II-positive ones act in antigen presentation; (2) activated microglia phagocytose damaged cells; (3) they produce substances such as the pro-inflammatory cytokines TNF-alpha and IL-6, which are pleiotropic and act either as neurotoxins or as neuroprotective agents, as well as neurotoxic substances, i.e., ROS, nitric oxide (reactive nitrogen species, RNS), and glutamate; and (4) they also produce neurotrophic substances such as the neurotrophin BDNF and cytokines that act neuroprotectively.

In the normal brain, many Kp1-positive resting microglia, which are non-toxic, are seen in the substantia nigra and putamen. In the PD ones, however, a large number of MHC class II-positive ramified, activated microglia are found in these regions compared with their number in normal controls. Furthermore, the number of MHC class II-positive microglia in the putamen in PD increases as the stage of PD advances. In the early stages of PD, MHC class II-positive microglia in the putamen and substantia nigra are associated with intensively TH-positive DA neurites showing no signs of degeneration. In the advanced stages, however, MHC class II-positive microglia in these areas are found with damaged TH-positive neurons and neurites. These results suggest that activated microglia in the substantia nigra and putamen may be non-toxic/neuroprotective or neurotoxic, depending on the stage of PD.

Imamura *et al.* (2003) observed that the number of MHC class II-positive activated microglia was significantly higher not only in the substantia nigra and putamen but also in various other brain regions such as the hippocampus, transentorhinal cortex, cingulate cortex, and temporal cortex in PD brains than in the control ones. Imamura *et al.* (2005) also observed activated microglia in the nigro-striatum and hippocampus in dementia with Lewy bodies (DLB), and compared them with those in PD. Neuronal degeneration in the putamen was observed in both PD and DLB, whereas neuronal loss in the hippocampus was observed in DLB but not in PD without dementia. In normal controls, neuronal loss, activated microglia, and alpha-synyclein-positive cells were not observed in the hippocampus (CA2/3 region), and neurons were strongly BDNF positive. In the hippocampus (CA2/3 region) in PD, the number of MHC class II-positive microglia was increased, which cells were also positive for ICAM-I (CD54), LFA-1, TNF-alpha, and IL-6. Alpha-synuclein-positive cells were also observed. BDNF-stained neurons were only slightly decreased in number in PD compared with those in controls. In the hippocampus (CA2/3 region) in DLB, the numbers of MHC class II (CR3/43)-positive microglia and alpha-synuclein-positive microglia, and alpha-synuclein-positive neurons were greater than those in PD, and the neurons were very weakly stained with anti-BDNF. These immunohistochemical data on the hippocampus (CA 2/3 region) indicate that the number of activated microglia increases in both PD and DLB and that the content of neurotrophic BDNF protein is markedly decreased in DLB but not in PD. Furthermore, in the hippocampus, mRNA levels of IL-6 and TNF-alpha were increased in both PD and DLB compared with the control levels; whereas the mRNA level of BDNF was greatly decreased in DLB, as compared with that in PD or normal controls. These different changes in the levels of mRNA and protein of BDNF, IL-6, or TNF-alpha in the hippocampus and putamen between PD and DLB suggest that activated microglia in these brain regions in PD and DLB are different in their properties and may secrete different kinds and different amounts of cytokines and neurotrophins such as BDNF and IL-6.

As other evidence supporting this concept of the presence of non-toxic/neuroprotective and neurotoxic microglia, we (Sawada *et al.*, 2006) separated two subsets of microglia from normal mouse brain by cell sorting based on profiles of intracellular ROS production induced by phorbol myristate acetate (PMA) stimulation: one subset of microglia producing a large amount of ROS and the other, just a minute amount of ROS. Furthermore, we obtained two cell lines of microglia, Ra2 cells and 6–3 cells, by spontaneous immortalization of mouse microglia in primary cultures. The Ra2 microglia cell line did not produce ROS upon PMA stimulation, whereas the 6–3 one produced ROS in large amounts in response to this stimulant. When co-cultured with N18 neuronal cells, Ra2 cells were neuroprotective, whereas 6–3 cells were neurotoxic. Furthermore, Sawada and co-workers found in a cell culture experiment a toxic change in activated microglia from neuroprotective to neurotoxic, caused by transduction of the cells with a lenti virus vector carrying HIV-1 Nef cDNA (Vilhardt *et al.*, 2002). It is speculated that a similar toxic change in activated microglia may occur *in vivo* in the PD brain as the second step, one caused by other factors such as invasion of serum, viruses, toxic substances, or inflammatory cells in some of the neuroprotective microglia in a specific brain regions, i.e., the nigro-striatum in PD. As a result of this toxic change, large amounts of cytotoxic factors such as ROS, NO, and RNS produced by NADPH oxidase, myeloperoxidase, cyclooxygenase 2 (COX 2), or nitric oxide synthase may promote the observed neuronal loss. The presence of reactive microglia in the substantia nigra years after MPTP exposure was detected in experimental monkeys (McGeer *et al.*, 2003) and in human patients (Langston *et al.*, 1999). These reactive microglia might have been produced by a toxic change in response to the exposure to MPTP. These results also suggest that a variety of causative agents of sPD, disappearing after having instituted long-lasting inflammatory changes, might cause progression of the disease.

Based on the results described earlier, Sawada *et al.* (2006) recently proposed a hypothesis of two-step activation of microglia *in vivo* in the PD brain. The observation on activated microglia associated with non-degenerating neurons and neurites in various brain regions such as the hippocampus in the early stage of PD suggests that microglia activated by the initiating factors of PD may be at first non-toxic and act for neuroprotection by producing neurotrophins, neurotrophic cytokines, and antioxidant substances in the first step. However, a toxic change in the activated microglia may occur as the second step to promote progression of the disease.

CONTRIBUTION OF JULIE AXELROD TO PD RESEARCH

Aside from his other numerous accomplishments, Dr. Julie Axelrod has made many great contributions also to PD research. One great contribution was his discovery of the reuptake of neurotransmitter catecholamines into the pre-synaptic nerve endings via membrane transporters and then from the cytoplasm to the synaptic vesicles via vesicular transporters (Axelrod *et al.*, 1959). This discovery provided a general principle for the termination of neurotransmission, and led to the identification of neurotransmitter transporters such as DA transporter (DAT) and NA transpoter (NAT), and vesicular monoamine transporters (VMAT), and to the

development of innovative drugs such as serotonin noradrenaline reuptake inhibitors as anti-depression drugs and of new diagnostic methods such as molecular imaging by PET (*p*ositron *e*mission *t*omography) or SPECT (*s*ingle *p*hoton *e*mission *c*omputed *t*omography) of synaptic function. Another great contribution is his discovery of catechol O-methyl transferase (COMT; Axelrod, 1957). Inhibitors of COMT are of great importance to the L-DOPA therapy of PD in combination with MAO B inhibitors such as deprenyl.

CONCLUSIONS AND FUTURE PROSPECTS

sPD is thought to be caused by the combination of a susceptible genetic background and various environmental factors. The biochemical analysis of postmortem brain from PD patients and neurotoxin-induced animal models indicates mitochondrial dysfunction and oxidative stress to be important. On the other hand, the causative genes of fPD indicate the accumulation of misfolded proteins due to UPS dysfunction to be important. It should be noted that both mitochondrial dysfunction and UPS dysfunction may be related to each other, and may trigger a common signal transduction pathway to programmed cell death. There are 2 types of programmed cell death, i.e., apoptosis and autophagy. Alpha-synuclein is degraded by both the ubiquitin–protesome pathway and the autophagy–lysosome pathway (Webb *et al.*, 2003). Much data on the pathogenesis of PD support the programmed cell death mechanism by apoptosis. However, this still remains controversial. The process of neuroinflammation may also be important, especially for the progression of PD. Sawada *et al.* (2006) has proposed a hypothesis of two-step activation of microglia in the brain and their toxic change in PD patients. In order to confirm this hypothesis, the following points remain to be proved: (1) the first and second causative stimuli must be identified; (2) a toxic change should be confirmed to occur *in vivo* in the nigro-striatum in PD models. A stimulus such as MPTP toxicity may directly produce degeneration of DA cells, and some signal from degenerating DA neurons may trigger activation of microglia, which may, due to the toxic change for producing neurotoxic cytokines, promote cell death of DA neurons, perpetuating a vicious circle. It is also possible that in fPD damaged DA neurons may send unknown signals to microglia to activate them. Thus, activated microglia producing neurotoxic cytokines may promote the progression of the disease in both sPD and fPD.

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Molecular Pathologies of and Enzyme Replacement Therapies for Lysosomal Diseases

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Abstract: Lysosomal diseases comprise a group of inherited disorders resulting from defects of lysosomal enzymes and their cofactors, and in many of them the nervous system is affected. Recently, enzyme replacement therapy with recombinant lysosomal enzymes has been clinically available for several lysosomal diseases. Such enzyme replacement therapies can improve non-neurological disorders but is not effective for neurological ones. In this review, we discuss the molecular pathologies of lysosomal diseases from the protein structural aspect, current enzyme replacement therapies, and attempts to develop enzyme replacement therapies effective for lysosomal diseases associated with neurological disorders, i.e., production of enzymes, brain-specific delivery and incorporation of lysosomal enzymes into cells.

Keywords: Lysosomal enzyme, lysosomal disease, Tay-Sachs disease, Sandhoff disease, Fabry disease, protein structure, enzyme replacement therapy, drug delivery.

INTRODUCTION

Lysosomes are cytoplasmic vesicles that contain lysosomal enzymes and their cofactors including activators and stabilizing proteins. Many lysosomal enzymes are exohydrolases that are involved in the degradation of cellular materials including glycoconjugates. Gene defects of lysosomal enzymes and their cofactors cause "lysosomal diseases", which result in the accumulation of undegraded substrates in lysosomes as reviewed by Sakuraba [1]. Lysosomal diseases comprise a group of more than 40 different disorders as shown in Table **1**. Although the clinical presentations of lysosomal diseases are very heterogeneous, many of them involve neurological disorders with the exceptions of Gaucher disease type 1, Fabry disease cardiac type, Pompe disease, and so on [1,2].

Efforts have been made to develop therapies for lysosomal diseases. Although various experimental approaches including bone marrow transplantation, enzyme replacement, substrate-depletion and normal gene transfer have been made [3-7], enzyme replacement therapy is thought to be clinically effective for lysosomal diseases at present. Recombinant enzymes for enzyme replacement therapy have been produced in cultured mammalian cells including Chinese hamster ovary (CHO) cells and human fibroblasts, and some of them can be produced in the milk of mammals. They are clinically available for enzyme replacement therapy for Gaucher disease involving hepatosplenomegaly, anemia, thrombocytopenia, bone disorders (types 1, 2 and 3), psychomotor delay, muscular weakness, hypotonia, pseudo bulbar palsy, laryngeal spasm, supranuclear gaze palsy and strabismus (types 2 and 3) [8,9], Fabry disease involving pain, angiokeratoma, hypohidrosis, corneal opacities, vascular disorders, renal involvement (the classic type) and cardiac involvement (the classic type and the cardiac type) [10-13], mucopolysaccharidosis (MPS) I involving corneal opacities, dysostosis multiplex, organomegaly and mental retardation [14], Pompe disease involving cardiomegaly, hepatomegaly (the classic type) and muscular weakness and hypotonia (the classic type and the late onset type [15,16], and MPS VI involving corneal opacities and dysostosis mutiplex [17] as shown in Table **2**. Furthermore, an application for MPS II involving dysostosis multiplex (the severe type and the mild type) and mental retardation (the severe type) [18] has been submitted.

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Table 1. Lysosomal Diseases

Disease	Defect
GM1 gangliosidosis	β-galactosidase
GM2 gangliosidoses	
Tay-Sachs disease (B-variant)	β-hexosaminidase A
Sandhoff disease (O-variant)	β-hexosaminidases A and B
GM2 activator deficiency (AB-variant)	GM2 activator protein
Fabry disease	α-galactosidase
metachromatic leukodystrophy	arylsulfatase A
Krabbe disease (globoid-cell leukodystrophy)	galactocerebrosidase
Gaucher disease	glucocerebrosidase
Niemann-Pick disease types A and B	sphingomyelinase
Farber disease	ceramidase
Walman disease and cholesteryl ester storage disease	acid lipase
Pompe disease	α-glucosidase
fucosidosis	α-fucosidase
α-mannosidosis	α-mannosidase
β-mannosidosis	β-mannosidase
sialidosis	lysosomal sialidase
aspartylglucosaminuria	aspartylglucosaminidase
Schindler disease and Kanzaki disease	α-*N*-acetylgalactosaminidase
mucopolysaccharidoses	
type I (Hurler, Scheie)	α-L-iduronidase
type II (Hunter)	iduronate sulfatase
type IIIA (Sanfilippo A)	heparan *N*-sulfatase
type IIIB (Sanfilippo B)	α-*N*-acetylglucosaminidase
type IIIC (Sanfilippo C)	acetyl CoA:α-glucosaminide acetyltransferase
type IIID (Sanfilippo D)	*N*-acetylglucosamine 6-sulfatase
type IVA (Morquio A)	galactose 6-sulfatase
type IVB (Morquio B)	β-galactosidase
type VI (Maroteaux-Lamy)	arylsulfatase B
type VII (Sly)	β-glucuronidase
I-cell disease and pseudo-Hurler polydystrophy	UDP-*N*-acetylglucosamine: lysosomal enzyme *N*-acetylglucosamine 1-phophotransferase
prosaposin deficiency	prosaposin
metachromatic leukodystrophy-like storage disease (saposin B deficiency)	saposin B
Gaucher-like disease (saposin C deficiency)	saposin C
Galactosialidosis	protective protein/cathepsin A
Niemann-Pick disease type C	NPC1, NPC2
multiple sulfatase deficiency	arylsulfatases A, B and C
neuronal ceroid lipofuscinosis	
infantile type	lysosomal thioesterase
classical late infantile type	pepinase
juvenile type	lysosomal membrane protein
adult type	at least eight genes are involved
Salla disease and infantile free sialic acid storage disease	sialin

Table 2. Recombinant Lysosomal Enzymes for Enzyme Replacement Therapy

Enzyme	Drug	Targeted Disease	Company	Stage (Country)
glucocerebrosidase	imiglucerase	Gaucher disease	Genzyme	Approved (EU, USA, Japan)
glucocerebrosidase	GA-GCB	Gaucher disease	Shire	Phase I/II (-)
α-galactosidase	agalsidase beta	Fabry disease	Genzyme	Approved (EU, USA, Japan)
α-galactosidase	agalsidase alpha	Fabry disease	Shire	Approved (EU)
α-iduronidase	laronidase	MPS I	Biomarin/Genzyme	Approved (EU, USA)
α-glucosidase	algucosidase alpha	Pompe disease	Genzyme	Approved (EU, USA)
acid sphingomyelinase	-	Niemann-Pick disease B	Genzyme	Preclinical (-)
iduronate-2-sulfatase	idurosulfase	MPS II	Shire	Submitted (EU, USA)
arylsulfatase B	aryplase	MPS VI	BioMarin	Approved (EU, USA)

However, the present enzyme replacement therapies are effective for the improvement of non-neurological disorders but not for that of neurological disorders. Intravenously administered enzymes cannot be incorporated into the central nervous system because of the blood-brain barrier.

To develop enzyme replacement therapies for lysosomal diseases affecting the nervous system, understanding of their molecular pathologies and targeting of lysosomal enzymes to neuronal tissues are required.

In this review, we discuss the molecular pathologies of lysosomal diseases from the protein structural aspect and attempts to develop enzyme replacement therapies effective for lysosomal diseases associated with neurological disorders.

MOLECULAR PATHOLOGIES OF LYSOSOMAL DISEASES

We describe here the molecular pathologies of GM2 gangliosidoses including Tay-Sachs disease (B-Variant) and Sandhoff disease (O-Variant) as models of lysosomal diseases associated with neurological disorders. Lysosomal β-hexosaminidase (Hex, EC 3.2.1.52) is a glycosidase that catalyzes the hydrolysis of terminal *N*-acetylhexosamine residues at the non-reducing ends of oligosaccharides of glycoconjugates [19,20]. There are two major Hex isozymes in mammals including man, Hex A (αβ, a heterodimer of α- and β-subunits) and Hex B (ββ, a homodimer of β-subunits), and a minor unstable isozyme, Hex S (αα, a homodimer of α-subunits). All these Hex isozymes can cleave off terminal β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) residues, while only Hex A and Hex S prefer negatively charged substrates and cleave off the terminal *N*-acetylglucosamine 6-sulfate residues in keratan sulfate. Hex A is essential for cleavage of the GalNAc residue from GM2 ganglioside in co-operation with GM2 activator protein [19-21].

Tay-Sachs disease and Sandhoff disease are autosomal recessive GM2 gangliosidoses caused by mutations of *HEXA*, which encodes the Hex α-subunit on chromosome 15q 23-24, and *HEXB*, which encodes the Hex β-subunit on chromosome 5q13, respectively [19,20]. The genes exhibit sequence homology, and the gene products exhibit 57% similarity in amino acid sequence. In Tay-Sachs disease, a genetic defect of *HEXA* causes a deficiency of Hex A with excessive accumulation of GM2 ganglioside mainly in the nervous system including neurons of the cerebrum, cerebellum, spinal cord, dorsal root ganglion and visceral organs, resulting in progressive neurological disorders. In Sandhoff disease, an inherited defect of *HEXB* leads to simultaneous deficiencies of Hex A and Hex B with accumulation of GM2 ganglioside in the nervous system and of oligosaccharides carrying terminal GlcNAc residues at their non-reducing ends, resulting in systemic manifestations including hepatosplenomegaly as well as neurological manifestations.

Tay-Sachs disease and Sandhoff disease exhibit a spectrum of clinical phenotypes ranging from a severe infantile form to a milder late onset form, and many mutations have been identified for each gene [19,20]. Patients with the severe infantile form of GM2 gangliosidoses develop progressive psychomotor delay, muscular weakness, hypotonia, visual disturbance, cherry-red spot, seizures, and macrocephaly. Patients with the milder late-onset form of GM2 gangliosidoses develop dystonia, ataxia, incoordination, muscle wasting and weakness [19,20]. The incidence of Tay-Sachs disease is predicted to be 1 in 3,900 births in Jewish people and 1 in 320,000 births in non-Jewish people. On the other hand, the incidence of Sandhoff disease is deduced to be 1 in 1,000,000 births in Jewish people and 1 in 309,000 births in non-Jewish people [19]. Mutations in the GM2 activator protein gene *(GM2A)* result in a rare form of GM2 gangliosidosis, GM2 activator deficiency (AB-Variant) exhibiting the same clinical manifestations as the severe form of Tay-Sachs disease and Sandhoff disease [19,20].

Recently, the crystal structure of human Hex B was determined by Mark *et al.* [22] and then by Maier *et al.* [23]. This information prompted us to examine Tay-Sachs disease and Sandhoff disease from the protein structural aspect.

1. Three-Dimensional Structure of β-Hexosaminidase

According to the reports of Mark *et al.* [22] and Maier *et al.* [23], the β-subunit of Hex comprises two domains (domain I and domain II). Domain I has an α/β topology, and domain II is folded into a $(\beta/\alpha)_8$-barrel with the active site pocket at the *C*-termini of the β-strands. An extrahelix that follows the eighth helix of the $(\beta/\alpha)_8$-barrel is located between domain I and the barrel structure. A structural model of human Hex A (αβ heterodimer) has been constructed on

Table 3. Human β-Hexosaminidase α- and β-Subunits

	α-Subunit	β-Subunit
Signal peptide	Met1-Ala22	Met1-Ala42
Domain I	Leu23-Pro165	Ala43-Pro198
Domain II	Arg166-Thr529	Arg199-Met556
Processing site	Ser75-His88	Phe108-Lys121, Arg312-Lys315
Catalytic site	Asp207, His262, Glu323	Asp240, His294, Glu355
Disulfide bond	Cys58:Cys104, Cys277:Cys328, Cys505:Cys522	Cys91:Cys137, Cys309:Cys360, Cys534:Cys551
Glycosylation site	Asn115, Asn157, Asn295*	Asn84*, Asn142, Asn190, Asn327*

*Asn residue linked mannose undergoing glycosyl phosphorylation.

the structure of human Hex B by means of homology modeling method [24]. The human Hex α- and β-subunits have corresponding catalytic sites and three disulfide bonds. The α-subunit is predicted to have one processing site and three glycosylation sites, and the β-subunit two processing sites and four glycosylation sites (Table 3).

2. Structural Defect in Tay-Sachs Disease

The modeled structure of the wild-type Hex A (αβ heterodimer) and localization of representative amino acid substitutions (R170W, R178H, W420C, C458Y, L484P, R499C/H, and R504C/H) are shown in Fig. **1**. Nine mutant structural models due to specific missense mutations were constructed, and compared with the wild-type model [24].

Among the mutations, R178H is deduced to affect the structure of the active site directly. The R178 residue is located close to the active site and is involved in substrate binding. R178H results in substitution of H for R178, which is an important residue for substrate binding. Moreover, R178H is thought to cause a conformational change of amino acid

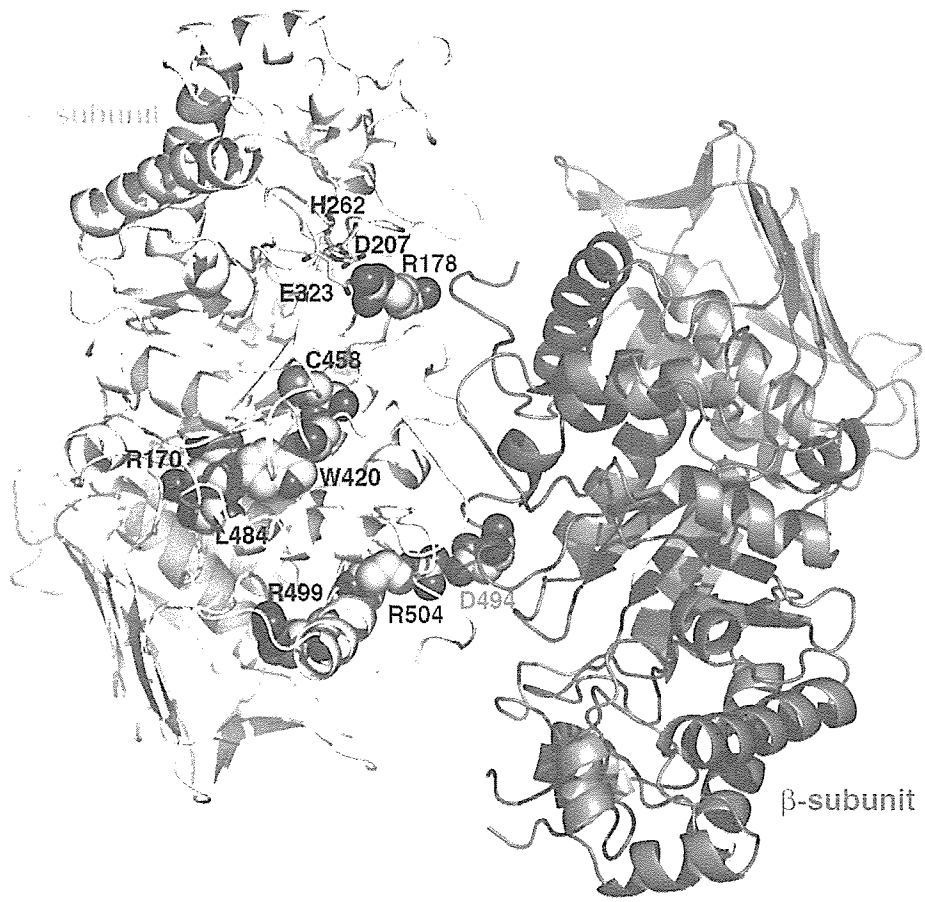

Fig. (1). Three-dimensional structure of human Hex A and residues involved in amino acid substitutions in the α-subunit.

A structural model of Hex A (αβ heterodimer) was constructed. Residues involved in the catalytic triad (D207, H262 and E323) are presented as ball-and-sticks models. Residues involved in amino acid substitutions in the α-subunit (R170, R178, W420, C458, L484, R499 and R504) and D494 in the β-subunit, which binds to R504 in the α-subunit, are presented as space-filling models.

residues responsible for the enzyme activity. The amino acid substitution is supposed to affect Hex A activity directly and to increase the apparent Km value for a substrate. The apparent Km value of this mutant was estimated to be 30 times higher than that in controls [24]. The R178H mutation has been identified in patients with B1-Variant GM2 gangliosidosis [25-29]. This variant form is known to be an unusual biochemical phenotype, and B1-Variant patients exhibit both Hex A and Hex B activities, as determined with 4-methylumbelliferyl *N*-acetyl-β-D-gluco-saminide (4-MUG), but the mutant Hex A cannot hydrolyze an artificial substrate, 4-methylumbelliferyl 6-sulfo-*N*-acetyl-β-D-glucosaminide (4-MUGS) [19,20]. Brown *et al.* [30] expressed a mutant Hex B with this mutation in COS cells, and confirmed that the mutant enzyme was catalytically inactive, although processing and stability were not affected. These data suggest that the R178H mutation causes a small conformational change inside the active site pocket.

The R504C/H mutations result in disruption of the interaction between the α- and β-subunits in Hex A as an αβ heterodimer. The R504 residue is located in the extrahelix in domain II. This amino acid residue points outside of the extrahelix and is exposed in the monomer. R504 in the α-subunit is thought to bind directly with the D494 residue in the β-subunit at the dimer interface in the αβ heterodimer. In the ββ homodimer, R501, which corresponds to R504 in the α-subunit, binds directly with D494 at the dimer interface. Substitution of R504 to C or H is deduced to cause disruption of the essential binding for dimerization. Paw *et al.* reported that cultured fibroblasts from a patient with R504H synthesized an α-subunit precursor but that the mutant α-subunit failed to associate with the β-subunit to form an active αβ heterodimer [31]. They also reported that R504C gave rise to a mutant α-subunit with the same biochemical defects as those in the case of R504H [32]. The expressed products were each secreted as an α-subunit monomer rather than a dimer of α-subunits. These biochemical results are consistent with the deduced structural information.

Mutations including R170W, W420C, C458Y, L484P and R499C/H were deduced to affect structural stability. The R170 and L484 residues are located in the region of domain II facing domain I, and they are adjacent to each other in the three-dimensional structure. The R170 residue forms hydrogen bonds with E141 in domain I. These hydrogen bonds are thought to contribute to stabilization of domains I and II. R170W is deduced to have a significant destabilizing effect on the domain interface. For the L484P mutation, introduction of P into the α-helix adjacent to domain I may destabilize it.

The W420 and C458 residues are located on the barrel structure enclosing the active site pocket. In contrast with R178H, the structural change caused by W420C and C458Y is large and they might destabilize the core barrel structure of domain II as well as the active site pocket. An expression study showed that the W420C mutation failed to give any catalytic activity with either a sulfated or nonsulfated substrate.

The R499 residue is present on the same extrahelix as R504. But R499 points inside the molecule between domain I and the barrel structure, and is thought to form hydrogen bonds with residues in domain I and the barrel structure. The R499 residue must be one of the important residues stabilizing the two domains. The R499C/H mutations are deduced to cause a loss of stability in this region, although the structural defects are moderate, and not to affect the active site. In cells from patients with R499C/H, residual Hex A activity was detected, and they clinically exhibited the late-onset moderate form of the disease.

3. Structural Defect in Sandhoff Disease

The positions of missense mutations causing Sandhoff disease (R505Q and C534Y) have been mapped in the wild-type structure of Hex B (ββ homodimer), as shown in Fig. **2**.

Among the mutations, C534Y has been identified in a patient with the early-onset severe form of the disease [33], and R505Q in a patient with the late-onset moderate one [34]. The C534 residue is located in the extrahelix between domain I and the barrel structure, and forms a disulfide bond with C551. Substitution of C534 to Y can cause disruption of the disulfide bond, which results in a large conformational change of the extrahelix stabilizing domains I and II. Furthermore, it may affect the dimerization, because the C551 residue is located at the dimer interface [35]. Western blot analysis showed a deficiency of the mature β-subunit and a reduction of the amount of the mature α-subunit [35]. These data suggest that C534Y causes a structurally unstable change in the β-subunit and secondary degradation of the α-subunit resulting from a failure to associate with the β-subunit.

R505Q results in a conformational change of the surface region, although it does not affect the active site. The conformational change caused by R505Q is smaller than that by C534Y. The expressed β-subunit with R505Q is partly processed to the mature form, and shows residual enzyme activity [35].

The structural defects well reflect biochemical and phenotypic abnormalities in the disease. Thus, dysfunctional and destabilizing defects in Hex α- and β-subunits result in Tay-Sachs disease and Sandhoff disease, respectively, and it is thought that enzyme replacement therapies for these diseases will be effective for improvement of the disorders, if the enzyme can be incorporated into neuronal cells.

PRODUCTION OF ENZYMES FOR ENZYME REPLACEMENT THERAPY

As described above, patients with lysosomal diseases accumulate substances to be degraded due to the absence of the enzymes responsible for their hydrolysis in lysosomes. Enzyme replacement therapy, which means administration of the responsible enzyme to the patients, was proposed by de Duve in 1964 [36]. However, large amounts of enzyme proteins are required for enzyme replacement. Enzyme replacement therapy for lysosomal diseases became a reality in early 1990s, when purified glucocerebrosidase derived from human placenta could be targeted to reticuloendothelial tissues, and its safety and effectiveness were demonstrated in Gaucher disease [37]. Now, some recombinant enzymes for lysosomal diseases, such as Gaucher disease [8,9], Fabry disease [10-13], MPS I [14], Pompe disease [15,16], and MPS VI [17], are available, and clinical trials with recombinant enzymes are ongoing for many lysosomal diseases (Table **2**). These recombinant enzymes have been produced in

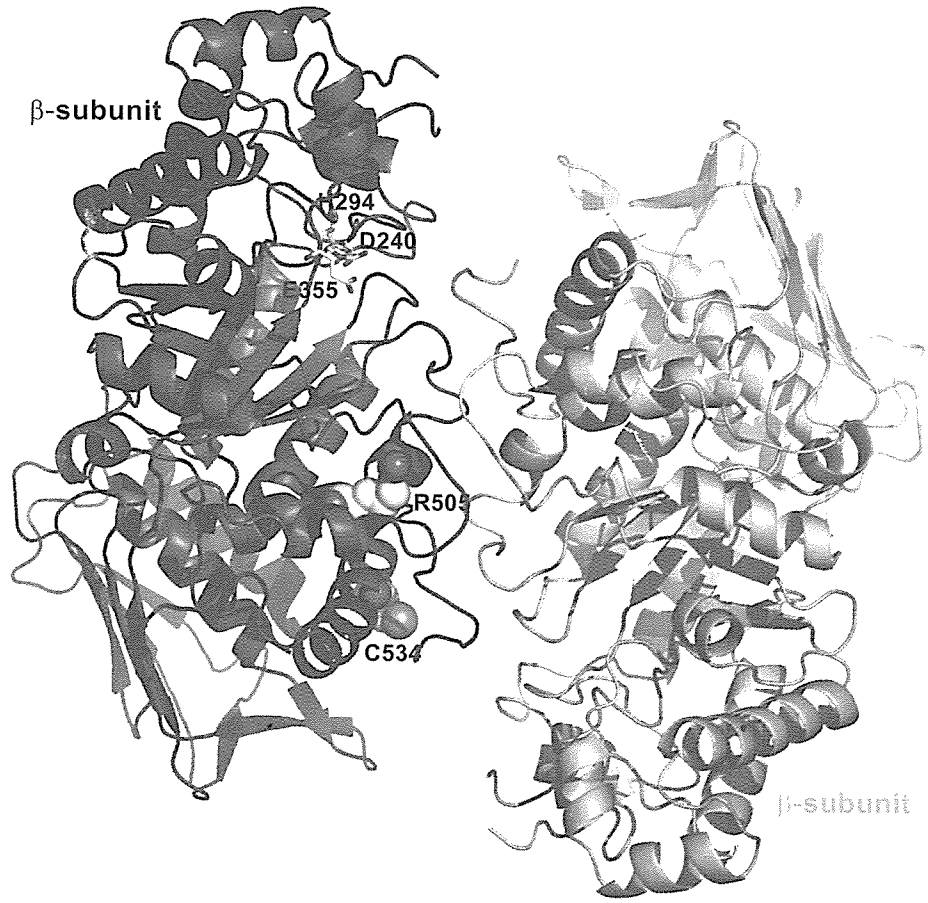

Fig. (2). Three-dimensional structure of human Hex B and residues involved in amino acid substitutions in the β-subunit.
The structure of Hex B (ββ homodimer) is shown. Residues involved in the catalytic triad (D240, H294 and E355) are presented as ball-and-sticks models. Residues involved in amino acid substitutions in the β-subunit (R505 and C534) are presented as space-filling models.

mammalian cells. Many lysosomal enzymes are glycoproteins, and mannose 6-phosphate (M6P) residues at the non-reducing ends of the sugar chains are required for efficient incorporation of the lysosomal enzymes into cells, although mannose residues are essential for incorporation of glucocerebrosidase into reticuloendothelial cells, which are the target cells in Gaucher disease. Lysosomal matrix enzymes are synthesized in rough endoplasmic reticulum and are modified by the addition of high-mannose oligosaccharides. These oligosaccharides are then subjected to the trimming reactions, and the nascent glycoproteins are transferred to the Golgi apparatus, where further modification including addition of M6P recognition maker and binding to M6P receptor occur, and subsequently are transported to endosomes/lysosomes. In some type of cells including fibroblasts, lysosomal enzymes may be transported from the extracellular milieu to lysosomes through M6P receptor-mediated endocytosis [38].

For example, for Fabry disease, two different human α-galactosidases are available: one from human fibroblasts (agalsidase alpha) [10,11] and one from CHO cells (agalsidase beta) [12,13]. Lee *et al.* reported biological and pharmacological comparison of these enzyme preparations [39]. Although the specific activity, *V*max and *K*m toward two different artificial substrates were almost the same for the two enzymes, agalsidase beta showed faster mobility on isoelectric focusing due to the modification of sialic acids on the sugar chains. They exhibited the same *N*-terminal amino acid sequences and *C*-terminal heterogeneity with truncated species lacking either one or two *C*-terminal amino acid residues. However, the predominant molecular species of agalsidase alpha lacked the *C*-terminal leucine, whereas the predominant species of agalsidase beta had the full-length sequence. The most significant difference between them was the pattern of modification of the sugar chains. Monosaccharide analysis indicated that the sialic acid/galactose ratio of agalsidase alpha (0.56) was lower than that of agalsidase beta (0.88), suggesting that the former had more asialo-complex type sugar chains than the latter. This may be due to the differences in host cells and cell culture conditions, and the characteristics of sugar chains should influence the clearance and biodistribution of the enzymes in the whole body. An additional difference in glycosylation between the two enzymes was the amount of M6P residues present on oligomannose side chains. Agalsidase beta had a significantly higher level of M6P (3.1 mol/dimer protein) on a molar basis than agalsidase alpha (1.8 mol/dimer protein). Human α-galactosidase contains three potential *N*-linked glycosylation sites per monomer. The M6P residues are responsible for the incorporation of the enzyme into lysosomes in the cell, because M6P receptors are located on the plasma membrane and play a role in endocytotic trafficking to the endosomes/lysosomes. Phosphorylated oligomannose type sugar chains

were attached to N192 and N215 in both enzymes. However, the amounts of phosphorylated sugar chains of agalsidase beta (72% and 91%) were much higher than those of agalsidase alpha (33% and 61%) for N192 and N215, respectively. These results well correlated with the ability of enzyme binding to the receptors and with that of cellular uptake. When the receptor binding was evaluated by means of surface plasmon resonance analysis to examine the interaction of α-galactosidase with immobilized M6P receptors, agalsidase beta bound to the receptors more than agalsidase alpha did. Cell uptake studies also indicated that the improved binding to the M6P receptors was correlated with the enhanced uptake of agalsidase beta into Fabry fibroblasts. The biodistribution data suggested that the majority of the injected dose was recovered in the liver, however, approximately twice as much agalsidase beta was detected in the kidneys, heart and spleen, compared to agalsidase alpha. These results indicated that the sugar moieties, including the monosaccharide composition, attachment sites and structures of the sugar chains, of the enzymes can influence the effect of enzyme replacement therapy for lysosomal diseases.

Since these recombinant enzymes are produced in mammalian cells, the production of recombinant enzymes is very expensive and careful monitoring for viral infection is essential. Therefore, alternative host cells are required to express an active enzyme at low cost in the near future. *Escherichia coli* [40] and insect cells [41] were used as alternative hosts for producing a recombinant α-galactosidase in early research attempts. However, the recombinant enzyme from *Escherichia coli* was not active and contained no sugar chains. More recently, methylotrophic yeast *Pichia pastoris* was used as a host to produce the α-galactosidase [42]. The expression level of α-galactosidase in this host was considerable (~ 30 mg/L) and the uptake by Fabry fibroblasts was almost the same as that by insect cells; however, the *N*-linked sugar chains of this glycoprotein have not been analyzed carefully and may not have M6P residues at the non-reducing ends. Moreover, it has been reported that *Pichia pastoris* produces a β-mannoside linkage in the associated mannan, which is antigenic for humans [43].

Chiba *et al.* have produced a recombinant α-galactosidase from yeast *Saccharomyces cerevisiae* cells [44]. In order to produce therapeutically effective glycoproteins for lysosomal diseases, the host strain should attach non-antigenic and highly phosphorylated sugar chains to enzyme proteins. Since *Saccharomyces cerevisiae* sometimes produces antigenic hypermannosylated sugar chains, Jigami and co-workers earlier developed a *Saccharomyces cerevisiae* YS132-8B strain that lacked three genes (*OCH1, MNN1,* and *MNN4*) responsible for the biosynthesis of the outer chains of yeast mannan [45,46]. However, since this strain lacked the ability of mannosylphosphorylation, Chiba *et al.* constructed a new disruptant, the HPY21G strain, with KK4 background to delete both *OCH1*, which encodes the initial α-1,6-mannosyltransferase, and *MNN1*, which encodes the terminal α-1,3-mannosyltransferase [44]. It has been reported that the *MNN4* gene encodes a positive regulator of the Mnn6 protein, which transfers mannose phosphate residues to *N*- and *O*-linked sugar chains [46]. The KK4 strain exhibited a higher level of mannosylphosphorylated cell surface than the other strains tested did. As the promoter region of the *MNN4* gene of the *Saccharomyces cerevisiae* KK4 strain had a mutation, the Mnn4 protein was produced constitutively by the KK4 strain. The recombinant α-galactosidase from the *Saccharomyces cerevisiae* HPY21G strain exhibited similar specific activity to that from insect cells, and its apparent molecular mass was a little bit smaller than that of α-galactosidase from mammalian cells. The α-galactosidase contained not only neutral-type sugar chains but also mono- and bis-phosphorylated acidic-type ones. The ratio of non-phosphorylated and phosphorylated sugars of the α-galactosidase was almost 1:2, suggesting that the constitutive expression of *MNN4* in the HPY21G strain may contribute to the increase in the level of phosphorylation of sugar chains of the recombinant α-galactosidase. Monosaccharide composition analysis also showed that the yeast-derived α-galactosidase contained a higher level of M6P (3.8 mol/dimer protein) on a molar basis [44].

Tong *et al.* showed that an uncovered M6P residue was important for the α-galactosidase to exhibit high affinity to the M6P receptor, because with a covered phosphate residue there was no effective binding with the receptor [47]. Since mannose residues covered the M6P residues in *Saccharomyces cerevisiae*, the terminal mannose residues attached through phosphodiester linkages should be removed. Chiba *et al.* have found, on screening, a new bacterium that produces an effective α-mannosidase that digests 'covered' mannose residues on the glycoprotein [44]. The bacterium was determined to be a *Cellulomonas* species. Treatment of the recombinant α-galactosidase with the *Cellulomonas* species α-mannosidase caused exposure of the M6P residues, which was confirmed by structural analysis of the sugar chains by high performance liquid chromatography and M6P receptor binding assaying. Uptake of the recombinant α-galactosidase by cultured Fabry fibroblasts was investigated. The enzyme activity in Fabry cells increased in response to the addition of the treated α-galactosidase and reached a normal level with a concentration of only 0.5 μg/ml in the culture medium. The uptake of the treated α-galactosidase was apparently inhibited by the addition of 5 mM M6P, suggesting that the uptake of the treated α-galactosidase largely depended on the M6P receptor. The effect of the incorporated α-galactosidase on the degradation of ceramide trihexoside accumulated in Fabry fibroblasts was also investigated. After cells had been treated with α-galactosidase for 18 hours, it was likely that the incorporated α-galactosidase was co-localized with ceramide trihexoside and the incorporated α-galactosidase degraded ceramide trihexoside accumulated in the Fabry cells [44].

Such production technology involving a yeast will be useful for producing lysosomal enzymes more economically than with the currently used technology. Efforts are being focused on developing a M6P type glycoprotein production system with a methylotrophic yeast *Ogataea minuta* and on trying to express other lysosomal enzymes including Hex A. The Hex A produced in *Ogataea minuta* exhibits catalytic activities toward synthetic substrates and native substrates under the conditions with a detergent [48].

BRAIN-SPECIFIC DELIVERY OF MEDICINES

To develop effective therapies for lysosomal diseases associated with neurological disorders, a brain-specific delivery system for lysosomal enzymes or their genes is required.

Microglia, macrophage-like cells in the brain, are multifunctional cells; they play important roles in the development, differentiation and maintenance of neuronal cells *via* their phagocytic activity and production of enzymes, cytokines and trophic factors [49]. Although activated microglia show similar phenotypes to macrophages in isolated conditions, they appear to exhibit different phenotypes from those of macrophages *in vivo* and *in vitro* [50-58]. Sawada *et al.* found that when intra-arterially injected into rats, isolated microglia exhibited higher affinity for and migrating activity toward the brain than macrophages did. Since intra-arterially injected microglia, which were labeled with fluorescent dye microparticles through their phagocytic activity, migrated specifically into the brain but were rarely found in the liver, this system could be used as a brain-specific delivery system for medicines or other bioactive materials, such as proteins or genes.

To investigate the possibility that microglia can deliver a gene of interest to the brain without any effect on other organs, a β-galactosidase gene expression vector was transfected into purified microglia from primary mixed brain cultures or Ra2 cells, immortalized microglial clone cells, the microglia then being injected into a vertebral artery in rats. For identification of these exogenous cells within the brain, the cells were tagged with a fluorescent dye specific for phagocytic cells, PKH26 [59,60]. PKH26 stained microglia efficiently; the purified microglia were stained an intensity of at least two-orders higher than the purified astrocytes. Forty-eight hours after the purified microglia had been injected intra-arterially, many fluorescent cells were observed in a brain section from a rat. A small portion of the fluorescent cells was observed in the brain capillaries, attached to the capillary walls. Two hours after the injection, migration of microglia into the brain parenchyma was observed. Exogenous fluorescently labeled microglia were observed to have adhered to a vessel in the medulla of the brain. Some microglia crossed the vessel wall and entered the parenchyma. Similar results were obtained with Ra2 cells. When a frozen brain section was stained with X gal as a substrate for exogenously introduced β-galactosidase, many lacZ-positive cells were observed in the brains of rats at 48 hours after Ra2 cells, which had been transfected with a lacZ-gene expression vector, had been injected intra-arterially. Similar results were obtained with purified microglia. Therefore, intra-arterially injected microglia and Ra2 cells can migrate to the brain, and can express the genes transfected *in vitro* and translate them into biologically active proteins in the brain.

The specificity of Ra2 cell migration was determined by measuring β-galactosidase activity in the brain and other tissues. Using a highly sensitive detection method for β-galactosidase activity involving a chemiluminescent substrate, Sawada *et al.* could detect β-galactosidase activity in frozen sections of the brain and other tissues. β-Galactosidase activity in tissues at 48 hours following the intra-arterial injection of Ra2 cells was highest in the brain, i.e., over 30-fold that in the liver and spleen, and was not detected in lung sections. The results indicated that most of the injected Ra2 cells migrated to the brain. On the other hand, purified macrophages migrated to the liver but were not found in the brain of normal rats [61]. The data indicate that microglia have characteristics differing from those of macrophages; the former exhibit specific affinity for and migrating activity toward the brain. The stability of gene expression in the brain was determined by measuring β-galactosidase activity in brain sections at 2, 9, 16 and 23 days after intra-arterial Ra2 cell injection. β-Galactosidase activity in brain sections was highest on the 2nd day and later gradually decreased. On the 23rd day the β-galactosidase activity was about half that on the 2nd day; the product of the transferred gene was still active. Twenty-three days after the injection, fluorescent Ra2 cells were still present in brain sections in similar numbers to as on day 2, although the fluorescence intensity of Ra2 cells was much weaker than that in the day 2 brain sections. Therefore, the decrease in β-galactosidase activity seemed to be due to a decrease in expression of the lacZ gene in Ra2 cells because it was transiently transfected. This means that if Ra2 cells that express the gene permanently are injected, genes of interest can be expressed in the brain for more than 20 days.

Many types of methods and techniques for *in vivo* gene transfer have been developed, some of which have already been applied in clinical trials. The retroviral system, the most widely accepted gene transfer method to date, allows highly efficient integration, providing the potential for permanent gene expression. However, the system has some major disadvantages, such as the typically low titer, instability of the viral vector obtained, and requirement for target cell division for integration and expression [62]. The adenoviral system allows more efficient gene transfer and greater stability of the virus, however, the difficulties in the control of target cells and re-administration necessitated by the strong antigenicity of the virus are serious problems [63]. *In vivo* electroporation has been demonstrated to allow highly efficient gene transfer into the brain [64]. But all of these methods require a major surgical procedure to transfer cells carrying genes, or the insertion of a stainless steel electrode.

A brain-targeting delivery system involving microglial cell line or related signal peptides which deliver enzyme proteins to the brain will facilitate the development of gene therapies and enzyme replacement therapies for lysosomal diseases including Tay-Sachs disease and Sandhoff disease.

INCORPORATION OF LYSOSOMAL ENZYMES IN TO CELLS

Itoh *et al.* established Chinese hamster ovary cell lines that simultaneously express the human *HEXA* and *HEXB* genes as well as the corresponding murine genes, *Hexa* and *Hexb*, respectively. Mice have the same gene organization as man, i.e., *Hexa* encoding the α-subunit on chromosome 9 and *Hexb* encoding the β-subunit on chromosome 13, and have the same Hex isozyme system [19,65,66]. The amino acid sequences deduced from *Hexa* and *Hexb* cDNAs are 55% identical, and each exhibits homology to the human counterpart, 84% and 75%, respectively [65]. Itoh *et al.* revealed the therapeutic effects of recombinant Hex A isozymes on the degradation of natural substrates including GM2 ganglioside and GlcNAc-oligosaccharides accumulated in cultured cells in Tay-Sachs disease and Sandhoff disease.

CHO cell lines CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb*s were established by co-introduction of the human *HEXA* and *HEXB* as well as the murine *Hexa* and *Hexb* genes, and drug resistance to hygromycin and neomycin derivatives (Itakura submitted). Other CHO cell lines, CHO-

HEXA, CHO-*HEXB* and CHO-*Hexb*, independently expressing the *HEXA*, *HEXB* and *Hexb* genes were also isolated as controls, respectively. As shown in Fig. **3A,B**, the intracellular total Hex activities toward the neutral substrate, 4-MUG for CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb* were similar to those for CHO-*HEXB* and CHO-*Hexb*, and 7~12-fold higher than those for the parent CHO and CHO-*HEXA*, while the Hex activity toward the anionic substrate 4-MUGS was significantly increased by 7- and 5.5-fold, respectively, compared to the levels for the control cell lines. As shown in Fig. **3C,D** the CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb* cell lines were also revealed to produce significant levels of Hex activities toward both 4-MUG and 4-MUGS in the conditioned media, although the levels varied among the cell lines.

Fig. **4** presents the results obtained on immunoblotting with anti-human placental Hex A serum. The CHO-*HEXA* and CHO-*HEXB* cell lines produced and secreted the precursor and mature forms of the corresponding α- (lanes hα) and β- (lanes hβ) subunits, respectively, although these bands were not observed for the parental CHO cells (lanes CHO). The CHO-*HEXA/HEXB* cells also expressed and secreted both the precursor and mature forms of the α- and β-subunits (lanes hαhβ). The molecular masses of the mature subunits were calculated to be 53 kDa for the α_m- and 25 kDa for the β_m-subunit, which were smaller than those of the human placental mature subunits (α_m: 55 kDa and β_m: 28 kDa) as standards. At present, it is unknown whether the difference in subunit size is involved in glycosylation, or it is connected with the difference in protein processing between human placenta and CHO cells.

To examine the corrective effects of the conditioned media containing the recombinant Hex isozymes secreted by the CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb* cell lines, they were administered to skin fibroblasts derived from a Sandhoff patient (SD572) and a Tay-Sachs patient (TS218). As shown in Fig. **5A**, the intracellular 4-MUG-degrading activities in the SD572 cells were restored to 80% and 116% of the control value in normal subjects, respectively. The 4-MUGS-degrading activities in the SD572 cells were also significantly increased by 35% and 67%, respectively, as shown in Fig. **5B**. However, the conditioned media from the CHO-*HEXB* and CHO-*Hexb* cell lines did not cause an increase in 4-MUGS-degrading activity, suggesting that the conditioned media from the CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb* cell lines contained not only the Hex B isozyme but also Hex A and/or Hex S, composed of allo-type Hex subunits. Incorporation of the recombinant Hex isozymes was inhibited in the presence of 5 mM M6P in the culture

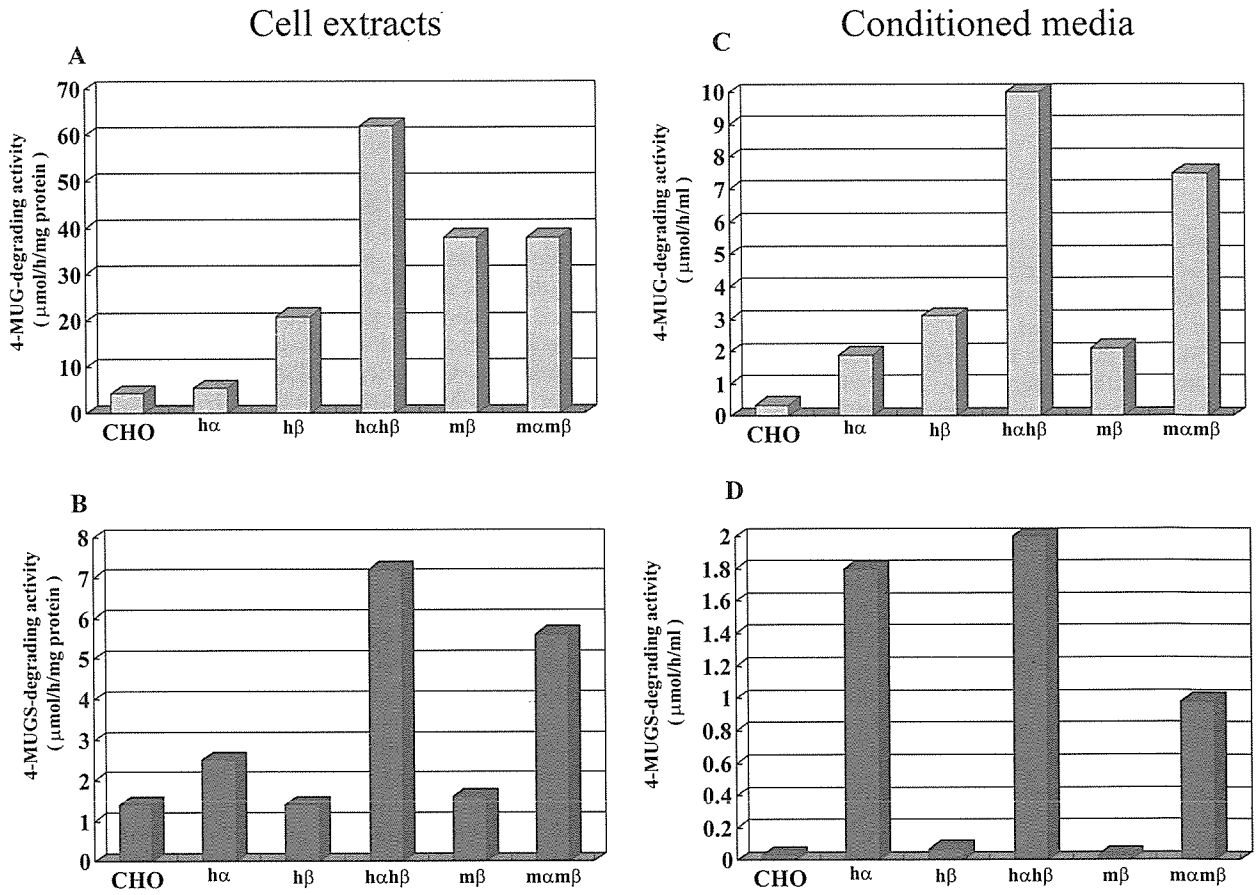

Fig. (3). Expression of Hex subunit genes in stably transformed CHO cell lines.

The intracellular (**A** and **B**) and secreted (**C** and **D**) Hex activities toward synthetic fluorogenic substrates 4-MUG (**A** and **C**) and 4-MUGS (**B** and **D**), respectively, were measured. In panels: CHO, parent CHO; hα, CHO-*HEXA*; hβ, CHO-*HEXB*; hαhβ, CHO-*HEXA/HEXB*; mβ, CHO-*Hexb*; mαmβ, CHO-*Hexa/Hexb*.

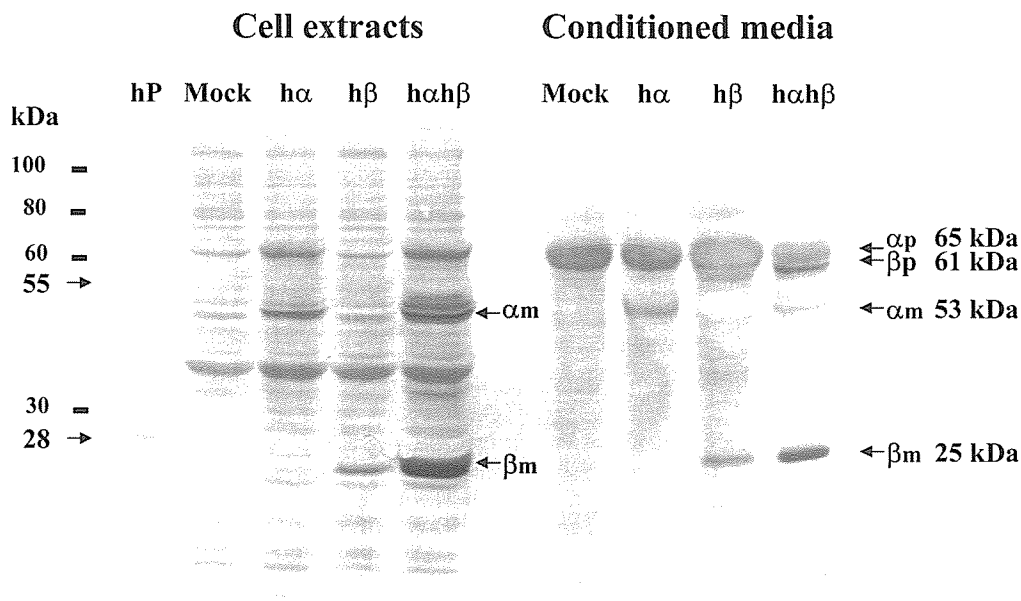

Fig. (4). Immunoblotting of Hex isozymes derived from transformed CHO cell lines stably expressing human Hex α- and β-subunit cDNAs. The isolated clones and conditioned media were harvested, and then aliquots of the cell extracts and conditioned media treated under reducing conditions were subjected to immunoblotting with rabbit anti-human placental Hex A serum, a biotinylated anti-rabbit IgG (Vector, Burlingame, CA), horseradish peroxidase-conjugated egg white avidin (ICN Pharmaceuticals, Inc., Aurora, OH), and chromogenic substrates (NBT and BCIP; GIBCO/LifeTech). In panels: hP, human placental Hex; Mock, parent CHO; hα, CHO-*HEXA*; hβ, CHO-*HEXB*; hαhβ, CHO-*HEXA/HEXB*. The molecular masses (kDa) of biotinylated standards (Cell Signaling Technology, Inc., Beverly, MA) are indicated. Arrows indicate the Hex α-subunit precursor (α_p), β-subunit precursor (β_p), mature α-subunit (α_m), and mature β-subunit (β_m).

medium (open columns), indicating that these Hex isozymes were taken up *via* M6P receptor on the surface of skin fibroblasts.

The enzyme replacement effect on the intracellular degradation of natural substrates accumulated in Sandhoff disease fibroblasts was also analyzed after continuous administration of the conditioned media containing the enzyme activity at 24-hour intervals for 3 days. As shown in Fig. **5C**, granular immunofluorescence due to GM2 ganglioside was observed in the SD572 cells (panel SD572). The accumulated fluorescence due to GM2 ganglioside disappeared after administration of the conditioned media from the CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb* cell lines (panels hαhβ and mαmβ) but not of the conditioned media from the parent CHO cells (panel Mock). The fluorescence due to GlcNAc-oligosaccharides also decreased after the addition of the conditioned media from the CHO-*HEXA/HEXB*, and CHO-*Hexa/Hexb* cell lines, and even the mock one, suggesting that the accumulated GlcNAc-oligosaccharides were easily degraded by the incorporated Hex B derived from man, mouse and hamster (data not shown).

Next, the conditioned media from the CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb* cell lines were administered to Tay-Sachs fibroblasts (TS218). As shown in Fig. **6A**, the 4-MUGS-degrading activities were also restored to 19% and 35% of the control value in normal subjects, which were higher than that (11%) when the conditioned medium from the CHO-*HEXA* cells was added to TS218 cells. The restoration of the intracellular 4-MUGS-degrading activities was also inhibited in the presence of M6P (data not shown).

Fig. **6B** shows the corrective effects of the conditioned media on the natural substrates accumulated in the TS218 cells. The granular immunofluorescence of GM2 ganglioside was also observed in the TS218 cells (panel TS218), while immunofluorescence of GlcNAc-oligosaccharides was absent (data not shown) because of the presence of endogenous Hex B. In contrast, the GM2 ganglioside immunofluorescence decreased after administration of the conditioned media from the CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb* cell lines (panels hαhβ and mαmβ), although the mock conditioned medium could hardly cause a decrease (panel Mock).

These results suggest that the secreted human and murine Hex A derived from the CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb* cell lines were also incorporated *via* M6P receptors to degrade the accumulated GM2 ganglioside in cooperation with GM2 activator protein in fibroblasts in Sandhoff disease and Tay-Sachs disease. Interestingly, the murine Hex A was clearly demonstrated to bind to the human GM2 activator protein to degrade the accumulated GM2 ganglioside in fibroblasts in GM2 gangliosidoses.

There has been recent progress in the development of enzyme replacement therapies for lysosomal diseases *via* cell surface receptors for recombinant lysosomal enzymes with oligosaccharide chains carrying M6P residues, as described above. The application of enzyme replacement therapies with recombinant human Hex isozymes produced by mammalian cells to GM2 gangliosidoses with neurological manifestations is also expected because they have oligosaccharides carrying M6P residues, although the disadvantage of the blood-brain barrier for enzyme replacement therapy still remains. Martino *et al.* reported the difficulty in cross-

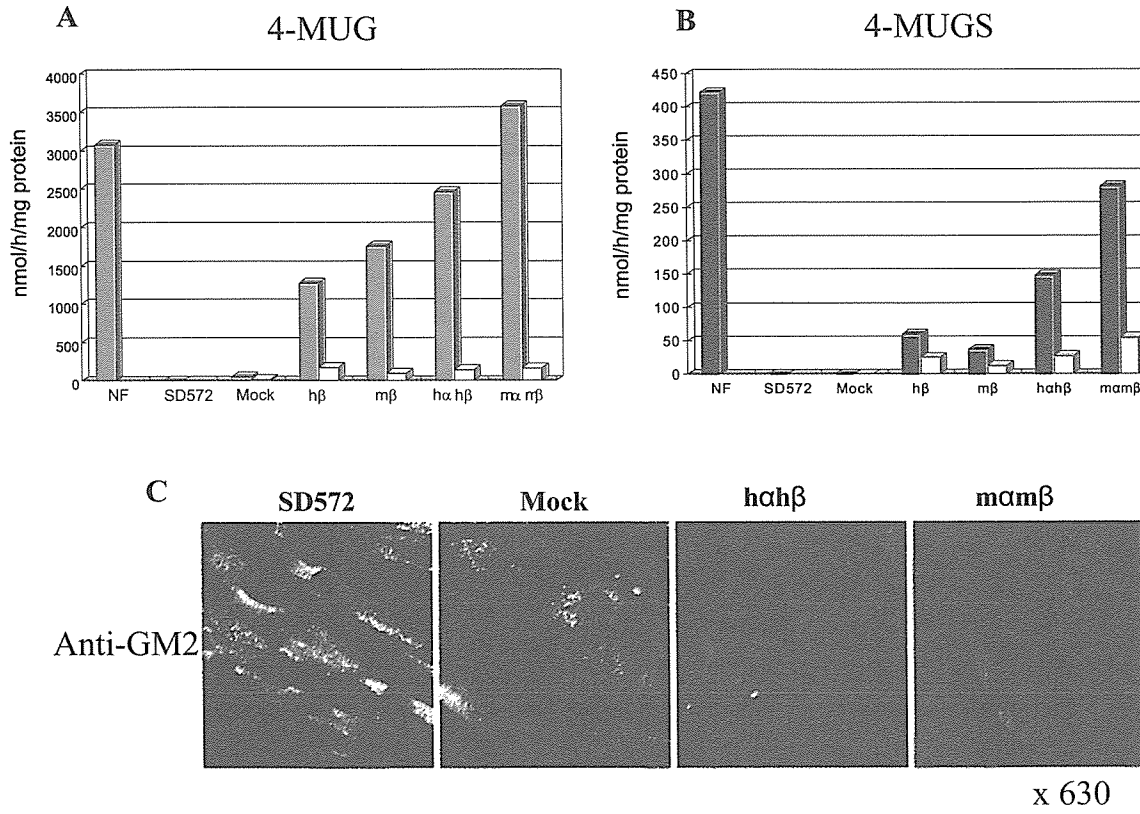

Fig. (5). Corrective effect of the recombinant Hex A on the accumulated natural substrates in Sandhoff fibroblasts.

(**A** and **B**) Concentrated conditioned media containing a definite level of 4-MUG-degrading activity (up to 1 mmol/h) were added repeatedly to the culture medium of Sandhoff fibroblasts (SD572; 1~2 x 10^5 cells/60-mm dish). After 3 days culture, fibroblasts were harvested, and then Hex activities toward synthetic 4-MUG (**A**) and 4-MUGS (**B**) in the cell extracts were measured. Each bar represents the mean for two independent experiments. Some experiments were performed in the presence of 5 mM M6P (open columns). (**C**) Degradation of intracellular GM2 ganglioside accumulated in Sandhoff fibroblasts (SD572) was evaluated by means of immunofluorescence with anti-GM2 ganglioside serum, and then a fluorescein-conjugated second antibody. Magnification, X 630. In panels: NF, normal fibroblasts; SD572, SD572 without the addition of conditioned medium; Mock, SD572 with the addition of conditioned medium from mock-transformed CHO; hβ, conditioned medium from CHO-*HEXB*; mβ, conditioned medium from CHO-*Hexb*; hαhβ, conditioned medium from CHO-*HEXA/HEXB*; mαmβ, conditioned medium from CHO-*Hexa/Hexb*.

correction in Tay-Sachs cells because recombinant Hex A incorporated into the cells could not efficiently degrade the intralysosomal GM2 ganglioside [67]. However, Itoh *et al.* demonstrated that administration of conditioned media derived from the CHO-*HEXA/HEXB* and CHO-*Hexa/Hexb* cell lines, each containing recombinant human and murine Hex A, respectively, to skin fibroblasts from Tay-Sachs and Sandhoff patients partly restored the intracellular 4-MUGS-degrading activities, and significantly decreased the accumulated GM2 ganglioside and GlcNAc-oligosaccharides. As the glycosylated recombinant Hex A containing M6P residues secreted by the CHO cell lines was revealed to be taken up *via* the cell surface M6P receptor, this specific receptor could be the target molecule for enzyme replacement therapies for Tay-Sachs disease and Sandhoff disease patients.

CONCLUSION

We have described the molecular pathologies of Tay-Sachs disease and Sandhoff disease as models of lysosomal diseases from the protein structural aspect, and have discussed enzyme production, targeting to the brain and incorporation into cells. Improvement of these methods and their combination will facilitate the development of efficient enzyme replacement therapies for lysosomal diseases.

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ABBREVIATIONS

CHO	=	Chinese hamster ovary
MPS	=	Mucopolysaccharidosis
Hex	=	β-Hexosaminidase
GlcNAc	=	*N*-Acetylglucosamine
GalNAc	=	*N*-Acetylgalactosamine
M6P	=	Mannose 6-phosphate
4-MUG	=	4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide