As to kidney tissues, immunofluorescence of CTH in the renal tubular cells was apparently decreased. Cleavage of the accumulated CTH in the glomeruli was not sufficient although immunofluorescence was slightly decreased (Fig. 4b). As to the heart, immunofluorescence of CTH accumulated in the tissue was apparently decreased after repeated injections of yr-haGal (Fig. 4c), as in the case of spleen (data not shown). In the dorsal root ganglia, no apparent degradation of the accumulated CTH was observed (Fig. 4d). These findings were essentially the same as for the 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 in wild type mice, and decreased it to 70% and 30% of the untreated Fabry mice levels in the kidneys and heart, respectively. The degree of degradation of the CTH deposited in these tissues was almost the same as in the case of agalsidase beta. The decrease in the accumulated CTH in the spleen on the administration of yr-haGal (to 30% of the untreated Fabry mice level) was smaller than that on the 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 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 derived from Fabry mice repeatedly injected with these preparations. The results are shown in Fig. 6. No significant antibodies unique to both enzyme preparations were found under the experimental conditions used.

Discussion

Compared with the production of recombinant human α-galactosidases in mammalian cells, that in yeast cells has considerable advantages, i.e., culture is easy and economical, and does not require fetal calf serum. Here, as a host we used a *Saccharomyces cerevisiae* mutant, in which two genes, *OCH1* and *MNN1* involved in *N*-linked mannan biosynthesis, were disrupted. The expressed glycoprotein in this cell line has mammalian-like *N*-linked high-mannose-type sugar chains and does not have any 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 resulted in the exposure of M6P residues at the non-reducing ends of sugar chains (Chiba

et al. 2002). As M6P residues are essential for the incorporation of α-galactosidase into human cells via M6P receptors on the cell membrane (Kornfeld and Sly 2001), 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. The productivity of the recombinant enzyme would be further improved by carefully choosing a cell strain in the future. We have preliminarily prepared a methylotrophic yeast cell line secreting a recombinant α-galactosidase into the culture medium at a concentration of 12 mg/L, and attempts to obtain abundant enzyme protein are underway involving the improved purification method described in this report.

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

Lee et al. reported that agalsidase beta has a higher percentage of phosphorylated oligomannose chains than agalsidase alfa has, which results in improved binding of agalsidase beta to M6P receptors, and higher

enzyme levels in the kidneys and heart, which are the mainly affected organs in Fabry disease, when tested at the same dose (Lee et al. 2003). Considering these results, we injected agalsidase beta into Fabry mice as a control in the experiment on the incorporation of yr-haGal into the organs of Fabry mice and its CTH-degrading activity. yr-haGal was successfully incorporated into the liver, kidneys, heart and spleen, and cleaved the CTH deposited in these organs as in the case of agalsidase beta. However, the degradation of CTH accumulated in the glomeruli by these recombinant enzymes was not sufficient, 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 the enzyme activity increase in the liver and spleen. This is probably due to differences in the compositions of their sugar chains. Asialylated complex-type oligosaccharides are involved in the uptake of lysosomal enzymes by hepatocytes in the liver via asialoglycoprotein receptors (Rosenfeld et al. 1986), but the recombinant α-galactosidase produced in yeast cells does not have any complex-type sugar chains although agalsidase beta has. Why agalsidase beta was incorporated more into the spleen than yr-haGal was 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 the administration of both the enzymes did not reduce the 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 the incorporation was apparently lower than that in the case of cultured Sandhoff fibroblasts (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 hardly incorporated into neural cells. Phase 3 clinical trials for agalsidase beta revealed that there were no significant differences in improvement of the pain in the peripheral extremities between a group of patients treated with agalsidase beta and the procebo 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 ones (Schiffmann et al. 2000; Eng et al. 2001). So, we examined the levels of antibodies against

yr-haGal in sera of recurrently injected Fabry mice by means of an ELISA, but no specific antibodies for yr-haGal were detected as in the case of agalsidase beta under the experimental conditions used.

In conclusion, we produced a recombinant α-galactosidase having M6P residues at the non-reducing ends of *N*-linked sugar chains in yeast cells. 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 not sufficient. As the production of the recombinant α-galactosidase in yeast is easy and economical, and does not require fetal calf serum, it is highly promising as an enzyme source for enzyme replacement therapy for Fabry disease.

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Figure legends

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

Figure 2. 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. Bars, 50 μm. Time-course (**a**) and dose-dependency (**b**).

Figure 3. Pharmacokinetics of the yeast recombinant human α-galactosidase (yr-haGal) and agalsidase beta. A single dose of yr-haGal or agalsidase beta was injected into Fabry mice, and then blood samples were collected and the time course changes in the α-galactosidase activity in plasma were determined. Each value represents the mean for two mice.

Figure 4. Immunohitochemical analyses of the accumulated CTH in organs of Fabry mice, and its degradation by the yeast recombinant human α-galactosidase (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. Bars, 50 μm. Liver (**a**), kidneys (**b**), heart (**c**), and dorsal root ganglia (**d**).

Figure 5. Morphological effects of repeated administration of the yeast recombinant human α-galactosidase (yr-haGal) on renal tubular cells of Fabry mice. yr-haGal was repeatedly injected into Fabry mice, and then kidney tissues were electron-microscopically examined. Bars, 2 μm. (**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.

Figure 6. Antigenicity of the yeast recombinant human α-galactosidase produced in yeast cells (yr-haGal) and agalsidase beta. ELISA to determine whether Fabry mice recurrently injected with yr-haGal (**a**) and agalsidase beta (**b**) produced antibodies to the enzymes or not was performed. Open circles, rabbit anti-α-galactosidase antibodies; closed circles, Fabry mouse sera treated with the enzymes (yr-haGal and agalsidase beta); open square, serum from an untreated Fabry mouse; open triangle, serum from an untreated wild type mouse.

Table 1.

Monosaccharide analysis.

	yr-haGal* (mol/mol protein)	agalsidase beta** (mol/mol protein)	agalsidase alfa** (mol/mol protein)
M6P	3.8±0.2	3.1±0.1	1.8±0.0
Galactose	ND	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

* Values are expressed as means±SD, n=3.

** Lee et al. 2003.

ND: not detected.

Table 2.

α-Galactosidase activities (nmol/h/mg protein) in organs from Fabry mice treated with a single dose of yr-haGal or agalsidas beta.

	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

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 hour 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.

Table 3.

Ceramide trihexoside levels (nmol/mg protein) in organs from Fabry mice treated with four doses of yr-haGal or agalsidase beta.

	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

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 then 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).