, 3, 5, 10, 20, 30, and 40 min after injection of the enzymes, and a time course of changes in α-galactosidase activity in plasma was determined. The mice were sacrificed at 1 h after administration of the enzymes, and their livers, kidneys, hearts, and spleens were then removed. Tissue samples were then homogenized in citrate-phosphate buffer, pH 4.6, and centrifuged. The resulting supernatants were assayed for α-galactosidase activity.

To examine cleavage of the CTH accumulated in organs, two groups of litter-matched Fabry mice, each consisting of three mice, were repeatedly injected with the recombinant α-galactosidase, 3.0 mg/kg body weight, and agalsidase beta, 2.0 mg/kg body weight, separately every week for four doses, and then sacrificed 6 days after the last injection. Their livers, kidneys, hearts, spleens, and dorsal root ganglia were then removed, and used as samples for biochemical and/or morphological analyses.

For immunohistochemical analysis, the mouse tissues were stored at −80°C before use, and then frozen sections of 10μm thickness were fixed with 4% paraformaldehyde in PBS for 5 min at room temperature. The specimens were incubated with PBS containing 5% (w/v) BSA for 30 min at room temperature to block nonspecific binding. Subsequently, the samples were treated with a mouse monoclonal anti-CTH antibody (culture supernatant; IgG isotype) for 1 h at room temperature, and then treated with fluorescent isothiocyanate conjugated goat anti-mouse IgG $F(ab')_2$ (diluted 1:200; Jackson Immuno Research). The stained tissues were examined under a confocal laser scanning microscope as described above.

For determination of CTH levels, tissues, including liver, kidney, heart, and spleen, were analyzed by means of thin-layer chromatography, followed by densitometry according to the method described previously (Takahashi et al. 2002).

For morphological examination, kidney tissues were cut into small pieces, and then fixed in cold 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The specimens were rinsed in PBS, and then postfixed with 2% osmium tetraoxide in 0.2 M sucrose in PBS for 1 h and dehydrated with graded concentrations of ethanol, 50% through absolute, and glycidyl *n*-butyl ether. Dehydrated specimens were then embedded in Epon 812 resin. Sections of 0.1μm thickness were prepared and stained with 2% uranyl acetate in 50% ethanol for 5 min, restained with Reynolds lead citrate for 3 min, and finally examined under an electron microscope (Hitachi H-7100; Hitachi, Tokyo, Japan).

Examination of the anti-α-galactosidase immune reaction

To determine whether or not Fabry mice injected with the enzymes produced antibodies against the enzymes, solid-phase enzyme-linked immunosorbent assay (ELISA) was performed. Serum samples were obtained from Fabry mice repeatedly injected with yr-haGal (3.0 mg/kg body weight), and agalsidase beta (2.0 mg/kg body weight) separately every week for four doses. Briefly, a 96-well flat bottom microplate for ELISA (Immulon 2 HB; Thermo Lab Systems, Flanklin, MA) was coated with 1.0 μg/ml of the enzymes in PBS. After washing 5 times with 1% BSA in PBS, 200 μl 1% BSA in PBS was added to each well as a blocking solution, followed by incubation for 1 h at room temperature. After removing the blocking solution, 100 μl of the mouse sera or rabbit anti-α-galactosidase antibodies (Ishii et al. 1994) diluted to various concentrations was added to each well, followed by incubation for 1 h. The wells were then washed, incubated in 100 μl peroxidase-conjugated anti-mouse IgG $F(ab')_2$ (diluted 1: 2,000; Jackson Immuno Research) for 45 min, washed again, and finally incubated in 100 μl *O*-phenylenediamine (Sigma) generated as 0.4 mg/ml 0.05 M citrate-phosphate buffer, pH 5.0. After incubation with the chromogenic substrate for 10 min, the optical density of each well was measured by means of an ELISA reader (Bio-Rad, Hercules, CA).

Results

Properties of yr-haGal

The new purification method described in this paper allowed us to treat a large volume of culture medium and obtain highly purified yr-haGal with 870-fold purifica-

Table 1 Monosaccharide analysis. *M6P* Mannose-6-phosphate, *yr-haGal* yeast recombinant human α-galactosidase

	yr-haGal[a] (mol/mol protein)	Agalsidase beta[b] (mol/mol protein)	Agalsidase alfa[b] (mol/mol protein)
M6P	3.8 ± 0.2	3.1 ± 0.1	1.8 ± 0.0
Galactose	ND[c]	8.0 ± 0.4	12.2 ± 1.0
Fucose	ND	1.8 ± 0.1	3.0 ± 0.3
N-Acetylglucosamine	0.8 ± 0.6	18.4 ± 0.4	22.5 ± 2.3
Mannose	53.8 ± 2.6	25.7 ± 1.8	27.6 ± 0.5
Sialic acid	ND	7.0 ± 1.0	6.9 ± 0.6

[a]Values expressed as means ± SD, $n = 3$
[b]Lee et al. 2003
[c]Not detected

tion. The amount of recombinant α-galactosidase secreted from the HPY21G strain, a *S. cerevisiae* mutant harboring a human α-galactosidase cDNA, into the culture medium was approximately 290 μg per 1 l culture. The recovery of the enzyme through the purification procedure was 30%. Before treatment with α-mannosidase the purified enzyme was detected as a single band on SDS-PAGE, and its apparent molecular mass was determined to be 51 kDa on MALDI/TOF-MS. The molecular mass changed to 46 kDa following α-mannosidase digestion. The HPLC profile on a reversed-phase column contained a single peak. However, the *N*-terminal amino acid sequence could not be determined, indicating that some modification occurred at the *N*-terminus of yr-haGal. The specific enzyme activity of yr-haGal was 2.0 mmol h^{-1} mg $protein^{-1}$, which was a little higher than that purified by the previously described method (1.7 mmol h^{-1} mg $protein^{-1}$).

Monosaccharide composition of yr-haGal

The monosaccharide composition of the recombinant α-galactosidase was determined, and then compared with those of agalsidase alfa and agalsidase beta, which have been reported elsewhere (Lee et al. 2003). The results are shown in Table 1. The recombinant α-galactosidase produced in yeast has high-mannose-type sugar chains and contains no fucose or galactose residues. The content of M6P residues is 3.8 mol/mol protein, this value being a little higher than those of agalsidase alfa (1.8 mol/mol protein) and agalsidase beta (3.1 mol/mol protein).

Effect of yr-haGal on cultured human Fabry fibroblasts

Fabry fibroblasts were cultured in culture medium containing the indicated concentrations of the recombinant α-galactosidase for 18 h. Uptake of the enzyme by the Fabry fibroblasts was then investigated. The results are shown in Fig. 1. The α-galactosidase activity in untreated Fabry fibroblasts was almost nil, but it increased in response to addition of recombinant α-galactosidase dose-dependently, reaching a normal level when the recombinant α-galactosidase was added to the culture medium at a concentration of 0.25–1.0 μg/ml. Uptake of yr-haGal was decreased to 15% of the control level by the addition of 5 mM M6P, suggesting that incorporation of the enzyme depends largely on the M6P receptor.

We also investigated incorporation of the recombinant α-galactosidase protein into cultured Fabry fibroblasts, and its effect on cleavage of accumulated CTH by means of double staining for α-galactosidase and CTH. At first, double staining for CTH and LAMP-1, a lysosomal marker, was performed, which revealed that the accumulated CTH was localized in lysosomes of untreated Fabry cells (data not shown). The results of time-course analysis are shown in Fig. 2a. The recombinant α-galactosidase was added to the culture medium at a concentration of 3.0 μg/ml. After a 1-day incubation, immunofluorescence of α-galactosidase was detected and that of CTH was apparently decreased. After 3 days of culture, the maximum immunofluorescence for α-galactosidase was observed and the accumulated CTH was completely degraded. Thereafter, the immunofluorescence of α-galactosidase gradually decreased, but the disappearance of deposited CTH was maintained for at least 7 days. Next, we added recombinant α-galactosidase to the culture medium of Fabry fibroblasts at various concentrations from 0.5 to 3.0 μg/ml, and then cultured the cells for 3 days. Double staining for α-galactosidase and CTH revealed that clearance of the accumulated CTH in response to incorporation of the enzyme occurred dose-dependently (Fig. 2b).

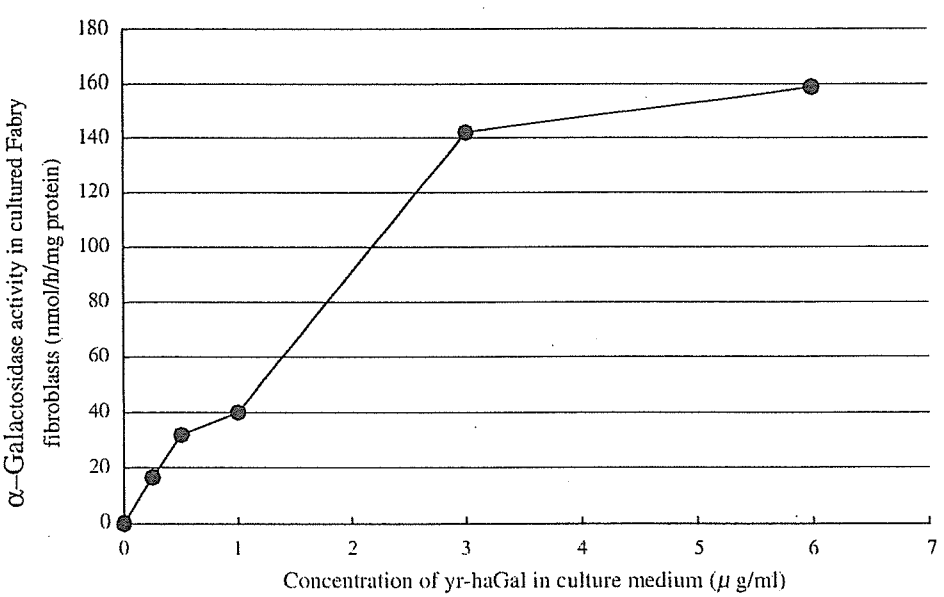

Fig. 1 Uptake by Fabry fibroblasts of the recombinant α-galactosidase produced in yeast cells (yr-haGal). Fabry fibroblasts were cultured in culture medium containing yr-haGal at concentrations of 0, 0.25, 0.5, 1.0, 3.0 and 6.0 μg/ml. After 18 h incubation, α-galactosidase activity in the cells was measured. The original α-galactosidase activity in Fabry fibroblasts was < 1 nmol h^{-1} mg $protein^{-1}$, and the normal range was 15–35 nmol h^{-1} mg $protein^{-1}$

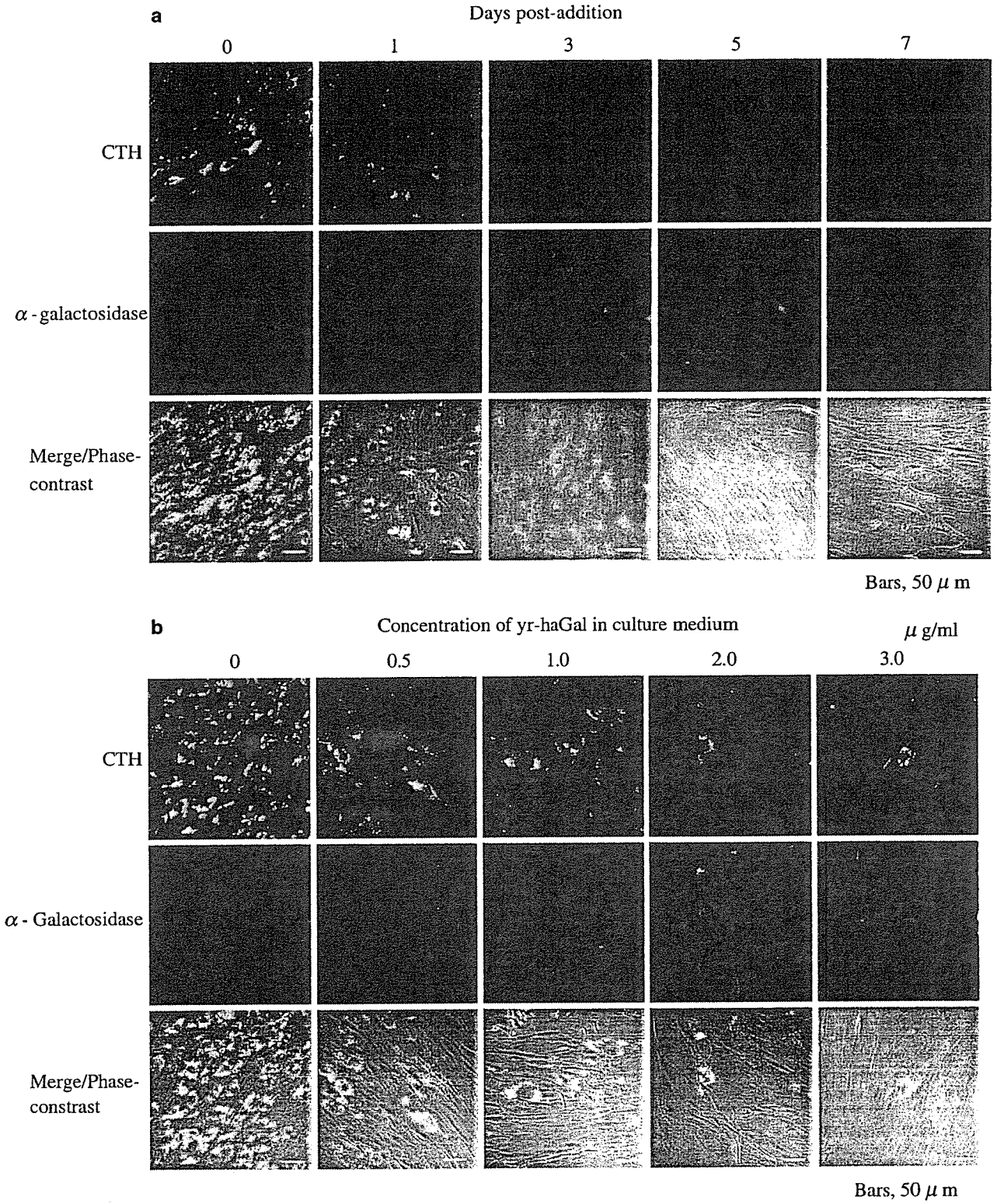

Fig. 2a,b Immunostaining for CTH and α-galactosidase in Fabry fibroblasts after addition of the recombinant α-galactosidase produced in yeast cells (*yr-haGal*). CTH, stained with an anti-CTH antibody (*green*); α-galactosidase, stained with anti-α-galactosidase antibodies (*red*); *Merge/Phase-contrast* overlapping images with these two fluorescent probes and phase-contrast images. Time-course (**a**) and dose-dependency (**b**). *Bars* 50μm

Effect of yr-haGal on Fabry mice

We injected recombinant α-galactosidase into Fabry mice to examine its therapeutic effect. We used agalsidase beta as a control enzyme because it has been reported that agalsidase beta is incorporated into affected organs in Fabry mice more than agalsidase alfa (Lee et al. 2003). As the specific enzyme activity in the yr-haGal we used (2.0 mmol h^{-1} mg $protein^{-1}$) was a little lower than that of the agalsidase beta sample (3.2 mmol h^{-1} mg $protein^{-1}$), we injected almost the same activity of the enzyme preparations into litter-matched Fabry mice for comparison.

After a single dose, 3.0 mg/kg body weight, of yr-haGal had been injected, its pharmacokinetics and biodistribution were examined and compared with those of agalsidase beta. The enzyme activity in plasma quickly increased, reaching a maximum level at 3 min after injection and then gradually decreasing. The pattern of the pharmacokinetics was essentially the same as that of agalsidase beta (Fig. 3). The biodistribution of yr-haGal and agalsidase beta after administration of a single dose is shown in Table 2. An apparent increase in α-galactosidase activity was observed in the organs of Fabry mice following yr-haGal administration. The degree of the enzyme activity increase in the kidneys and heart was almost the same as in the case of agalsidase beta, although that in the liver and spleen was a little lower than with agalsidase beta. The effect of yr-haGal on the degradation of tissue CTH was examined after repeated administration of the enzyme at 3.0 mg/kg body weight every week for four doses, followed by killing of the mice 6 days after the last injection. Immunohistochemical analysis revealed that the CTH deposited in the liver was cleaved (Fig. 4a). In kidney tissues, CTH immunofluorescence in the renal tubular cells was apparently decreased. Cleavage of the accumulated CTH in the glomeruli was insufficient although immunofluorescence was slightly decreased (Fig. 4b). In the heart, immunofluorescence of CTH accumulated in the tissue was apparently decreased after repeated injection of yr-haGal (Fig. 4c), as was also the case in the spleen (data not shown). In the dorsal root ganglia, no apparent degradation of accumulated CTH was observed (Fig. 4d). These findings were essentially the same as those found following administration of agalsidase beta. The results of quantitative analysis of CTH are shown in Table 3. Repeated administration of yr-haGal decreased the accumulated CTH in the liver to the level found in wild type mice, and decreased it to 70 and 30% of the levels in untreated Fabry mice in the kidneys and heart, respectively. The degree of degradation of CTH deposited in these tissues was almost the same as in the case of agalsidase beta. The decrease in accumulated CTH in the spleen upon administration of yr-haGal (to 30% of the untreated Fabry mice level) was smaller than that seen upon administration of agalsidase beta (to 10% of the untreated Fabry mice level). Morphological analysis revealed that many lamellar inclusion bodies, exhibiting accumulation of CTH, were present in the renal tubular cells of untreated Fabry mice (Fig. 5a), and that their number was markedly decreased after repeated administration of yr-haGal (Fig. 5b).

Immunoreactivity

The antigenicity of yr-haGal and agalsidase beta was examined by analyzing the cross-reactivity of antisera

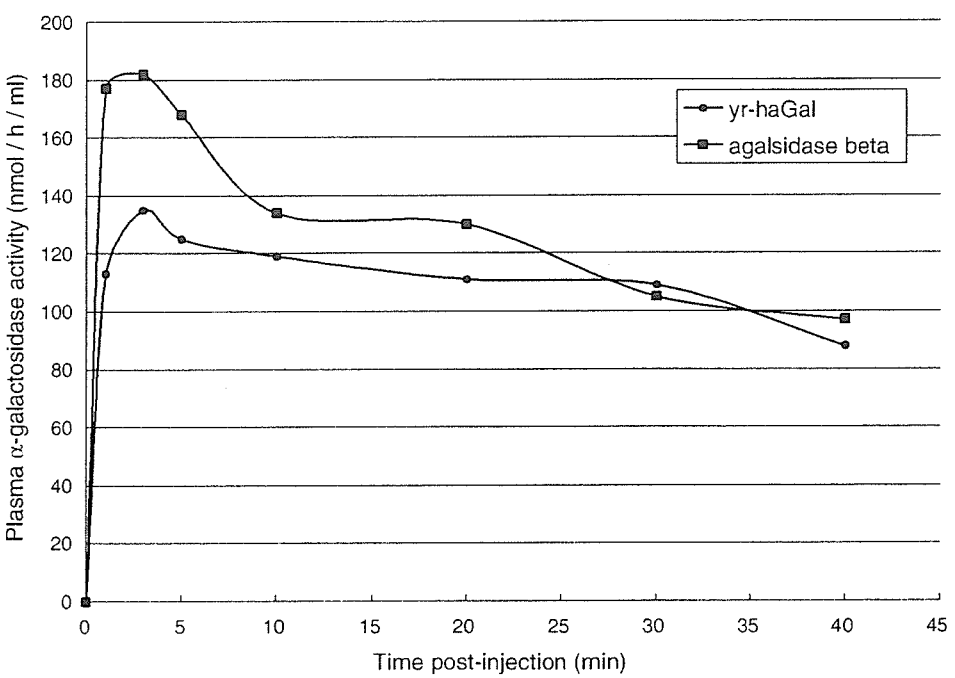

Fig. 3 Pharmacokinetics of yr-haGal and agalsidase beta. A single dose of yr-haGal or agalsidase beta was injected into Fabry mice; blood samples were collected and time courses of changes in plasma α-galactosidase activity were determined. Each value represents the mean from two mice

Table 2 α-Galactosidase activities (nmol h^{-1} mg $protein^{-1}$) in organs from Fabry mice treated with a single dose of yr-haGal or agalsidase beta. Fabry mice were injected with a single dose (almost the same enzyme activity) of yr-haGal (3 mg/kg body weight) or agalsidase beta (2 mg/kg body weight), and then were sacrificed 1 h after the administration. *Wild type* Wild type mice, *Fabry* untreated Fabry mice, *yr-haGal* Fabry mice treated with yr-haGal, *agalsidase beta* Fabry mice treated with agalsidase beta. Values are expressed as the means for two mice

	Liver	Kidney	Heart	Spleen
Wild type	30	11	7	29
Fabry	1	1	1	1
yr-haGal	57	52	90	42
agalsidase beta	174	87	76	100

derived from Fabry mice repeatedly injected with these preparations. The results are shown in Fig. 6. No significant antibodies unique to either enzyme preparation were found under the experimental conditions used.

Discussion

Production of recombinant human α-galactosidases in yeast cells has considerable advantages over production in mammalian cells, i.e., culture is easy, economical, and does not require fetal calf serum. Here, we used as a host a *S. cerevisiae* mutant in which two genes involved in *N*-linked mannan biosynthesis, *OCH1* and *MNN1*, are disrupted. The glycoprotein expressed in this cell line has mammalian-like *N*-linked high-mannose-type sugar chains and has no β-linked mannoside residues that are antigenic in humans (Chiba et al. 1998, 2002). As previously reported, treatment of the recombinant α-galactosidase expressed in this cell line with a bacterial α-mannosidase results in the exposure of M6P residues at the non-reducing ends of sugar chains (Chiba et al. 2002). As M6P residues are essential for incorporation of α-galactosidase into human cells via M6P receptors on the cell membrane (Kornfeld and Sly 2001), an α-galactosidase having many M6P residues would be beneficial for enzyme replacement therapy for Fabry disease. The yeast cell line also has a mutation in the *MNN4* gene, which regulates mannosylphosphorylation (Odani et al. 1996), resulting in the production of recombinant α-galactosidase with highly phosphorylated sugar chains. Productivity of the recombinant enzyme could be further improved in the future by careful choice of a host cell strain. We have preliminarily prepared a methylotrophic yeast cell line secreting recombinant α-galactosidase into the culture medium at a concentration of 12 mg/l, and attempts to obtain abundant enzyme protein using the improved purification method described in this report are underway.

The recombinant α-galactosidase added to the culture medium of Fabry fibroblasts was well incorporated into the cells. The accumulated CTH was cleaved, and the disappearance of deposited CTH held for at least 7 days. Incorporation of the enzyme was strongly inhibited in the presence of M6P. This finding allowed us to examine the effect of the recombinant α-galactosidase on Fabry mice.

Lee et al. (2003) reported that agalsidase beta has a higher percentage of phosphorylated oligomannose chains than agalsidase alfa, which results in improved binding of agalsidase beta to M6P receptors, and higher enzyme levels in the kidneys and heart, which are the organs most affected in Fabry disease, when tested at the same dose. Considering these results, we injected agalsidase beta into Fabry mice as a control in the experiment on the incorporation of yr-haGal into organs of Fabry mice, and its CTH-degrading activity. yr-haGal was successfully incorporated into the liver, kidneys, heart and spleen, and the CTH deposited in these organs was cleaved as in the case of agalsidase beta. However, degradation by these recombinant enzymes of CTH accumulated in the glomeruli was insufficient, although that in renal tubular cells was almost complete. Clinical trials using agalsidase beta have revealed that clearance of the CTH accumulated in podocytes was more limited than that observed in other cell types in kidney tissues (Thurnberg et al. 2002), suggesting that uptake of the recombinant α-galactosidases by podocytes is very low. There was a difference between yr-haGal and agalsidase beta in the degree of enzyme activity increase in the liver and spleen. This is probably due to differences in their sugar chain compositions. Asialylated complex-type oligosaccharides are involved in the uptake of lysosomal enzymes by hepatocytes in the liver via asialoglycoprotein receptors (Rosenfeld et al. 1986). Unlike agalsidase beta, the recombinant α-galactosidase produced in yeast cells does not have any complex-type sugar chains. Why agalsidase beta was incorporated into the spleen more than yr-haGal remains obscure. However, as Fabry disease does not affect the liver or spleen, the relatively low uptake of yr-haGal is thought not to be disadvantageous for enzyme replacement therapy for Fabry disease. Immunohistochemical analysis revealed that administration of either enzyme did not reduce granular immunofluorescence in the dorsal root ganglia from Fabry mice. Recently, we found that recombinant human β-hexosaminidases A and B, which are the lysosomal enzymes responsible for Sandhoff disease, produced in CHO cells, could be incorporated into cultured Schwann cells derived from dorsal root ganglia and adjacent peripheral nerves from Sandhoff mice via M6P receptors, but incorporation was apparently lower than that in the case of cultured Sandhoff fibroblasts

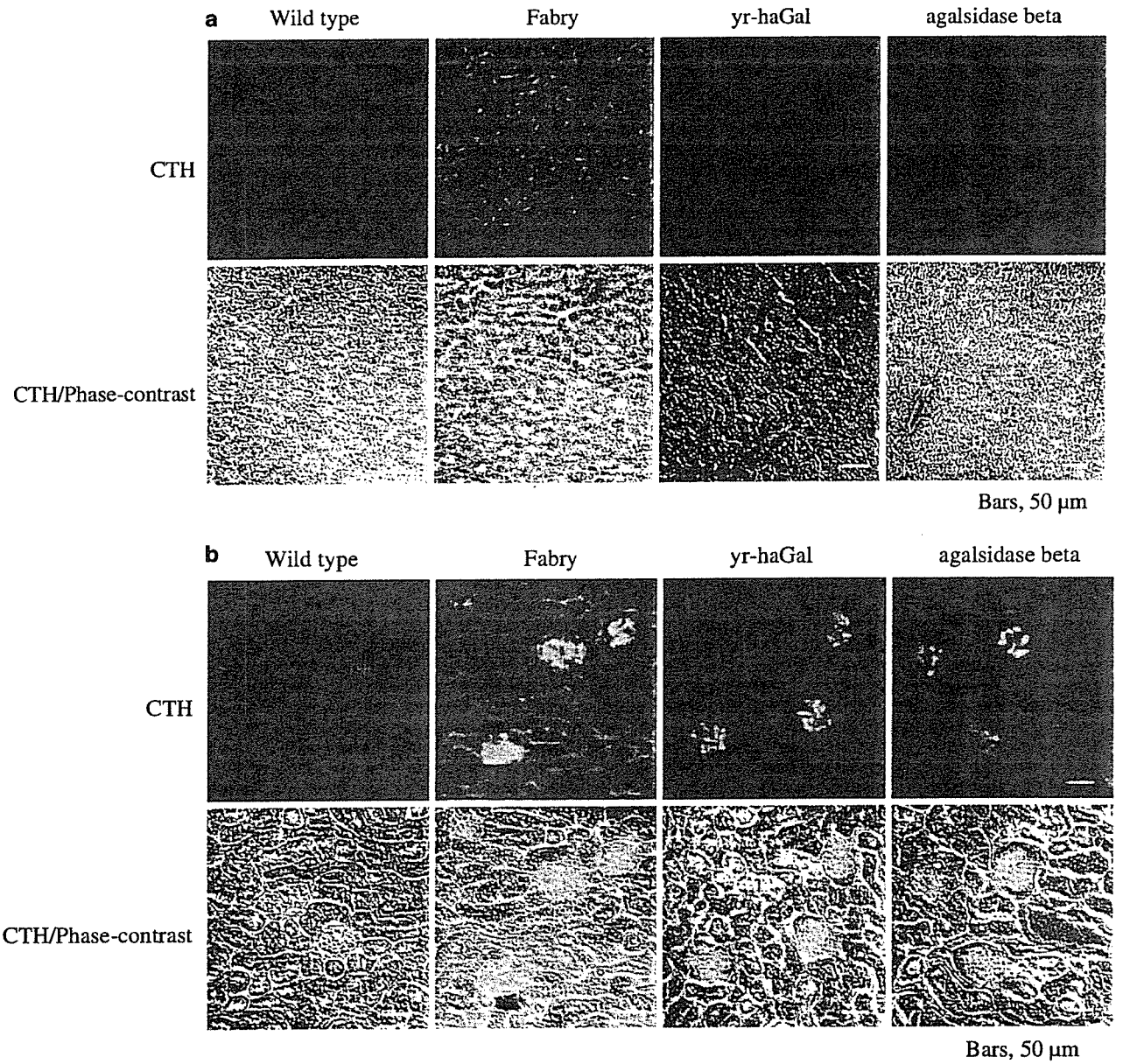

Fig. 4 Immunohistochemical analyses of the accumulated CTH in organs of Fabry mice, and its degradation by yr-haGal and agalsidase beta. Fabry mice were repeatedly injected with yr-haGal and agalsidase beta separately, and then immunostaining for CTH was performed. *CTH* Stained with an anti-CTH antibody (*green*), *CTH/Phase-contrast* overlapping CTH and phase-contrast images, *Phase-contrast* phase-contrast images. *Wild type* A wild type mouse, *Fabry* an untreated Fabry mouse, *yr-haGal* a Fabry mouse treated with yr-haGal, *agalsidase beta* a Fabry mouse treated with agalsidase beta. **a** Liver, **b** kidneys, **c** heart, **d** dorsal root ganglia. *Bars* 50 μm

(Ohsawa et al. 2005). The total number of M6P receptors on the surface of neural cells might be less than that on non-neural cells. Intravascularly administered lysosomal enzymes are barely incorporated into neural cells. Phase 3 clinical trials for agalsidase beta revealed that there were no significant differences in improvement of pain in the peripheral extremities between a group of patients treated with agalsidase beta and the placebo group (Eng et al. 2001). Some improvement is required for targeting of the enzyme to neural cells, i.e., the production of a recombinant α-galactosidase with abundant M6P residues.

It is known that the administration of agalsidase alfa and agalsidase beta frequently causes infusion reactions, mainly allergic reactions (Schiffmann et al. 2000; Eng et al. 2001). Thus, we examined the levels of antibodies against yr-haGal in sera of recurrently injected Fabry mice by means of ELISA, but no specific antibodies for yr-haGal were detected, as was also the case of agalsidase beta under the experimental conditions used.

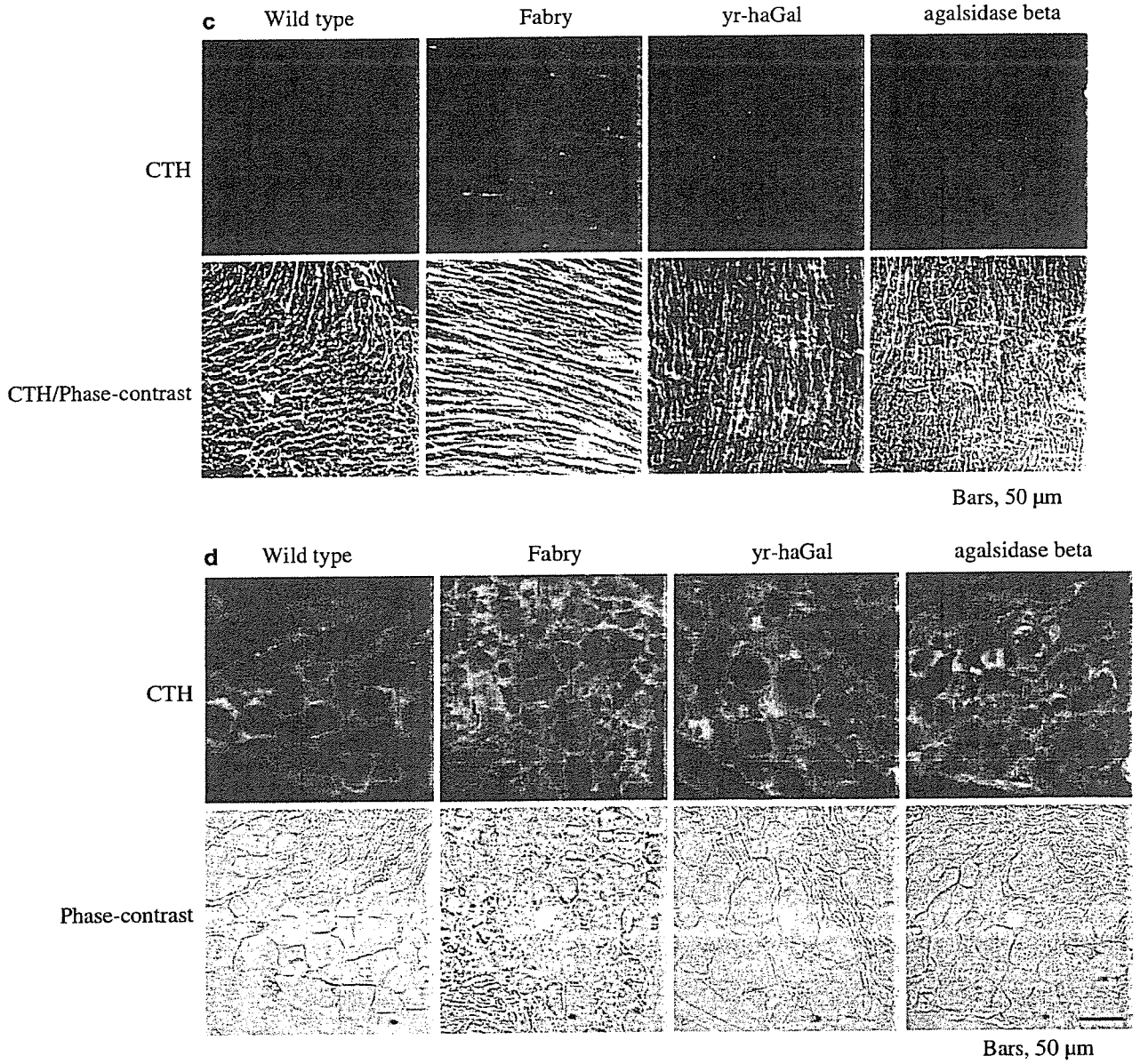

Fig. 4 (Contd.)

Table 3 Ceramide trihexoside (CTH) levels (nmol/mg protein) in organs from Fabry mice treated with four doses of yr-haGal or agalsidase beta. Fabry mice were injected with almost the same enzyme activity of yr-haGal (3 mg/kg body weight) or agalsidase beta (2 mg/kg body weight) separately every week for four doses, and were sacrificed 6 days after the last injection. *Wild type* Wild type mice, *Fabry* untreated Fabry mice, *yr-haGal* Fabry mice treated with yr-haGal, *agalsidase beta* Fabry mice treated with agalsidase beta. Values are expressed as means ± SEM ($n = 3$)

	Liver	Kidney	Heart	Spleen
Wild type	0.07 ± 0.01	0.78 ± 0.10	0.00 ± 0.00	0.34 ± 0.11
Fabry	1.40 ± 0.41	5.36 ± 0.08	2.01 ± 0.21	3.05 ± 0.33
yr-haGal	0.03 ± 0.02	3.89 ± 0.36	0.58 ± 0.16	0.96 ± 0.41
agalsidase beta	0.09 ± 0.06	3.39 ± 0.14	0.67 ± 0.34	0.37 ± 0.06

Fig. 5a,b Morphological effects of repeated administration of yr-haGal on renal tubular cells of Fabry mice. yr-haGal was repeatedly injected into Fabry mice; kidney tissues were then examined by electron microscopy. **a** An untreated Fabry mouse. **b** A Fabry mouse treated with yr-haGal. Many lamellar inclusion bodies can be seen in the renal tubular cells of the untreated Fabry mice, and the number of lamellar inclusion bodies is apparently decreased after repeated administration of yr-haGal. *Bar* 2 μm

In conclusion, we produced in yeast cells a recombinant α-galactosidase with M6P residues at the non-reducing ends of *N*-linked sugar chains. This recombinant enzyme was incorporated into the liver, kidneys, heart and spleen, and degraded the accumulated CTH in these tissues, although cleavage of the CTH accumulated in the dorsal root ganglia was insufficient. As production of recombinant α-galactosidase in yeast is easy and economical, and does not require fetal calf serum, yr-haGal is highly promising as an enzyme source for enzyme replacement therapy for Fabry disease.

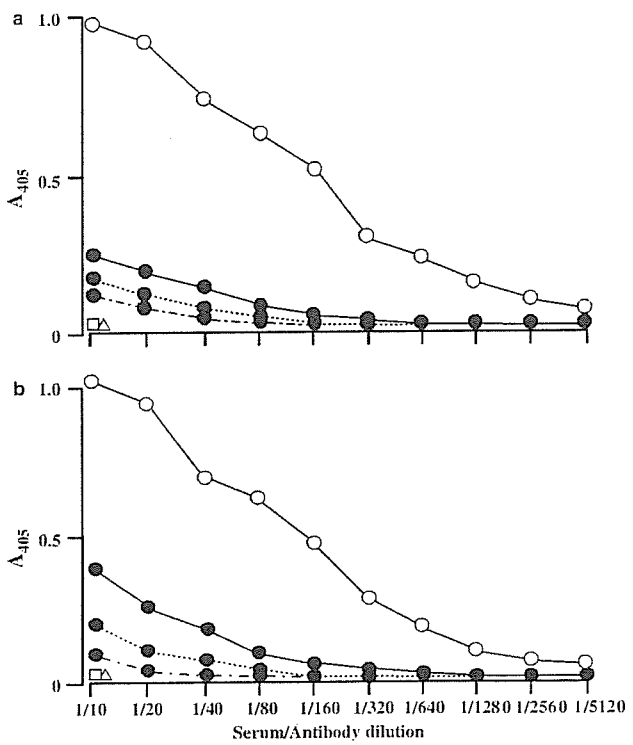

Fig. 6 Antigenicity of yr-haGal and agalsidase beta. ELISA was performed to determine whether Fabry mice recurrently injected with yr-haGal (**a**) or agalsidase beta (**b**) produced antibodies to the enzymes or not. *Open circles* Rabbit anti-α-galactosidase antibodies, *closed circles* Fabry mouse sera treated with the enzymes (yr-haGal and agalsidase beta), *open squares* serum from an untreated Fabry mouse, *open triangles* serum from an untreated wild type mouse

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Research Report

Ex vivo cell-mediated gene therapy for metachromatic leukodystrophy using neurospheres

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ABSTRACT

Metachromatic leukodystrophy (MLD) is an autosomal recessive disease caused by mutations in the gene encoding the lysosomal enzyme arylsulfatase A (ASA). In MLD, accumulation of the substrate, sulfated glycoprotein, in the central and peripheral nervous systems results in progressive motor and mental deterioration. Neural progenitor cells are thought to be useful for cell replacement therapy and for cell-mediated gene therapy in neurodegenerative diseases. In the present study, we examined the feasibility of ex vivo gene therapy for MLD using neural progenitor cells. Neural progenitor cells (neurospheres) were prepared from the striatum of E14 embryo MLD knockout mice or GFP transgenic mice and were transduced with the VSV pseudotyped HIV vector carrying the ASA gene (HIV-ASA). For in vivo study, neurospheres from GFP mice were transduced with HIV-ASA and inoculated into the brain parenchyma of adult MLD mice. HIV vector-transduced progenitor cells retained the potential for differentiation into neurons, astrocytes and oligodendrocytes in vitro. Expression of ASA in neurospheres transduced with HIV-ASA was confirmed by spectrophotometric enzyme assay and Western blotting. In vivo, GFP-positive cells were detectable 1 month after injection. These cells included GFAP- and MAP2-positive cells. Immunohistochemistry using anti-ASA antibody demonstrated localization of ASA in both GFP-positive and -negative cells. Partial clearance of accumulated sulfatide was confirmed in vivo in MLD knockout mice. The present findings suggest that ASA enzyme is released from migrated neurospheres and is able to digest sulfatide in surrounding cells. Our results suggest the potential of genetically engineered neural progenitor cells (neurospheres) for ex vivo therapy in MLD.

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1. Introduction

Metachromatic leukodystrophy (MLD) is an autosomal recessive inherited lysosomal disorder caused by a deficiency of the lysosomal enzyme arylsulfatase A (ASA; EC3.1.6.8), which catalyzes the degradation of galactosyl-3-sulfate ceramide (sulfatide), a major sphingolipid of myelin. This disease is characterized pathologically by degeneration of myelin in both the central and peripheral nervous systems, and clinically by progressive motor and mental deterioration, and finally lethal neurological symptoms.

Three types of MLD are clinically recognized based on age at disease onset and symptom severity. The most common type, the late infantile type, appears from 18 to 24 months of age and is diagnosed based on gait disturbances from infancy, and ataxia, spastic quadriplegia and optical atrophy are subsequently observed. In the juvenile type, the disease appears between the ages of 4 and 16 years. In the adult type, some patients show psychiatric symptoms (Von Figura et al., 2001; Gieselmann et al., 1998; Gieselmann, 2003). No effective therapy, other than allogeneic bone marrow transplantation (BMT), has yet been reported. Moreover, it is crucial to perform BMT at presymptomatic stages of MLD in order to achieve the best neurological outcome (Krivit et al., 1990).

Enzyme replacement therapy is very effective in a limited number of disorders, including type-1 Gaucher disease and Fabry disease (Barton et al., 1991; Schiffmann et al., 2001; Eng et al., 2001; Brady and Schiffmann, 2000). However, this therapy is not available for lysozomal diseases in which the central nervous system is widely affected, such as MLD and Krabbe disease. This has encouraged investigators to seek other strategies to treat these disorders.

Gené therapy experiments for MLD using various viral vectors have been undertaken (Matzner et al., 2000, 2002; Wei et al., 1994; Consiglio et al., 2001). Improvement of neurological symptoms was reported with retroviral transduction of bone marrow progenitor cells with the ASA gene in MLD knockout mice (Matzner et al., 2000; Matzner et al., 2002). Direct injection of a lentiviral vector containing the ASA gene into brains of MLD knockout mice resulted in protection of cells in the CNS and improvement in movement disturbance (Consiglio et al., 2001).

An alternative strategy for neurological disorders, neural cell replacement therapy using brain tissue from aborted embryos, has been studied mainly for Parkinson's disease, and the effectiveness of this therapy has been discussed (Piccini et al., 1999; Bjorklund and Lindvall, 2000). Multipotential neuronal cells have been also studied (so-called neurospheres). These are isolated from mammalian CNS cells and can differentiate into neurons and glial cells in vitro (Reynolds and Weiss, 1992).

Currently, transplantation of cells transduced with a therapeutic gene is promising as a novel therapeutic strategy for genetic diseases characterized by enzyme deficiency. The present study was designed to determine the feasibility of cell-mediated gene therapy for MLD using multipotent neurospheres as carrier cells.

2. Results

2.1. *Lentiviral-vector-transduced NPCs retained the potential for differentiation into MAP2+, GFAP+ and GalC+ cells in vitro*

Cells harvested from the corpus striatum of mouse embryo at 13–15 days of gestation were cultured in specific medium, as described above, and formed neurospheres after 6–8 days. Neurospheres derived from MLD knockout mice were transduced with lentivirus vector containing the GFP gene as a marker. Neurospheres showed very high sensitivity to lentiviral vectors, as reported previously (Falk et al., 2002; Ostenfeld et al., 2002; Hughes et al., 2002) (Fig. 1a). Transduced cells were maintained on polyethylenimine-coated dishes with addition of 10% fetal bovine serum (FBS) as neuroproliferative medium. Three days later, cells were stained with MAP2 antibody (neuron marker), GFAP antibody (astrocyte marker) and GalC antibody (oligodendrocyte marker). Cells positive for each antibody were observed (Fig. 1b). Phenotypically, the various types of cells expanded continuously for as long as 7 weeks in vitro, thus demonstrating that neurospheres contained neural stem cells and neural progenitor cells and that they possessed pluripotency to differentiate into neurons and glial cells. Moreover, these cells showed that transduction with the lentivirus vector did not change morphology and that differentiation potential was preserved.

2.2. *Expression of ASA on neurospheres from MLD knockout mice after lentiviral transduction*

Neurospheres derived from MLD knockout mice were transduced with lentivirus vector containing the ASA gene. ASA enzyme activity was measured by colorimetric assay after transduction. ASA activity of transduced neurospheres was elevated to 5-fold the level of that in non-transduced MLD mice (transduced, 254.42 ± 49.0 nmol/h/mg protein; non-transduced, 50.45 ± 11.6 nmol/h/mg protein; C57-BL6 (normal control), 322.65 ± 12.25 nmol/h/mg protein), which was as high as 80% of that in normal controls (Fig. 2a). On Western blotting analysis, the 50-kDa band corresponding to ASA protein was confirmed after lentivirus transduction with the ASA gene (Fig. 2b).

2.3. *Neurospheres from GFP mice were transduced with HIV-ASA and inoculated into brains of MLD mice*

Neurospheres derived from GFP mice were transduced with lentiviral vector having the ASA gene. ASA activity of transduced cells increased by about 5-fold when compared with that in wild-type cells (3722 ± 1202 and 570 ± 288 nmol/h/mg protein, respectively) (Fig. 2c). These ASA-expressing neurospheres (5×10^4) were stereotaxically inoculated into the hippocampus of MLD knockout mice. Four weeks after transplantation, numerous ASA-transduced GFP-positive cells were detected at the site of injection and also continuously along with the corpus callosum (Fig. 3b). A few GFP positive cells were found at

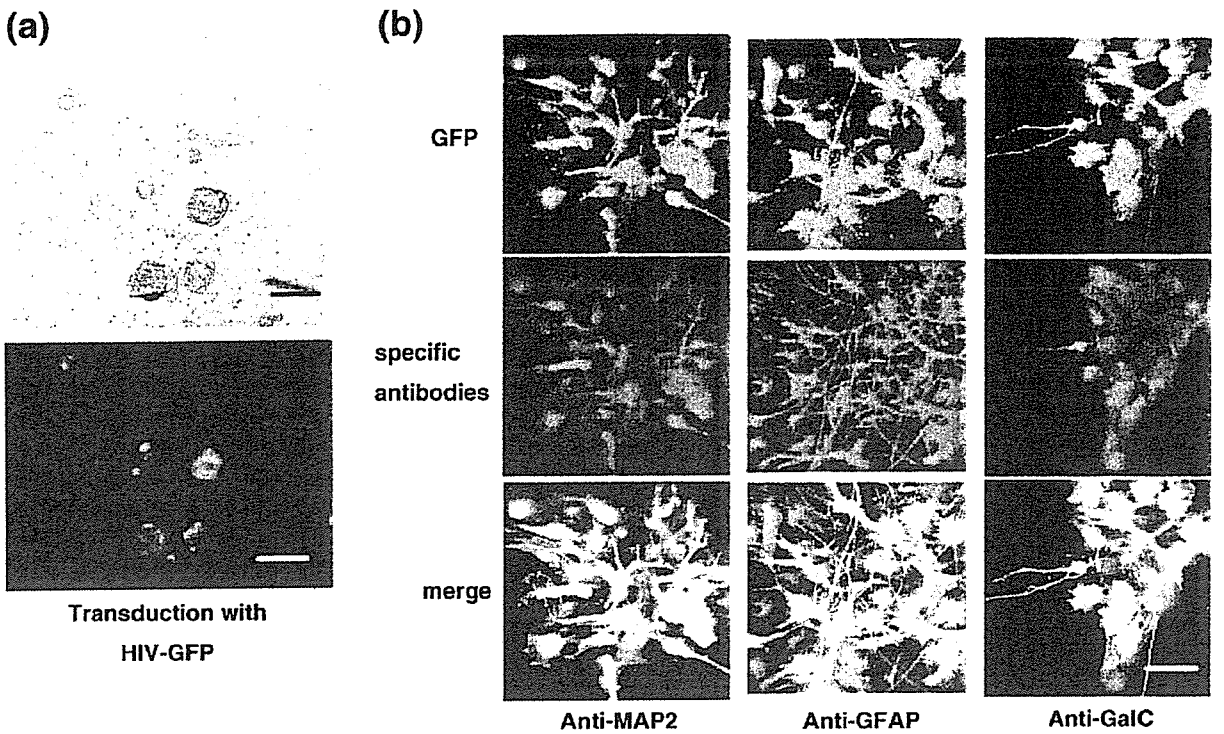

Fig. 1 – (a) Neurospheres derived from MLD knockout mice were transduced with lentivirus vector containing the GFP gene as a marker. Neurospheres showed very high sensitivity to lentiviral vectors (scale bar = 100 μm). (b) HIV vector-transduced neurospheres retained the potential for differentiation into MAP2+, GFAP+ and GalC+ cells in vitro. Immunostaining with Abs against neuron-specific MAP2, oligodendrocyte-specific GalC and astrocyte-specific GFAP (scale bar = 40 μm).

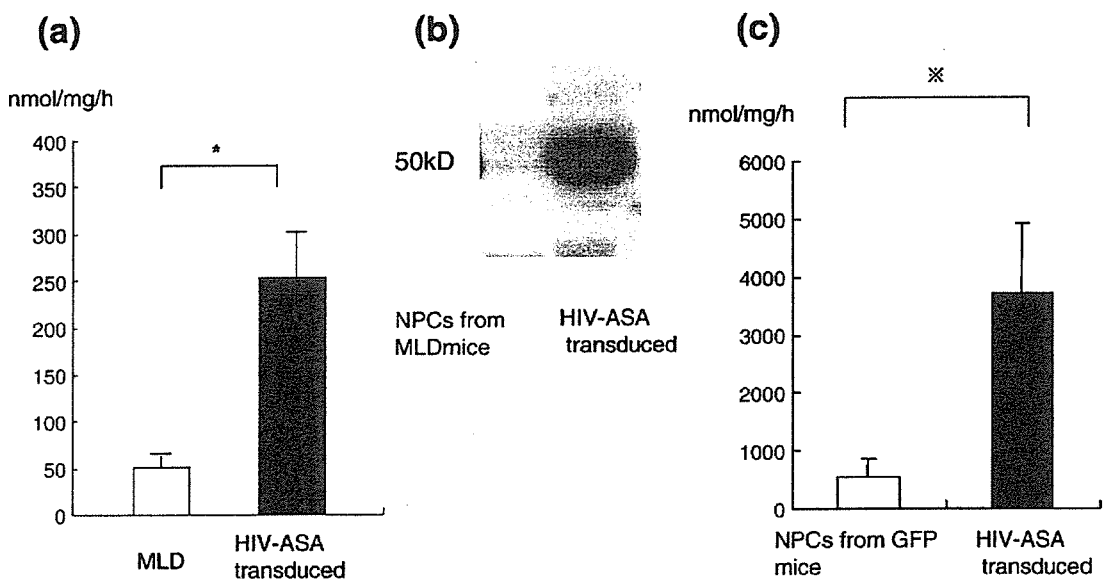

Fig. 2 – ASA was expressed in neurospheres from MLD knockout mice after HIV ASA transduction. Enzyme activity was 5-fold higher than in untransduced neurospheres (MLD-KO mice, 50.45 ± 11.57 nmol/mg/h; HIV-ASA-transduced mice, 254.42 ± 49 nmol/mg/h. $^{*}P < 0.025$ ($n = 3$) by t test between MLD and transduced group. (b) 50-kDa protein band corresponding to ASA protein was confirmed on Western blotting. (c) Neurospheres derived from GFP mice were transduced with HIV-ASA gene. ASA activity of transduced cells increased by about 5-fold when compared with that of wild-type cells (3722 ± 1202 and 570 ± 288 nmol/h/mg protein, respectively. $^{*}P < 0.001$ ($n = 3$) by paired t test.

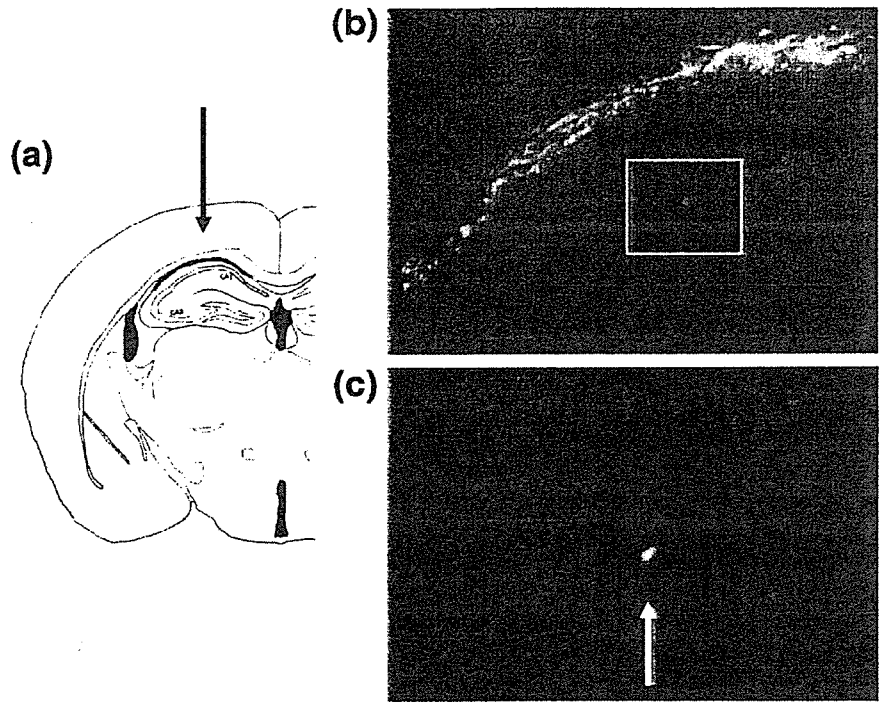

Fig. 3 – NPCs transduced with HIV-ASA were stereotaxically inoculated into the hippocampus of MLD knockout mice. Four weeks after transplantation, numerous ASA-transduced GFP-positive cells were detected at the site of injection and along with the corpus callosum. (a) Schematic representation of the brain indicating the area shown in (b) (highlighted in red). (b) GFP+ cells were detectable at various sites in the brain at 1 month after injection (×200). (c) Magnified image of square in (b). Some neurospheres were thus confirmed to migrate throughout the brain.

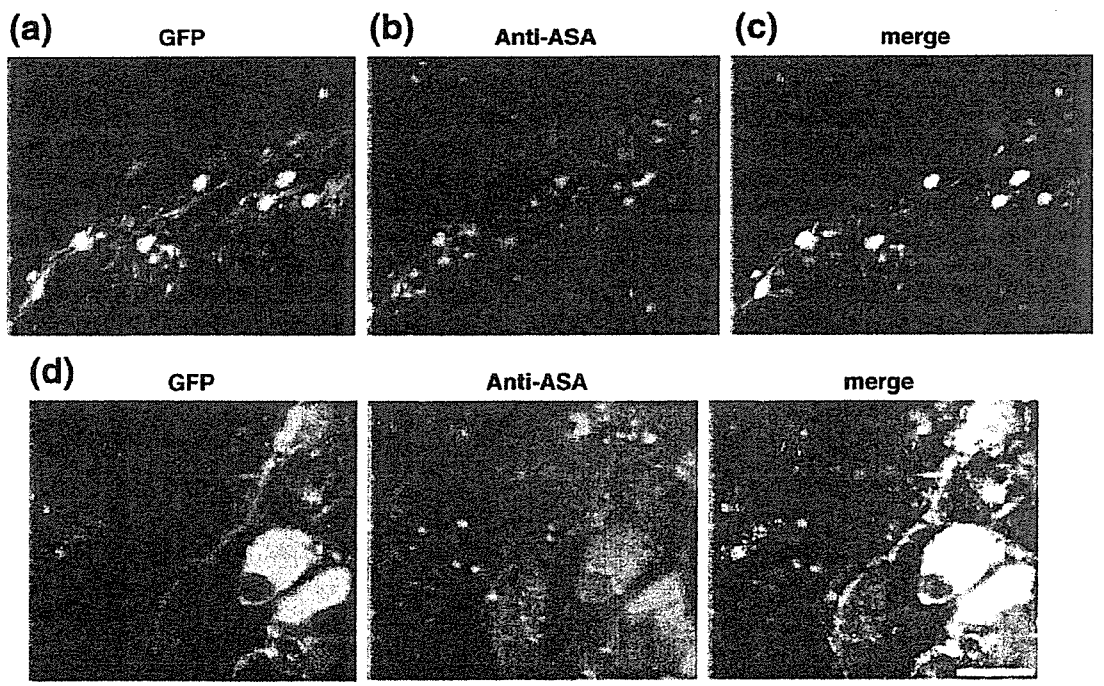

Fig. 4 – (a) Migrated GFP+ cells were detected in brain at 1 month after inoculation. ASA expression was confirmed by immunohistochemical staining using anti-ASA antibody. (c) ASA protein was observed in both GFP-positive and -negative cells (×400). (d) Confocal analysis (scale bar = 20 μm). ASA expression was observed by immunohistochemical staining using anti-ASA antibody in both GFP-positive and -negative cells.

the corpus striatum, suggesting that the cell migrated to distant sites (Fig. 3c). ASA expression was observed by immunohistochemical staining using anti-ASA antibody in both GFP-positive and -negative cells (Fig. 4). This suggests that ASA was secreted from ASA-transduced GFP-positive cells and that ASA was taken up by surrounding GFP-negative cells.

We then studied the differentiation of neurospheres in recipient brains. Immunostaining using CNS cell-specific antibodies revealed that some GFP-positive cells were labeled with anti-MAP2 and anti-GFAP antibodies, respectively, suggesting that donor neurospheres differentiated into neurons and astrocytes within 1 month of transplantation (Fig. 5). Cells positive for GalC antibody, an oligodendrocyte marker, were not detected.

2.4. *Partial clearance of accumulated sulfatide by inoculation of neurospheres transduced with ASA*

At 3 months after inoculation, injected GFP-positive cells were not detectable. However, accumulated sulfatides, which were stained with toluidine blue, were partially cleared. At the injection side, toluidine-blue-positive granules were decreased, both in total count and spot size (Fig. 6a). Toluidine-blue-positive spot count was reduced to 75.0 ± 9.0% of untreated controls after inoculation with GFP-positive cells (Fig. 6b). Total area where granules exhibited toluidine-blue-positive spots was also reduced to 60.4 ± 8.0% of untreated controls after inoculation with GFP-positive cells (Fig. 6c). This finding suggests that the ASA enzyme was released from migrated neurospheres and digested sulfatide in surrounding cells.

3. Discussion

In this study, we evaluated the efficacy of cell-mediated gene therapy for MLD using neural progenitor cells (neurospheres). The effectiveness of enzyme replacement therapy (ERT) for some lysosomal diseases has been reported and, at present, many patients with these diseases regularly receive ERT (Barton et al., 1991; Schiffmann et al., 2001; Eng et al., 2001). However, ERT is not effective in patients with diffuse CNS involvement, such as in Gaucher disease type 2, MLD or Krabbe

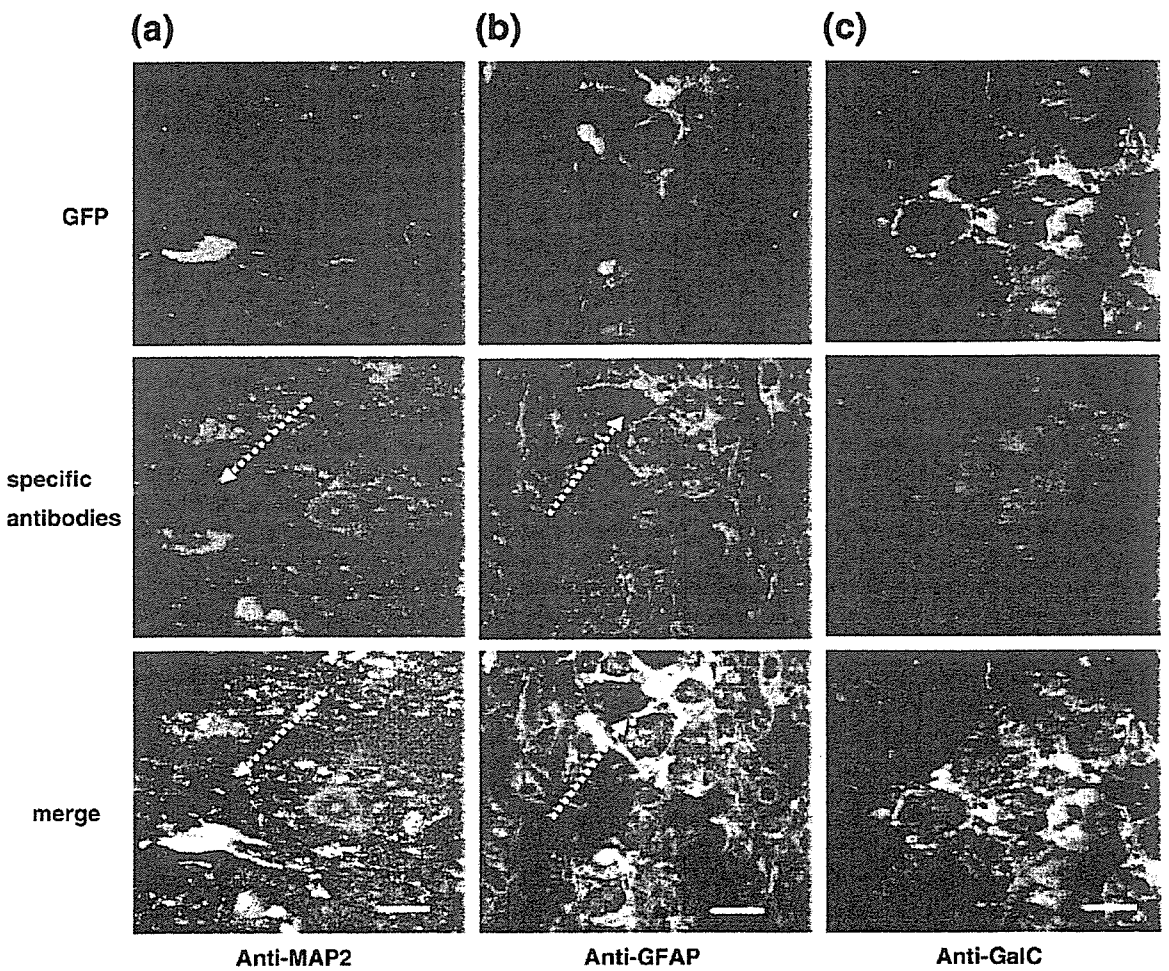

Fig. 5 – GFP-positive cells were labeled with anti-MAP2 (a), anti-GFAP antibodies (b) and anti-GalC antibodies (c), thus suggesting that donor neurospheres differentiated into neurons and astrocytes within 1 month of transplantation (scale bar = 20 μm). Cells positive for GalC antibody, an oligodendrocyte marker, were not detected.

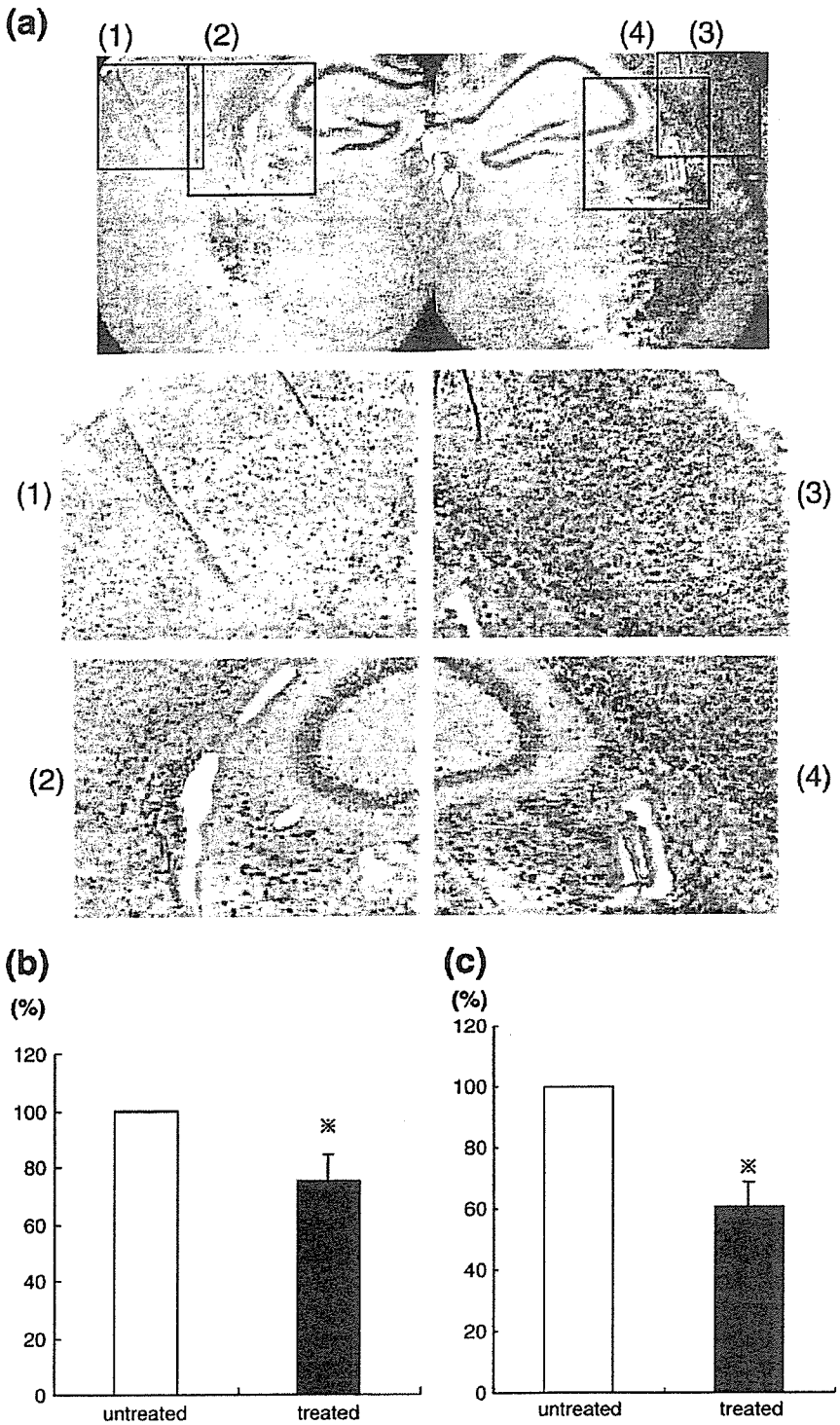

Fig. 6 – (a) Three months after inoculation with neurospheres, GFP+ cells were not detectable, but accumulated sulfatide was partially cleared in injected mice, (1) and (2), as compared with uninjected mice, (3) and (4) (upper, ×40; middle and lower, ×100). (b) Reduction of toluidine-blue-positive spot count, which visualized accumulation of sulfatide, was observed after injection of GFP-positive cells (75.0 ± 9.0% of untreated controls; $P < 0.001$ ($n = 5$) by paired t test). Reduction of total area of toluidine-blue-positive spots was observed after injection of GFP-positive cells (60.4 ± 8.0% of untreated controls; $P < 0.001$ ($n = 5$) by paired t test).

disease because the administered enzyme cannot adequately cross the blood–brain barrier (Altarescu et al., 2001; Migita et al., 2003; Kakkis et al., 2001). The only effective therapy to date is hematopoietic stem cell transplantation prior to onset of irreversible neural damage as hematopoietic stem cells can migrate into brain and secrete the deficient lysozomal enzyme, which is then taken up by CNS cells via mannose-6-phoshate receptor (Kornfeld, 1992). In current clinical practice, there are limitations in adapting allogeneic hematopoietic stem cell transplantation due to a lack of HLA-matched donors and the risk of graft-versus-host disease. Moreover, the critical issue for success of this therapy is timing as effective treatment can only be anticipated when it is performed before the onset of CNS symptoms (Krivit et al., 1990; Shapiro et al., 1995).

Several alternative strategies have been studied for these diseases, for example, direct enzyme replacement into brain parenchyma (Zirzow et al., 1999), gene delivery to brain parenchyma (Bosch et al., 2000), cell transplantation as an enzyme pump and neural stem cell transplantation for regeneration of damaged cells in CNS (Yandava et al., 1999). Until recently, CNS neurons were considered to lack the ability to regenerate after birth, and, therefore, once damaged by disease or trauma, clinical recovery was not anticipated. However, multipotential neuronal cells, so-called neurospheres, have been reported, and these can differentiate into neurons and glial cells in vitro (Reynolds and Weiss, 1992). This strategy has facilitated investigation into the regenerative capacity of CNS cells.

Recently, successful cell transplantation therapies have been reported for disorders involving neuronal damage, such as multiple sclerosis (Ben-Hur et al., 2003), mucopolysaccharidosis VII (Buchet et al., 2002) and Parkinson's disease (Piccini et al., 1999; Bjorklund and Lindvall, 2000). In particular, cell replacement therapy has been performed in Parkinson's disease using cells from the brain tissue of aborted embryos and was reported to be effective (Piccini et al., 1999; Bjorklund and Lindvall, 2000).

Several viral vectors have been investigated for gene transfer to cells in the CNS. Various types of viral vector have been extensively used to transfer genes into stem cells, but clinical problems remain. One major obstacle to success is thought to be the insufficient transduction efficiency of the retroviral vectors currently in use. Non-dividing cells like neurons are resistant to retroviral gene transfer (Karlsson, 1991). Adenovirus vectors are capable of transferring into non-dividing cells (Ohashi et al., 1997), but gene expression is only transient, and high titer adenoviral vectors can cause severe inflammation. The lentiviral vector used in this study is known to be capable of transducing therapeutic genes into quiescent stem cells.

In this study, substantial enhancement of ASA enzyme activity was observed both in neurospheres derived from MLD mice originating from 129SV mice and GFP mice originating from C57black 6 mice after lentiviral transduction with the ASA gene (Figs. 2a, c). The overall ASA activity levels of GFP mice were much higher than those in MLD mice, irrespective of gene transduction. In our study, the transduction protocol was the same in both MLD mice and GFP mice, and the lentiviral vector used in these experiments was concentrated to the degree in both experiments. One possible reason for the differences in ASA activity is the difference in mouse strain. MLD knockout mice originated from the 129SV strain, while GFP mice originated from the C57black 6 strain. Alternatively, Cα-formylglycine generating enzyme has been reported to be essential for catalytic activity of sulfatases in multiple sulfatase deficiency (Dierks et al., 2003). It is not clear whether this enzyme is well maintained in the MLD knockout mice used in this study, but differences in enzyme activity may have been caused by similar mechanisms in these experiments.

In order to achieve clinically efficient gene therapy for MLD, widespread and sustained expression of ASA is required. Direct vector injection is thought to mediate gene transfer into only a limited area, whereas demyelination and accumulation of metachromatic granules occur in all areas of the brain in MLD patients. On the other hand, in stem or progenitor cell transplantation, provided that the implanted neuronal stem cells can proliferate and differentiate into neurons and glial cells, they have the potential to migrate and spread widely throughout the brain. Considering these potential advantages of transduction with a lentiviral vector system and cell therapy, the lentiviral vector/ASA gene-mediated cell therapy approach for MLD using neurospheres is a promising strategy.

In this disease, storage of sulfatide itself affects myelin or myelin-forming cells, oligodendroglia, which leads to degeneration of myelin in both the central and peripheral nervous systems. If neural progenitor cells could be efficiently differentiated into oligodendrocytes, the injected cells might be expected to act as both enzyme-releasing pumps and as myelin-producing cells. However, in this study, we did not confirm that inoculated neurospheres differentiated into oligodendroglial cells. Cells implanted into the CNS have been reported to differentiate into oligodendroglia and form myelin, remyelinating demyelinated axons, in a multiple sclerosis mouse model (Pluchino et al., 2003). In the case of bone-marrow-derived cells, hematopoietic stem or progenitor cells can efficiently differentiate and migrate in specific conditions where tissues are damaged or if there is supplementation of cell growth factors (Brazelton et al., 2000; Mezey et al., 2000). This MLD mouse model is not reported to exhibit demyelination when compared with human MLD patients. If a model with severe demyelination was used in these experiments, it is possible that neurospheres would have differentiated into oligodendroglia. However, at this point, the reason the cells did not differentiate into oligodendrocytes remains unclear. When neurospheres from GFP mice were transduced with HIV-ASA and inoculated into the brain parenchyma of adult MLD mice in this study, GFP-positive cells were detectable at 1 month after injection but were not detectable at 3 months, while partial clearance of sulfatide was observed. Disappearance of GFP-positive cells may be due to cell death, promoter shut down or immunological rejection. It has been reported that the implanted cells required the essential growth factors, which were supplied in vitro, in adult MLD knockout mice (Schumm et al., 2004).

In ongoing studies, we plan to assess the clinical improvement in the motor and mental states of treated mice. We anticipate that the present ex vivo gene-mediated cell therapy approach will be applicable to the future treatment of MLD.

4. Experimental procedures

4.1. *Vector construction*

The Pbeh/HT14-CP8 plasmid containing human ASA cDNA was a generous gift from Dr. Gieselmann (University of Kiel, Germany) (Stein and Gieselmann, 1989). The ASA cDNA fragment was isolated from Pbeh/HT14-CP8 by EcoRI and was blunt-ended. The resulting fragment was modified with an *Xhol* linker. The lentiviral vector construct used in this study was derived from the plasmid pHIV-CS-CAG-PRE(ppt), which was constructed by inserting the central polypurine tract and the central termination sequence (ppt) fragment from HIV-1 downstream of the 5′-LTR of pHIV-CS-CAG-PRE (Igarashi et al., 2003). The vector expressing human ASA (pHIV-CS-CAG-ASA-PRE(ppt)) was constructed by inserting the *Xhol* ASA fragment into pHIV-CS-CAG-PRE(ppt). The vector plasmid expressing EGFP (pHIV-CS-CAG-EGFP-PRE(ppt)) was constructed in a similar manner.

4.2. *Lentiviral vector production*

293T cells (embryonic kidney cells) (1×10^6) were plated on thirty 10-cm plates and were cultured for 16–24 h. Cells were then co-transfected with 7 μg of the envelope plasmid pCMVΔR8.2, 7 μg of the packaging plasmid pMDG and 7 μg of the transfer plasmid pHIV-ASA or pHIV-GFP by calcium phosphate precipitation. After incubation for 4–6 h at 37 °C, the medium was removed and fresh DMEM medium was added. Cells were cultured for an additional 3–4 days. Viral supernatant was harvested and passed through a 45-μm filter. The resulting medium was centrifuged to a volume of 5 ml at room temperature using CENTREPREP (Millipore Corporation, Bedford, MA) at 3000 rpm with a CH3.8 rotor (Beckman Coulter, Boulevard, PO). Five milliliters of viral supernatant was concentrated to a pellet by ultracentrifugation, and the pellet was finally diluted with 400 μl of phosphate-buffered saline (PBS). To determine the titer of the HIV vector encoding EGFP (HIV-EGFP), HELA cells ($2–3 \times 10^5$ cells/well in 6-well plates) were transduced for 3 days with this vector. EGFP-positive cells were then evaluated by fluorescence-activated cell sorting (FACS) analysis. The titer of the final vector stock was approximately 1×10^7 to 1×10^8 transduction units (TU)/ml.

4.3. *Animals*

Female MLD knockout mice (age, 1 year; 18–20 g; generous gift from Dr. Gieselmann) were used in this study (Hess et al., 1996). The MLD mice used in this study have a mixed C57BL6/129 genetic background. In MLD young mice, galactosyl sulfatide is not accumulated yet. Therefore, 1-year-old MLD mice were used. Neurospheres were inoculated into nine MLD mice brains in this study and three of them were analyzed in detail. GFP mice are originated from C57/BL6 (Okabe et al., 1997).

All stereotaxic surgical procedures were performed aseptically under anesthesia with nembutal (25 mg/kg). All surgical procedures were performed in accordance with the regulations set by the Ethics Committee for use of Laboratory Animals at Nippon Medical School.

4.4. *Establishment of neural progenitor cells (NPC), neurospheres transduction of NPC*

The corpus striatum was removed from several mouse embryos derived from one pregnant mouse at 13–15 days of gestation and was dissociated mechanically with a Pasteur pipette in F12/Dulbecco's Modified Eagle Medium (DMEM, Life technologies, Rockville, MD). Isolated cells were suspended at a concentration of 2×10^6 cells/ml in F12/DMEM supplemented with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 0.6% glucose, 3 mM sodium bicarbonate, 2.5 mM glutamine, 25 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 60 μM putrescine and 30 nM sodium selenite (modified N2 medium) (Bottenstein and Sato, 1979; Suzuki et al., 2002) and were then placed in 25-cm^2 tissue culture flasks. EGF (20 ng/ml; Peprotech, Rocky Hill, NJ) and βfibroblast growth factor (βFGF, 20 ng/ml; Peprotech) were added to these cultures. The cells formed spheres after 6–8 days and expanded continuously in the medium (Reynolds et al., 1992). To transduce NPC with lentivirus vector, 6×10^5 cells were plated in 1 ml of culture medium and 50 μl of lentivirus vector (multiple of infection; MOI = 1). Neurospheres established from MLD knockout mice were transduced with GFP-lentiviral vector in order to study the expression of GFP and their potential for differentiation into neurons and glial cells in vitro. The expression of ASA was studied in neurospheres transduced with HIV-ASA vector by ASA enzyme assay and Western blotting analysis with anti-ASA antibody.

4.5. *Immunocytological analysis*

Neurospheres transduced with GFP gene were cultured on polyethylenimine-coated glass cover slips in F12/DMEM supplemented with 10% FBS. At 3 days after induction by 10% FBS, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min and then washed three times with phosphate-buffered saline. Cells fixed on glass coverslips were stained with primary antibodies, which included rabbit anti-microtubule associated protein 2 (MAP2) antibody (1:100; CHEMICON) for neurons, rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:2000; Dako) for astrocytes and rabbit anti-galactocerebroside (GalC) polyclonal antibody (1:100; Sigma) for oligodendrocytes for 16 h at 4 °C. After washing three times with PBS, cells were stained with Texas-red-conjugated secondary antibodies, including donkey anti-rabbit IgG (1:200 dilution). Sections were then examined using a confocal scanning laser microscope (Leica TCSSP, Heidelberg, Germany), with the collected signals undergoing digital color enhancement before superimposition.

4.6. *Direct injection of neural progenitor cells*

Initially, cells harvested from the corpus striatum of mouse embryo at 13–15 days of gestation formed spheres in dishes after 6–8 days, and these cells were dissociated mechanically to be transduced with lentiviral vectors. Three days after

transduction, neurospheres were dissociated with a single pass through a 23-gauge needle. For injection of neurospheres into mouse brain, the skin over the skull was incised under anesthesia. The injection site was 2 mm posterior and 2 mm lateral to the bregma. A small hole was drilled in the skull. Neurospheres (5×10^4 cells in 2 μl of PBS) were slowly injected through the hole into the hippocampus 2 mm below the duramater using a stereotaxic apparatus. At least three animals were analyzed under each experimental condition. The direct injection experiment was repeated three times. Mice were perfused via the left ventricle with cold 0.9% NaCl followed by 4% paraformaldehyde in PBS (pH 7.4) under deep anesthesia. Brains were removed and fixed overnight at 4 °C and were then transferred to PBS solution containing 20% sucrose until equilibration and placed in embedding medium, which consisted of 100% optimal cutting temperature (OCT) compound. Approximately 200 microsections (20-μm thick) were prepared from each mouse brain with a Coldtome (Sakura, Tokyo, Japan). To identify cell types, brain sections were stained immunohistochemically using specific antibodies. Primary antibodies were as follows: rabbit anti-microtubule associated protein 2 (MAP2) antibody (CHEMICON) (diluted 1:200 in PBS containing 0.3% Triton-X 100 and 10% donkey serum) for neurons; rabbit anti-glial fibrillary acidic protein (GFAP) (Dako) (diluted 1:2000 in PBS containing 0.3% Triton-X 100 and 10% donkey serum) for astrocytes; rabbit anti-galactocerebroside (GalC) polyclonal antibody (Sigma) diluted 1:100 in PBS containing 0.3% Triton-X 100 and 10% donkey serum for oligodendrocytes; and rabbit anti-ASA antibody (diluted 1:100 in PBS containing 0.3% Triton-X 100 and 10% donkey serum). Microsections were incubated with primary antibodies overnight at 4 °C, washed with PBS containing 0.3% Triton-X 100 and stained with the Texas-red-conjugated secondary antibodies, including donkey anti-rabbit IgG for MAP2, GFAP, GalC and ASA at 1:200 dilution in 2% mouse serum. After three cycles of washing, sections were mounted and coverslipped. Fluorescent signals were observed under a confocal laser-scanning microscope (Leica TCSSP, Heidelberg, Germany).

4.7. *Measurement of ASA enzyme activity*

Cultured cells were harvested and washed twice in cold PBS. They were then sonicated in double-distilled water and centrifuged to eliminate cellular debris. After quantifying protein using the Bradford protein assay (BIO-RAD, Tokyo), ASA enzyme activity was measured by colorimetric assay utilizing p-nitrocatecholsulfate (Sigma, St. Louis, MO) as a substrate, as described previously (Baum et al., 1959). Activity was described as nmol/mg total protein per hour.

4.8. *Western blotting analysis*

Neurospheres transduced with lentiviral vector containing ASA were cultured in neuromedium. Three days later, sodium dodecyl sulfate (SDS) buffer was added to cells, and cells were heated at 95 °C for 2 min. Arylsulfatase or 10 μl of media was resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The transfer membrane was incubated with anti-rabbit ASA antibody and with secondary antibody (anti-rabbit IgG HRP conjugate, Promega, Madison, WI). The blot was then immersed in an enhanced chemiluminescence solution (ECL plus; Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to Kodak KAR5R film.

4.9. *ASA antibody*

Antisera against human ASA were generated by immunizing rabbits with KLH-conjugated peptide (OPERON Biotechnologies, Japan). The peptide sequence was LLGTGKSPRQSLFFY for human ASA.

4.10. *Transplantation into brain*

The ASA-lentiviral vector used in this study did not contain a marker gene, and in order to demonstrate the efficacy of ex vivo gene therapy using neural progenitor cells for MLD, neurospheres derived from GFP mice were transduced with lentiviral vector with the ASA gene for donor cells. These ASA-expressing neurospheres (5×10^4) were stereotaxically injected into the hippocampus of MLD knockout mice as recipients. At 4 weeks or 12 weeks after transplantation, implanted brains were removed as described above and were sliced to 20-μm sections and immunostained with antibodies. In addition, to examine storage and clearance of sulfatide, samples sliced at 10 μm were stained with toluidine blue for 4 h followed by washing three times. The effect of cell-mediated gene therapy on accumulation of sulfatide in brain tissue was determined by measuring the count and areas of toluidine-blue-positive inclusion bodies in microscopic sections using WIN ROOF software (MITANI CORP, Tokyo, Japan). At least three sections from each of three different animals were examined in this analysis.

4.11. *Statistical analysis*

All data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using Student's t test. A P value of less than 0.05 denoted the presence of a statistically significant difference.

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