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Therapeutic Potential of Intracerebroventricular Replacement of Modified Human β-Hexosaminidase B for GM2 Gangliosidosis

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To develop a novel enzyme replacement therapy for neurodegenerative Tay-Sachs disease (TSD) and Sandhoff disease (SD), which are caused by deficiency of β-hexosaminidase (Hex) A, we designed a genetically engineered *HEXB* encoding the chimeric human β-subunit containing partial amino acid sequence of the α-subunit by structure-based homology modeling. We succeeded in producing the modified HexB by a Chinese hamster ovary (CHO) cell line stably expressing the chimeric *HEXB*, which can degrade artificial anionic substrates and GM2 ganglioside *in vitro*, and also retain the wild-type (WT) HexB-like thermostability in the presence of plasma. The modified HexB was efficiently incorporated via cation-independent mannose 6-phosphate receptor into fibroblasts derived from Tay-Sachs patients, and reduced the GM2 ganglioside accumulated in the cultured cells. Furthermore, intracerebroventricular administration of the modified HexB to Sandhoff mode mice restored the Hex activity in the brains, and reduced the GM2 ganglioside storage in the parenchyma. These results suggest that the intracerebroventricular enzyme replacement therapy involving the modified HexB should be more effective for Tay-Sachs and Sandhoff than that utilizing the HexA, especially as a low-antigenic enzyme replacement therapy for Tay-Sachs patients who have endogenous WT HexB.

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INTRODUCTION

Tay-Sachs disease (TSD) and Sandhoff disease (SD) are neurodegenerative GM2 gangliosidoses caused by mutations in the *HEXA* and *HEXB* genes encoding the α- and β-subunits of human lysosomal β-hexosaminidase (Hex) (EC 3.2.1.52), respectively. They involve HexA (αβ-heterodimer) deficiency, lysosomal accumulation of GM2, mainly in neural tissues, and neurological symptoms.[1,2] There are two major Hex isozymes, HexB (ββ-homodimer) and HexA, in mammals. Thermostable HexB can degrade neutral substrates, whereas HexA can cleave both neutral and anionic substrates, although only HexA can degrade GM2 in cooperation with GM2 activator protein (GM2AP).

So far, little effective treatment for these GM2 gangliosidoses has been developed, although several experimental and clinical trials have been performed.[3–6] In recent years, intravenous enzyme replacement therapy (ERT) involving recombinant enzyme drugs produced by mammalian cell lines stably expressing human lysosomal enzyme genes has been clinically applied for lysosomal storage diseases (LSDs), including Gaucher disease,[7,8] Fabry disease,[9] Pompe disease,[10,11] and mucopolysaccharidoses I,[12] and II[13] involving peripheral dysfunctions such as hepatosplenomegaly, vascular disorders, cardiomegaly, and dysostosis multiplex. Intravenous ERT is based on incorporation of a recombinant enzyme via cell surface glycan receptors, including MR[7,8] and CI-M6PR/insulin-like growth factor type II receptor,[9–13] into the target organs, and delivery to intracellular lysosomes and degradation of the accumulated substrates. However, intravenous ERT is ineffective for LSD patients with neurological symptoms because of the presence of the blood brain barrier, although delivery through the cerebrospinal fluid as a potential route to the central nervous system has been examined to develop intracerebroventricular (*icv*) and intrathecal ERT for LSD patients with the central nervous system affected.[14–16] In addition to above issues, enzyme functions, including substrate preference, efficiency of uptake and maintenance of intracellular catalytic activity, are important factors for therapeutic effectiveness on intravenous and *icv* ERT. Furthermore, an adverse outcome of intravenous ERT, immune responses to therapeutic enzymes due to the production of neutralizing antibodies in LSD patients, has been reported.[17] For example, such neutralizing antibodies abrogate the therapeutic effect in Pompe disease patients.[11]

In this study, we designed and developed a modified HexB with GM2-degrading activity and binding ability as to GM2AP, based on the amino acid sequence homology between the Hex

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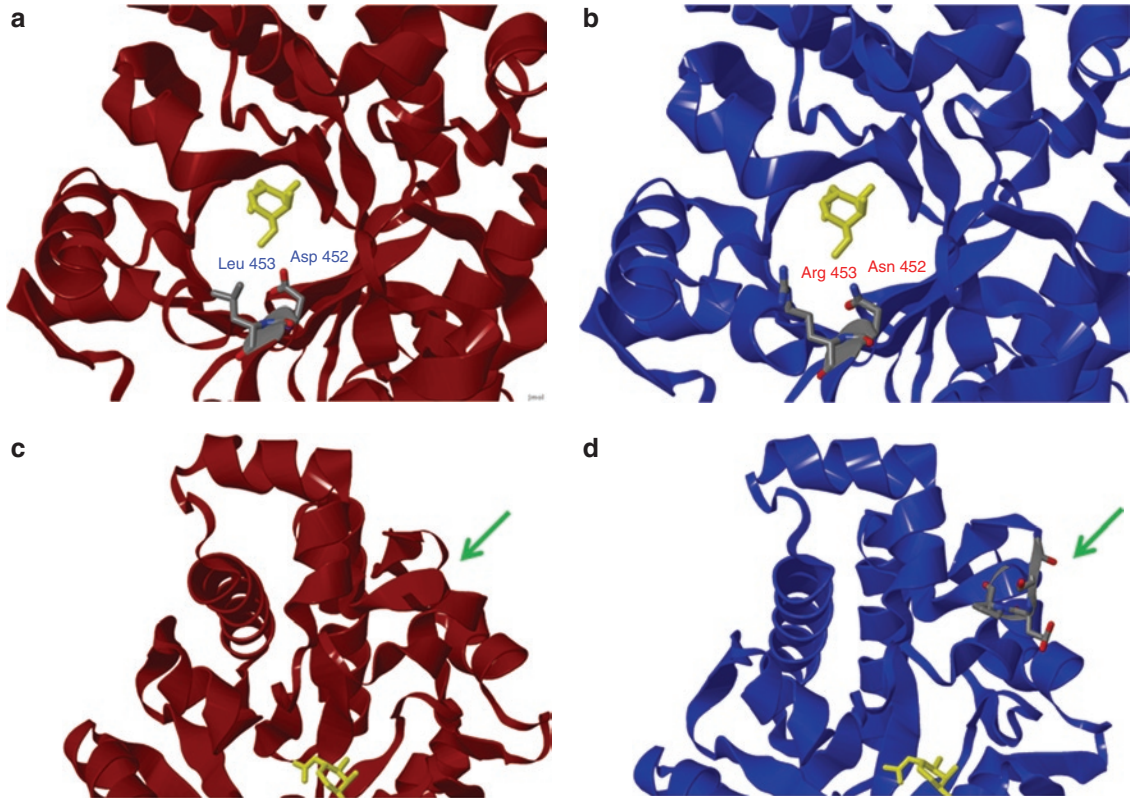

Figure 1 Structure of wild-type HexB (WTHexB) and modified HexB. (**a**,**c**) The active site and loop structure of WTHexB and (**b**,**d**) those of the modified HexB are shown as ribbon models. Each structure is presented as a complex with a substrate analog inhibitor. βD452 and βL453 are corresponding to αN423 and αR424, which are important for determining the substrate specificity. Green arrows indicate the position of the loop structure.

α- and β-subunits. Here, we show that therapeutic effects of modified HexB of *icv* replacement for GM2 gangliosidosis model mouse. The modified HexB could be a potentially low-antigenic therapeutic enzyme for *icv* ERT for TSD patients who have an intrinsic Hex β-subunit.

RESULTS

Structure-based design of a modified HexB β-subunit

A homology model of the modified human Hex β-subunit was built on the basis of the X-ray crystallographic data for the human Hex β-subunit (protein Databank code, 1now) and the amino acid sequence for human Hex α-subunit, which exhibits 56% amino acid identity with the human Hex β-subunit. In order to create a modified Hex β-subunit with an α-subunit-like substrate preference and binding ability as to GM2AP, the difference in the solvent accessible surface area value of each residue between the apo form of Hex β-subunit and its complex with the substrate analog inhibitor was calculated at first. As a result, 13 residues (R211, H237, D240, H294, D354, E355, W405, W424, Y450, D452, L453, W489, and E491) were defined as residues involved in the active pocket of β-subunit. **Figure 1** shows structural comparison of the wild-type (WT) β- and modified β-subunits. Among the pocket residues, only D452 and L453 are not conserved in the α- and β-subunits, indicating that βD452 and βL453 are important as to determining the substrate specificity.

The loop (280-283, GSEP) sequence in the α-subunit is required for GM2AP to interact with HexA, and essential for the degradation of GM2 by HexA. In total, the functions of HexA including its binding ability as to GM2AP and substrate specificity may be due to these six residues (G280, S281, E282, P283, N423, and R424) of the α-subunit. Therefore, we replaced R312, Q313, N314, K315 (**Figure 1c,d**), D452 and L453 (**Figure 1a,b**) of the β-subunit with the corresponding residues, G, E, S, P, N, and R of the α-subunit, respectively, to change the substrate preference to anionic substrates and the binding ability as to GM2AP of the WT β-subunit, and thereby designed a modified β-subunit.

Expression and purification of modified HexB with an altered substrate specificity and a loop sequence for interacting with GM2AP

Each of WT*HEXB* and the modified *HEXB* cDNA bearing the R312G, Q313S, N314E, K315P, D452N, and L453R substitutions in the β-subunit was introduced into Chinese hamster ovary (CHO) cells. The conditioned medium (CM) of each cell line was collected, and the recombinant enzymes were purified by three-step column chromatography. **Figure 2a,b** show the results obtained on native- and sodium dodecyl sulfate polyaclylamide gel electrophoresis, respectively. Modified HexB migrated to the same position as WTHexB, indicating that the molecular mass and electrostatic properties of the modified HexB were similar to those of WTHexB (**Figure 2a**). **Figure 2b** shows intracellular processing of β-subunit. The mature form of WT β-subunit is composed of three polypeptides and they are connected by disulfide bond. WT precursor β-subunit was detected at the 63 kDa and mature ones

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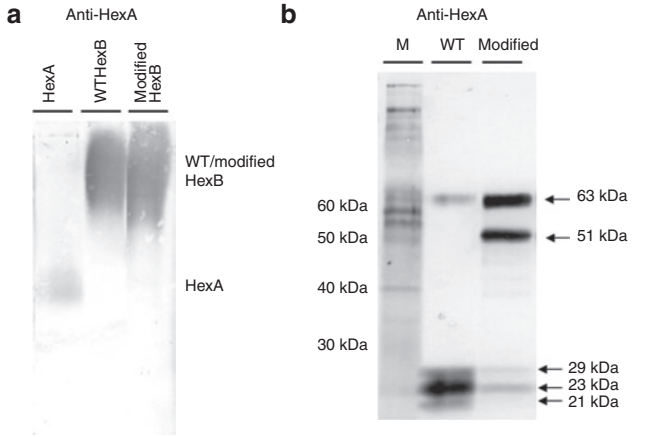

Figure 2 Immunoblotting of the Hex isozyme. (**a**) The native form of the modified HexB, wild-type HexB (WTHexB) and HexA were separated by native-polyacrylamide gel electrophoresis (PAGE), and (**b**) the WT- and modified HexB were separated by sodium dodecyl sulfate-PAGE, and then immunoblotting analysis with anti-HexA antibodies was performed. Each lane contained 750 nmol/hour 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MUG)-degrading activity.

were detected at the 29, 23, and 21 kDa under the experimental condition, respectively. On the other hand, the major β-subunit moieties of modified HexB migrated to the 63 and 51 kDa, and minor ones migrated to the 29, 23, and 21 kDa, respectively. These results indicate different processing pattern between WT- and modified β-subunit.

Enzymological characteristics of modified HexB

The kinetic parameters for degradation of 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MUG) and 4-methylumbellifery-6-sulfo-β-D-glucosaminide potassium salt (4-MUGS) of WT- and modified HexB were measured. The K_m value for 4-MUG of the modified HexB was slightly decreased, whereas that for 4-MUGS was greatly decreased compared with those for WTHexB (**Table 1**). The specific activities of WT- and modified HexB toward the 4-MUG were 3.3×10^{-3} mmol/minute/mg protein and 1.5×10^{-3} mmol/minute/mg protein, and those of WT- and modified HexB toward the 4-MUGS were 5.0×10^{-5} mmol/minute/mg protein and 5.0×10^{-3} mmol/minute/mg protein, respectively. The thermostability of the modified HexB in murine plasma was examined *in vitro*. The modified HexB retained higher stability than that of WTHexB 7 days after incubation (**Table 2**).

GM2-degrading activity of modified HexB *in vitro* and in TSD fibroblasts

To examine the GM2-degrading activity of modified HexB, GM2 micelles were incubated with Hex isozymes, followed by thin-layer chromatography. As shown in **Figure 3a**, modified HexB degraded GM2 in a similar manner as HexA, but there was no degradation of GM2 by WTHexB. Next, the effects of amino acid substitutions in the Hex β-subunit were evaluated on enzyme replacement in fibroblasts (F218) derived from a TSD patient. The WT- or modified HexB was added to the cultured medium, followed by incubation for 4 days. Then restoration of the Hex activity and reduction of the accumulated GM2 were examined with anti-GM2 antibodies after treatment with the modified HexB by means of immunofluorescent microscopy. As shown in **Figure 3b**, significant restoration of 4-MUGS-degrading activity was observed in TSD fibroblasts on treatment with the modified HexB, which was superior to WTHexA. The recovery of Hex activity on treatment with the modified HexB was significantly inhibited in the presence of 5 mmol/l M6P. These results indicate that the modified HexB was taken up via CI-M6PR. As shown in **Figure 3c**, remarkable accumulation of GM2 due to HexA deficiency was detected in the TSD fibroblasts. WTHexA reduced the accumulated GM2. The modified HexB also degraded GM2, although no reduction was observed in the TSD fibroblasts on treatment with WTHexB. There seemed to be little difference in GM2-degrading activity between the modified HexB and WTHexA. The reduction of GM2 was also inhibited in presence of 5 mmol/l M6P, indicating that uptake of the modified HexB carrying M6P-type N-glycans was mediated by CI-M6PR.

Table 1 Kinetic parameters of Hex isozymes

	4-MUG		4-MUGS	
Enzyme	Specific activity	K_m/V_{max}	Specific activity	K_m/V_{max}
Modified HexB	1.5×10^{-2}	$1.1/1.5 \times 10^{-2}$	5.0×10^{-2}	$0.6/6.7 \times 10^{-3}$
WTHexB	3.3×10^{-3}	$1.1/3.3 \times 10^{-3}$	5.0×10^{-5}	$5.5/6.7 \times 10^{-5}$
WTHexA	1.0×10^{-2}	$1.7/1.2 \times 10^{-2}$	8.3×10^{-3}	$1.3/3.3 \times 10^{-3}$

Abbreviations: 4-MUG, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide; 4-MUGS, 4-methylumbellifery-6-sulfo-β-D-glucosaminide potassium salt; Hex, β-hexosaminidase; WT, wild type.
Specific activity: (mmol/minute/mg protein); K_m: (mmol/l); V_{max}: (mmol/minute/mg protein).

Table 2 Analysis of enzyme thermostability in plasma

	Residual activity	
Enzyme	2 days	7 days
WTHexA	47.7	27.0
WTHexB	94.3	85.3
Modified HexB	96.6	91.4

Abbreviations: Hex, β-hexosaminidase; WT, wild type.
Means indicate percent of starting enzyme activity in mice plasma. Values indicate percentages of starting enzyme activity in the presence of murine plasma.

Replacement effects of the modified HexB on Sandhoff mice

The replacement effects of modified HexB on *icv* injection into the lateral cerebral ventricles of 10-week-old SD mice were further examined. One week after injection of the modified HexB, the Hex activity (4-MUGS) in each region (fore-, mid-, and hindbrain) of SD mice had recovered to above 40% of that observed in the WT mice. Three weeks after injection, the residual Hex activity was maintained at above 15% of the level in the WT mice, it being especially about 40% in the hindbrain of SD mice (**Figure 4a**). The effect of *icv* administration of modified HexB on the recovery of the Hex activity was greater than that of WTHexA (**Figure 3b** and **Supplementary Figure S1**).

The effects of *icv* administration of the modified HexB on reduction of the accumulated substrates in the brain regions of SD mice were also analyzed by immunohistochemistry 3 weeks

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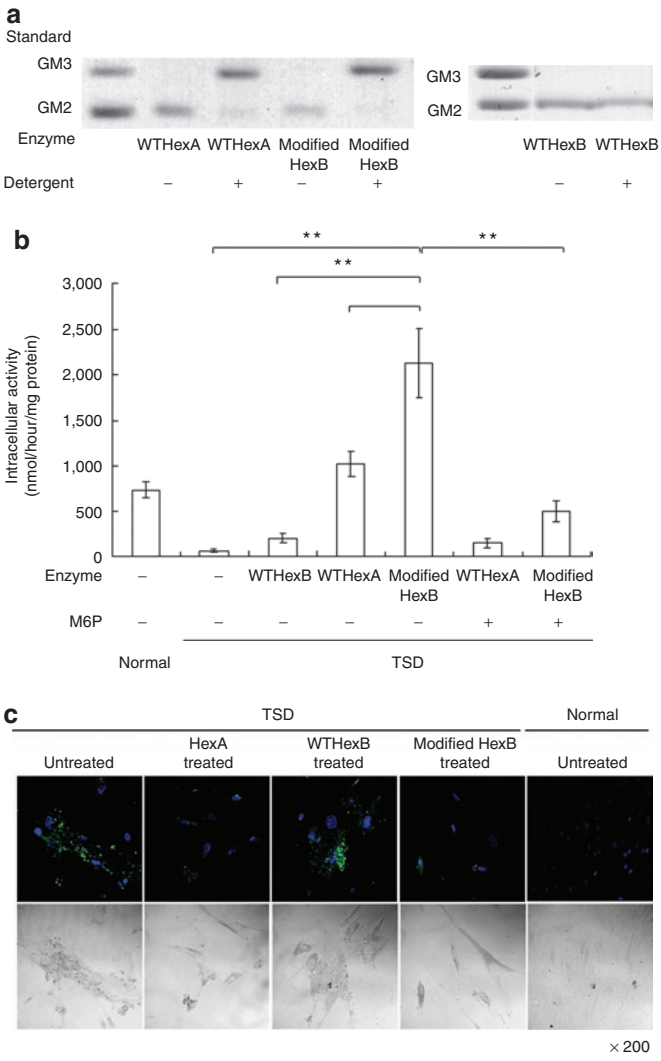

Figure 3 Degradation of GM2 by modified HexB in the presence of a detergent. (**a**) Five micrograms of GM2 was incubated with wild-type HexA (WTHexA), WTHexB and the modified HexB, respectively, in the presence or absence of 2 mmol/l taurodeoxycholate hydrate (–, absence; +, presence), and the GSLs were purified and subjected to thin-layer chromatography (TLC). The TLC plate was sprayed with orcinol reagent. (**b**) The uptake of enzymes by fibroblasts was analyzed as described under "Materials and Methods." Before enzyme treatment, fibroblasts were treated (+) or not treated (–) with 5 mmol/l M6P. Error bars represent means ± SD ($n = 4$) (**$P < 0.01$). (**c**) Cultured fibroblasts derived from the Tay-Sachs disease (TSD) patient were treated with WTHexA, WTHexB and the modified HexB, and then immunostaining for GM2 was performed as described under "Materials and Methods."

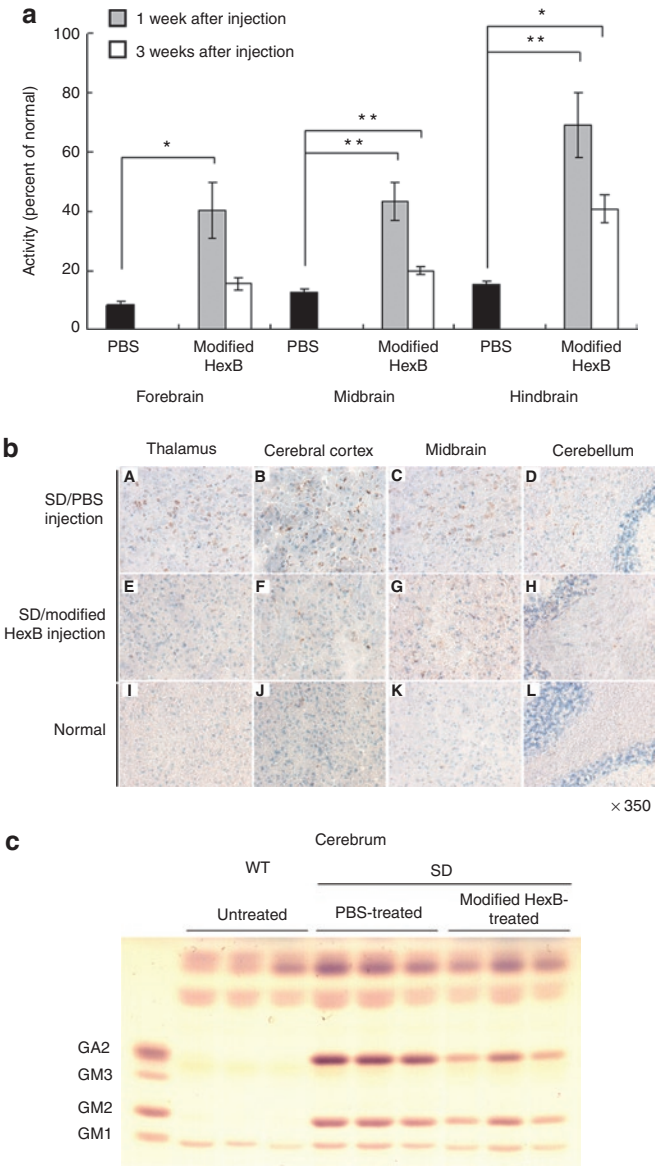

Figure 4 Hex activity and accumulated GSL level in brains from Sandhoff disease (SD) ($Hexb^{-/-}$) model mice treated with a single administration of the modified HexB. The modified HexB was administered (20,000 nmol/hour/25 µl) to the lateral ventricle of $Hexb^{-/-}$ mice, and then the mice were killed after 1 and 3 weeks after administration. (**a**) The Hex activity in an extract of each brain part (fore-, mid-, and hindbrain, respectively) of $Hexb^{-/-}$ mice was measured. Error bars represent means ± SD ($n = 3$) (*$P < 0.05$, **$P < 0.01$). (**b**) Immunohistochemical analysis of the accumulated GM2 in the brains of $Hexb^{-/-}$ mice was performed 3 weeks after administration of modified HexB was performed, and then counter-stained with hematoxylin. (**c**) Thin-layer chromatography analysis of GSLs was performed 1 week after administration of the modified HexB. PBS, phosphate-buffered saline; SD, Sandhoff disease; WT, wild type.

after injection. In the brains of SD mice treated with phosphate-buffered saline (PBS) as a control, marked GM2 accumulation was observed throughout the brain regions (**Figure 4b**A–D). In contrast, on treatment with the modified HexB, remarkable reductions of GM2 were observed in the thalamus and cerebral cortex (**Figure 4b**E,F), although it was less in the cerebellum (**Figure 4b**G,H).

To characterize the glycosphingolipids (GSLs) in the SD mice brains after the modified HexB treatment, thin-layer chromatography analysis was performed (**Figure 4c** and **Table 3**). Excessively accumulated GM2 and GA2 due to the deficiency of HexA activity were observed in the brains of SD mice. In the modified HexB-treated group, the levels of GM2 and GA2 in the brain had markedly decreased 1 week after injection, lower levels being maintained, compared to in control mice, for 3 weeks after injection. These results indicated that the modified HexB can degrade the accumulated substrates *in vivo*. We also examined

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Table 3 Comparison of the relative level of GSLs in the brain present in mouse brain

GSL	PBS-treated control	Modified HexB-treated
GM2		
Cerebrum	1.00	0.77 ± 0.02**
Cerebellum	1.00	0.57 ± 0.04*
GA2		
Cerebrum	1.00	0.66 ± 0.04**
Cerebellum	1.00	0.48 ± 0.04**

Abbreviations: HexB, β-hexosaminidase B; PBS, phosphate-buffered saline; SD, Sandhoff disease; TLC, thin-layer chromatography.
The relative levels of GM2 and GA2 extracted from mouse brains after enzyme treatment were determined by TLC. These values were calculated from the ratio of the band intensities on TLC and normalized on the basis of those for SD mice treated with PBS as controls. Mean ± SD.
**$P < 0.001$: PBS versus modified HexB, *$P < 0.05$: PBS versus modified HexB.

whether *icv* administered modified HexB distribute to periphery or not. One week after injection, the 4-MUGS-degrading activity in the liver of SD mice treated with modified HexB significantly increased to 363 ± 108.4 % of that of WT mice (**Supplementary Figure S2a**), and the levels of GM2 and GA2 in the liver had markedly decreased (**Supplementary Figure S2b** and **Supplementary Table S2**). These finding suggested that icv administration is effective for reducing the accumulated GSL levels not only in the brain, but also in the peripheral organs.

DISCUSSION

ERT is a clinically available treatment for LSDs involving visceral manifestations. However, it has several disadvantages, including high-frequency of administration, ineffectiveness for LSDs involving neurological symptoms and the production of neutralizing antibodies in LSD patients because of the continuous administration of relatively high doses of the enzyme drugs at above 1 mg protein/injection.[17]

In order to develop a novel therapeutic enzyme for overcoming the disadvantages of the present one, we performed the drug design of a genetically engineered human HexB for the future ERT for TSD and SD in this study. First, we designed a modified Hex β-subunit with partial amino acid substitutions compared to in the α-subunit on the basis of homology modeling. It was predicted that βD452 and βL453 should be changed to αN423 and αR424, to alter the substrate specificity, respectively, as well as the βRQNK (312-315) sequence corresponding to αGSEP (280-283), to introduce the α-specific loop structure for binding as to GM2AP into the β-subunit.

It was reported previously that αN423 and αR424 stabilize not only the negatively charged sialic acid moieties of GM2 but also neutral substrates,[18,19] and mutant HexA without the loop structure of αGSEP (280-283) exhibits no GM2-degrading ability in TSD fibroblasts because HexA can degrade GM2 through binding with GM2AP via the loop sequence specific for the α-subunit.[20]

Here, we demonstrated that the modified HexB produced by a CHO cell line stably expressing the engineered *HEXB* encoding the human Hex β-subunit bearing with the amino acid substitutions (R312G, Q313S, N314E, P315K, D452N, and L453R) had acquired the catalytic activities toward 4-MUGS and GM2 *in vitro* in the presence of a detergent. The modified HexB was also efficiently taken up by fibroblasts derived from a TSD patient via CI-M6PR and degraded the GM2 accumulated in lysosomes as the result that the incorporated enzyme could interact with GM2AP. On *icv* administration to SD model mouse, it was successfully incorporated into the brain parenchyma, restored the Hex activity, and cleaved the GM2 and GA2 accumulated in neural cells.

Recent experimental and clinical attempts to develop *icv* and intrathecal ERTs for LSDs with neurological manifestations[14–16] are expected to lead to central nervous system–directed ERTs using the CI-M6PR expressed on neural cells as a delivery target molecule that can be clinically applied for these diseases in the near future. The present modified HexB has several advantages for the future ERT for TSD and SD. First, it has HexA-like GM2-degrading activity to reduce the GM2 accumulated in the brain parenchyma on *icv* administration to SD mice. The *icv* ERT is also expected to have therapeutic effects on TSD and SD patients involving neurological symptoms.

Second, modified HexB is considered to be superior to WTHexA as for uptake by target cells expressing CI-M6PR on their cell surface because homodimeric HexB should have four M6P-carrying N-glycans per molecule whereas the heterodimeric WTHexA should have three. It was reported that the human Hex β-subunit contains two N-glycans carrying M6P but the α-subunit only contains one.[21] Akeboshi *et al.* reported that a higher number of M6P residues on N-glycans of a recombinant human HexA derived from a methylotrophic yeast *Ogataea minuta* strain causes more efficient uptake of the enzyme into TSD and SD cells, leading to improvement of the replacement effect.[22] These studies suggested that the number of M6P-type-N-glycans attached to a replacement enzyme contributes to the efficiency of uptake and the therapeutic effectiveness. Consistent with this evidence, we also demonstrated the more efficient uptake of the modified HexB by TSD fibroblasts and neural cells in the brain parenchyma of SD mice and recovery of Hex activity in comparison with WTHexA.

Third, the modified HexB retained extremely high stability at 37 °C in the presence of plasma similar to WTHexB, and maintained high Hex activity in the brains of $Hexb^{-/-}$ mouse during 3 weeks after *icv* injection. These enzymatic characters are also advantageous as to not only stable industrial production of the recombinant enzyme drug but also stability in the circulation system and in intracellular of the patient after administration. Previously, we founded that intracerebroventricularly administered enzyme can distribute to peripheral organs (data not shown). In fact, the modified HexB administered intracerebroventricularly to SD mouse distributed to periphery, and incorporated into the liver and then reduced the accumulated GSL levels. These evidences suggest that the modified HexB excreted from the CSF on *icv* ERT should contribute to recover the Hex activities in the visceral organs and ameliorate the peripheral manifestations.

Recently, it has become more important to avoid the immune response caused by repetitive administration of therapeutic enzymes on continuous intravenous ERT. In the case of Fabry disease, the clinical demand for an application of ERT for patients has been increasing, however, repeated administration of recombinant α-galactosidase to patients cause infusion reactions, mainly an allergic one, because most male Fabry disease patients do not have

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any α-galactosidase protein.[9,23] The induction of immune tolerance using an immunosuppressive regimen, cyclosporine and azathiopurine, improves the effectiveness of a recombinant enzyme,[24] although these reagents have side effects. From these aspects, we would like to propose and emphasize the clinical use of modified HexB instead of WTHexA to avoid the immune response to HexA containing α-subunit for future ERT for TSD patients who have the endogenous WTHexB. The evidence we obtained in this study suggests that the modified HexB should be clinically advantageous as a novel therapeutic enzyme, and that low-immunogenic *icv* and intravenous ERTs for TSD patients can be expected in the future.

MATERIALS AND METHODS

Materials. 4-MUG, M6P, nutrient mixture Ham's F-10, EX-CELL ACF CHO Medium, dimethyl sulfoxide, BCIP/NBT liquid substrate system and bovine serum albumin were purchased from Sigma (St Louis, MO). GenomeLab DCIT-Quick Start Kit, CEQ Separation Gel-LPAI and CEQ separation buffer were purchased from Beckman Coulter (North Harbor, CA). Wizard plus midipreps DNA purification kit, dNTPs, and hygromycin were purchased from Promega (Madison, WI). PepstatinA and leupeptin were purchased from Peptide Institute (Osaka, Japan). Quick gel extraction kit and purification kit were purchased from QIAGEN (Germantown, MA). 4-MUGS was purchased from Calbiochem (San Diego, CA). Fetal bovine serum was from Biological Industry (Tokyo, Japan). KOD plus polymerase was from TOYOBO (Osaka, Japan). DNA ligation kit ver. 1 was purchased from TaKaRa (Otsu, Japan). G418 was purchased from Wako (Osaka, Japan). Unifecter was purchased from B-Bridge International (Mountain View, CA). D_C protein assay kit was purchased from Bio-Rad (Hercules, CA). Viva pure mini spin column Q type was purchased from VIVA SCIENCE (Stonehouse, UK). Amicon ultra was purchased from Millipore (Carrigtwohill, Ireland). HiTrap DEAE HP, Phenyl HP and Concavalin A-Sepharose were purchased from GE Healthcare BioSciences (Piscataway, NJ and Uppsala, Sweden, respectively).

Mice. Sandhoff (*Hexb*$^{-/-}$) mice (C57BL/6x129sv) were kindly provided by Dr Proia (National Institutes of Health). Mice were bred by mating with WT C57BL/6 mice (SLC, Shimazu, Japan), and were housed under standard housing conditions approval of the Animal Care Committee of the University of Tokushima.

Cells. Cultured skin fibroblasts from a patient with TSD (F218), and a normal control subject (F592) were obtained with informed consent, and the study was approved by the ethical committee of our institution. The fibroblasts were cultured in Ham's F-10 medium containing 10% fetal bovine serum and antibiotics at 37 °C in a humidified incubator continuously flushed with a mixture of 5% CO_2–95% air.

Antibodies. Mouse monoclonal antibodies were kindly donated by Dr Tai (Department of Tumor Immunity, The Tokyo Metropolitan Institute of Medical science, Tokyo, Japan); GMB28 (anti-GM2; immunoglobulin M) against GM2.

Design of a modified human Hex β-subunit. To determine the amino acid residues comprising the active site pocket of the Hex β-subunit, the solvent accessible surface area value of each amino acid residue was calculated both in the apo form of Hex β-subunit and its complex with the substrate analog inhibitor. Then, the amino acid residues whose solvent accessible surface area values decrease from the apo form to the complex are defined as amino acid residues comprising the pocket. The calculations were conducted using the program ACCESS,[25] which is an implementation of the Lee and Richards algorithm.[26] The three-dimensional coordinates of the human Hex β-subunit was obtained from the Protein Data Bank (protein Databank code: 1now).[27]

To explore the difference between the Hex α- and β-subunits, comparison of their protein sequences was performed using BLAST.[28] The protein sequences of the Hex α- and β-subunits were obtained from the ExPASy Homepage (http://au.expasy.org/).

Structural modeling of a modified Hex β-subunit was performed using molecular modeling software TINKER.[29–32] Again, the crystal structure of the Hex β-subunit (protein Databank code: 1now) was used as a template. Then, energy minimization was performed and the root-mean-square gradient value was set at 0.05 kcal/mol/Å.

Construction of a vector plasmid and establishment of CHO cell lines stably expressing the modified HEXB. DNA fragments 1, 2, and 3 were amplified using primer sets as summarized in **Supplementary Table S1**, and the pCXN$_2$-*HEXB-Neo* vector previously constructed in our laboratory,[33] as a template, under the conditions indicated in **Supplementary Table S1**. Fragments 4 and 5 were amplified using sets of amplified fragments (fragment 1 and 2, and 2 and 3, respectively) as templates by two-step PCR.

Fragment 5 was digested with BamHI and BglII, and then ligated into a pFLAG-CMV$_2$-*Neo* derivative digested with the same endonucleases. The resultant vector plasmid was designated as pFLAG-CMV$_2$-*modified HEXB*-Neo. Fragment 4 was digested with BamHI and XhoI, and then ligated into pFLAG-CMV$_2$-*modified HEXB*-Neo digested with the same endonucleases. The resultant vector plasmid was digested with *Xho*I and *Bgl*II, and then ligated into the pCXN$_2$-*Neo* vector digested with the same endonucleases. The resultant vector plasmid was designated as pCXN$_2$-*modified HEXB-Neo*.

One day before transfection, seed 2.0–4.0 × 10^5 cells per ϕ 60 mm dish in 2 ml of Ham's F-10 without antibiotics to attain 80–90 % confluence at a time of transfection. For a transfection sample, prepare DNA-Lipofectamine 2000 complexes as follows: dilute 4 μg DNA and 20 μg Lipofectamine 2000 in 250 μl Ham's F-10 without serum, respectively, and mix gently and then let for 20 minutes at room temperature. Cells washed with serum-free medium, added 1.5 ml of medium and then added 500 μl of DNA-Lipofectamine 2000 complexes. Incubate the cells at 37 °C in a humidified incubator with 5% CO_2 for 24 hours and add 2 ml of medium containing serum. Neomycin-resistant cell lines were selected by the limited dilution method, and designated as CHO/*modified HEXB* cell lines.

Purification of recombinant Hex isozymes. Each CHO cell line stably expressing and secreting the WTHexA, WTHexB, or modified HexB, respectively, was cultured in EX-CELL ACF CHO medium in an incubator containing 5% CO_2. CM derived from each cell line was collected. For acid precipitation, HCl added and adjusted to pH 4.5 and gently rotated at 4 °C for 2 hours and then centrifuged at 6,000*g* for 30 minutes at 4 °C. The resultant supernatant corrected and NaOH added to a final pH at pH 7.0. For lectin affinity chromatography, $CaCl_2$ and $MnCl_2$ were added to the final concentrations of 1 mmol/l. CM was applied to a Concavalin A-Sepharose column equilibrated with 1 mmol/l $CaCl_2$/1 mmol/l $MnCl_2$/20 mmol/l HEPES (pH 7.2) (HEPESB). After washing the column with 50 column volumes of 0.5 mol/l NaCl/HEPESB, the bound proteins were eluted with 0.5 mol/l methyl α-D-mannopyranoside/0.1 mol/l NaCl/HEPESB. The eluate containing Hex activity as collected, and then $(NH_4)_2SO_4$ was added to a final concentration of 1 mol/l. The eluate containing Hex activity was applied to a HiTrap Phenyl HP column. Bound proteins were eluted by increasing the proportion of sodium phosphate buffer (NaPB). The eluate containing Hex activity was applied to a HiTrap DEAE HP column. Flow-through fractions containing WTHexB and modified HexB, and elution fractions containing WTHexA were collected. The eluate containing Hex activity was collected and dialyzed against PBS (pH 7.4), and then concentrated with Amicon ultra (30,000 MWCO; Millipore, Billerica, MA). The purified Hex isozymes were stored at −80 °C before use.

Characterization of recombinant Hex isozymes and analysis of processing of the β-subunit. The cultured CHO cell line stably expressing the modified Hex β-subunit gene on ϕ 60 mm dishes were grown to

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confluency. After washing with PBS, they were harvested, and then suspended in 10 mmol/l NaPB containing protease inhibitors. The cell suspensions were sonicated, and then centrifuged at 13,200*g* for 15 minutes at 4 °C, the resultant supernatants being stored as cell extracts. To characterize the intracellular and secreted Hex isozymes, aliquots of the cell extracts and CM were subjected to native-PAGE on a 7.5% acrylamide gel. To analyze the intracellular processing of the β-subunit, aliquots of HexB fractions of cell extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, on a 10% acrylamide gel. The proteins were transferred to polyvinylidine difluoride membranes. On immunoblotting, each membrane was incubated with 50% (v/v) Blocking One (Nacalai Tesque, Japan) in Tris-buffered saline (pH 7.4) overnight at 4 °C. The membrane was treated with anti-human HexA polyclonal antibodies[33] diluted with Blocking One/Tris-buffered saline (1:1,000 dilution) at room temperature. After washing, biotin-conjugated anti-rabbit immunoglobulin G antibody (1:1,000 dilution) was used as secondary antibodies. After washing, the membrane was treated with alkaline phosphatase-conjugated streptavidin (1:1,000 dilution) for 1 hour. The protein bands were visualized with the BCIP/NBT liquid substrate kit system. A kinetic experiment was performed with the modified HexB to determine the V_{max} and K_m values using human WTHexA and WTHexB as controls.

In vitro *stability of the modified HexB.* Each enzyme was added to 10 mmol/l NaPB (pH 6.0) containing 30% murine plasma, followed by incubation at 37 °C. Taking the Hex activity at time 0 as 100%, the stabilities of each enzyme was calculated as the ratio (%) of the enzyme activity at the incubation time point to the value at time zero.

Degradation of GM2* in vitro *by modified HexB. Micellar form of GM2 was incubated with each Hex isozyme (modified HexB and WTHexA: 3,000 nmol/hour 4-MUGS-degrading activity/well; WTHexB: 3,200 nmol/hour 4-MUG-degrading activity/well, respectively) in the presence or absence of 2 mmol/l sodium taurodeoxycholate hydrate in 200 µl of 10 mmol/l citrate buffer (pH 4.2) at 37 °C for 24 hours. After the incubation, the reaction was stopped by heating the tube in a bath of boiling water for 3 minutes, GSLs were isolated using a C18 Sep-Pak Cartridge. Aliquots of samples were spotted on a Silica gel plate and developed with chloroform/methanol/0.2 % $CaCl_2$ (60:40:90 (v/v)). To reveal GSLs, the thin-layer chromatography plate was sprayed with orcinol reagent and heated at 120 °C for 5 minutes.

Assay for enzyme replacement effects of the modified HexB on TSD fibroblasts. A fibroblastic cell strain (F218) from a TSD patient was seeded onto a type I collagen-coated 96-well plate (2×10^3 cells/well), and then the isolated WT-, modified HexB and WTHexA fractions derived from CM (modified HexB and WTHexA: 4-MUG-degrading activity 1,500 nmol/hour/well; WTHexB: 4-MUG-degrading activity 3,200 nmol/hour/well, respectively) were added, and followed by incubation for 3 days. The medium was removed, and the cells were washed with PBS and then treated with NaPB containing 1% NP-40/protease inhibitors. The microplate was vibrated on an EM-36 Micro Mixer for 1 hour at 4 °C, following pipetting, and then the intracellular activities were assayed. In some experiments, M6P was added to a final concentration of 5 mmol/l, and followed by incubation for 1 hour prior to the addition of an enzyme fraction. The TSD (F218) cells were seeded onto a type I collagen-coated 96-well plate (3×10^3 cells/well), and then the isolated WTHexB, modified HexB and WTHexA fractions derived from CM (modified HexB and WTHexA: 1,500 nmol/hour 4-MUG-degrading activity/well; WTHexB: 3,200 nmol/hour 4-MUG-degrading activity/well, respectively) were added, and followed by incubation for 3 days. F218 cells were seeded onto 8-well Lab-Tek chamber slides (Nunc, Rochester, NY). After administration of the WTHexB, modified HexB and WTHexA fractions to the cultured media, the fibroblasts were incubated for 3 days and then fixed with cold 4% paraformaldehyde. After washing with PBS, intracellular GM2 was detected by anti-GM2 mouse monoclonal antibodies according to the method described previously.[34] The specimens were viewed under a confocal fluorescent microscope.

Enzyme assays. Hex isozyme activities in cell extracts and CM toward 4-MUG and 4-MUGS were measured in 30 µl of 0.1 mol/l sodium citrate buffer (pH 4.5 and 4.2, respectively) by incubation at 37 °C for 30 minutes followed by addition of 375 µl of a 0.2 mol/l glycine-NaOH (pH 10.7). K_m and V_{max} value were determined by the method of Lineweaver and Burk plot at various substrate concentrations (4-MUG, 0.0625–2 mmol/l and 4-MUGS, 0.0625–4 mmol/l, respectively).[35] Protein levels were assayed with the D*c* protein assay kit with bovine serum albumin as a standard.

Intracerebroventricular administration of recombinant modified HexB and analysis of the enzyme replacement effect. SD mice at the age of 10 weeks were anesthetized with sodium 5-ethyl-5-(1-methylbuthyl) barbiturate (32.4 mg/kg body weight). Modified HexB (20,000 nmol/hour 4-MUG-degrading activity/25 µl) as well as PBS, as an enzyme-untreated control, in a total volume of 25 µl, were injected (0.5 mm caudally from the bregma, 1.0 mm laterally from the central line, and 2.0 mm from the top of the skull) into each ventricle with a two-step needle attached to a glass syringe. After the injection, the needle was held at the site for 1 minute to prevent reverse flow. One and three weeks after injection, $Hexb^{-/-}$ mice were killed, and their brains and livers were obtained after PBS perfusion. The brains were cut into three sections (fore-, mid-, and hindbrain, respectively). The sections were homogenized by sonication in 10 mmol/l NaPB containing protease inhibitors. After centrifugation at 13,200*g*, the supernatants were collected as tissue extracts. For immunohistochemical analysis, each brain hemisphere was embedded in O.C.T. compound and frozen at −30 °C. Slices (10 µm thick) were prepared with a cryostat, placed on silane-coated glass slides, and then air-dried for 2 hours. The brain slices were fixed with 4% paraformaldehyde/PBS for 2 days at 4 °C. After washing with PBS, the specimens were treated with 0.03% H_2O_2/PBS and washed with PBS. They were then treated with GMB28 mouse monoclonal antibodies diluted with 5% goat serum/1% bovine serum albumin/PBS (1:20 dilution) overnight at 4 °C. After washing with 0.05% Tween20/PBS, the brain slices were treated with anti-mouse immunoglobulin M antibodies diluted with 5% goat serum/1% bovine serum albumin/PBS (1:1,000 dilution) for 1 hour at room temperature. After washing, the brain slices were treated with horseradish peroxidase-conjugated streptavidin for 30 minutes at room temperature. After washing, the immuno-positive cells were visualized with diaminobenzidine (DAB) reagent (0.012% H_2O_2/0.0133% DAB/50 mmol/l Tris-HCl pH 8.0). The specimens were counter-stained with hematoxylin.

Analysis of the glycosphingolipid accumulated in mouse brains and livers by thin-layer chromatography. The extraction of glycolipids was performed as previously described.[36] For determination of GSL pattern, the silica gel plate was heated at 120 °C for 30 minutes on the hot plate before sample spotting. Aliquots of samples were spotted on a silica gel plate and developed with chloroform/methanol/0.2% $CaCl_2$ (55:45:10 (v/v)) for 50 minutes. To reveal GSLs, the silica gel plate was sprayed with orcinol reagent and heated at 120 °C for 5 minutes on the hot plate.

Statistical analysis was performed using Turkey's *post hoc* test.

SUPPLEMENTARY MATERIAL

Figure S1. Hex activity in the brain from SD mice treated with a single administration of enzymes.

Figure S2. Hex activity and accumulated GSL level in the liver from SD mice treated with a single administration of the modified HexB.

Table S1. Primer sets and PCR conditions.

Table S2. The relative levels of GM2 and GA2 extracted from mouse livers after enzyme treatment were determined by TLC.

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