るものでは治療にも関わらず予後不良であった事実も神経症状が治療抵抗性であることを示している。しかし、A群では臓器症状や、血液学的所見の改善、ADLの維持が認められることからある程度の治療効果は望めると考えられる。

E. 結論

現在、ゴーシェ病の治療法の主流となっているERTにおいては、1型においては十分な効果が限られているが、2型、3型においては、その効果は十分とは言えず、ケミカルシャペロン療法などの他の治療選択肢を考慮にいれて、治療方針を決定すべきと考えられる。

F. 健康危険情報

なし

G. 知的所有権の取得状況

なし

別添4

厚生労働科学研究費補助金（こころの健康科学研究事業）
分担研究報告書

クラッベ病の欠損酵素galactocerebrosidaseの生化学的特性について

分担研究者　酒井規夫　大阪大学大学院医学系研究科・講師

研究要旨

リソソーム病の１つであるクラッベ病は根本的な治療法のない遺伝性脱髄疾患である。分子シャペロン法の応用の可能性を調べるために、欠損酵素galactocerebrosidaseの生化学的な特徴について解析した。

A. 研究目的

galactocerebrosidase の正常蛋白としての特性を調べる。

変異蛋白の特徴について患者細胞、発現実験によって調べる。

シャペロン物質 NOEV の患者皮膚線維芽細胞における酵素活性に対する効果を調べ、有効な細胞株を検索する。

B. 研究方法

galactocerebrosidase の正常蛋白としての特性；至適 pH、G_{M1} による阻害実験、温度感受性などについて検討した。

変異蛋白の特徴；変異蛋白の in vitro 活性、細胞内局在、発現実験による活性比較などを検討した。

シャペロン物質 NOEV の患者皮膚線維芽細胞における酵素活性に対する効果；患者皮膚線維芽細胞の培養上清にシャペロン物質 NOEV を添加し、４日間培養後、細胞を回収し細胞内酵素活性を測定した。線維芽細胞は９種類を用い、その遺伝子変異についても確認した。また、galactocerebrosidase の活性化因子として知られる SAP 蛋白質の添加による効果についても検討した。

なお、患者細胞の遺伝子解析、細胞利用に関しては、患者から書面による説明と同意を得ている。

C. 研究結果

galactocerebrosidase の正常蛋白としての特性；精製した galactocerebrosidase の至適 pH は天然基質を用いた TC 系では 4.2、蛍光基質を用いた TC 系では 4.4 であることが確認された。また G_{M1} ganglioside とは競合阻害を示し、HMGal とは非競合阻害を示すことが確認された。

変異蛋白の特徴；患者の変異をリンパ球と培養皮膚線維芽細胞で確認し、いくつかは COS1 細胞での発現実験を行った。発現蛋白質の局在はリソソーム内のみでなく細胞質に広がり、輸送に障害ある可能性が疑われた。

シャペロン物質 NOEV の患者皮膚線維芽細胞における酵素活性に対する効果；９個の患者皮膚線維芽細胞において NOEV を 0.5, 2.0, 6.0□M の濃度において付加したが、明らかに活性が上昇する細胞株は認められなかった。また、活性化因子 SAP を 2–2000 nM 付加して同じ実験を行ったが、同様の結果であった。

D. 考察

精製蛋白の特徴から酵素活性の測定条件に留意する必要があること、変異蛋白の発現実験から変異により細胞内輸送に障害がある変異がある可能性があり、これが今後の実験に検討する際に問題になると考えられた。NOEVのガラクトセレブロシダーゼに対する阻害効果は大変強いものであったが、調べた細胞株での残存活性が上昇するものは見いだせなかった。

E. 結論

NOEVの効果について更に多くの細胞株を用いて、測定条件などを最適化した上で検討が必要と考えられた。

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G. 知的所有権の取得状況

なし

別添4

厚生労働科学研究補助金（こころの健康科学研究事業）
分担研究報告書

ファブリー病患者由来変異酵素の細胞内局在とシャペロン治療に関する研究

分担研究者　石井　達　帯広畜産大学畜産学部・教授

研究要旨

ファブリー病患者由来変異酵素(R301Q, L166V)の細胞内局在性をPercoll密度勾配遠心と免疫蛍光染色により、平常状態ではERに局在することが確認された。次に1-デオキシガラクトノジリマイシン添加の影響を検討したところ、リソソームへの移行が確認され、細胞内輸送の正常化が認められた。また、糖脂質蓄積を検討するためのGb3測定の簡便法としてHisタグを付けたShiga toxin Bサブユニットを使ったBinding assay法を確立した。

A．研究目的

先に我々は残存活性を有するファブリー病患者由来変異酵素をCOS-7細胞で一過性に発現させ、これらに対する活性部位特異的シャペロンである1-デオキシガラクトノジリマイシン(DGJ)添加により顕著な酵素活性の上昇を確認し、これらの変異酵素の多くは機能的には正常であるが、熱安定性が低いフォールディング異常酵素であることを明らかにした。そこで今回これら変異酵素の細胞内局在について検討し、DGJがこれら酵素の細胞内局在性に影響することを検討する。

また、ファブリー病の発症には酵素欠損に起因した糖脂質の蓄積が直接関わっており、患者組織では特にグロボトリアオシルセラミド(Gb3)が蓄積する。そこで今回、シャペロン療法の治療効果判定に不可欠な組織Gb3含量の簡便な定量法の開発も合わせて行なった。

B．研究方法

古典型ファブリー病患者で認められたアミノ酸変異(L166V)と古典型及び亜型患者の両者で認められている変異 (R301Q)について、COS-7細胞で一過性に発現させ、酵素の細胞内局在性に対する20 μM DGJ添加の影響を検討した。Percoll密度勾配遠心法による分画と免疫蛍光染色により酵素の細胞内分布を調べた。

また、Gb3の測定法としてはHisタグを付けたShiga toxinのBサブユニット(1B-His)とHisProbe-HRP (Pierce Biotechnology Inc.)を用いた2段階Binding assayの検討を行った。

C．研究結果

Percoll密度勾配遠心による分画で正常酵素は、高密度画分と低密度画分の2相性分布を示したが、患者由来変異酵素(L166V, R301Q)はいずれも低密度画分でのみ認められ、ERのマーカータンパク質であるBiPを含む画分と一致していた。ところが、DGJ存在下で培養した細胞では、これら変異酵素は高密度画分でも認められるようになり、リソソームのマーカー酵素であるβ-hexosaminidaseの分布と一致した。また、免疫蛍光染色においてもDGJ非添加ではR301Qの局在はBiPと一致していたが、DGJ添加により一部LAMP2(リソソームのマーカータンパク質)の分布と一致するようになった。

1B-Hisを用いたBinding assayはGb3 (1~100 ng)において最終生成物による吸光度と良好な対応が得られた。また、Gb4に対して約6％の反応交差性を示したが、培養細胞や組織Gb3量の測定には影響ないことが明らかとなった。

D．考察

変異酵素は機能的には正常であるが、熱安定性の低さに見られるフォールディング異常により細胞内、特にERでの品質管理機構によりERに留まり、分解されると考えられる。この時、DGJの添加により酵素のフォールディング異常が矯正され、ERからリソソームへの細胞内輸送も正常化すると考えられる。

Gb3定量の簡便法としては、すでにShiga toxin Bサブユニットを使ったELISA法が報告されているが、今回Hisタグを付けてBサブユニットを作成することで、その生成を簡便化するとともに測定を2段階で行うことができるようになったことで今後汎用性が高まると考えられる。

E. 結論

DGJを用いたシャペロン療法では細胞内の酵素活性の上昇とともに、変異酵素のERからリソソームへの細胞内輸送の正常化が生じていることが確認され、治療応用への可能性が高まった。そこで次に、DGJにより糖脂質の蓄積を抑えることを証明する必要があるが、今回Gb3の簡便な測定法が確立した。しかし、現時点では糖脂質の蓄積が見られるファブリー病に対するシャペロン療法検討用モデルマウスは存在しないことから、今後そのための検討が必要である。

F. 研究発表

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G. 知的財産権の出願・登録状況

特許取得　なし
実用新案登録　なし
その他　なし

別添４

厚生労働科学研究費補助金（こころの健康科学研究事業）
分担研究報告書

立体構造予測と分子シミュレーションによるヒトβ-ガラクトシダーゼの機能解析

分担研究者 榊原康文　慶應義塾大学理工学部・教授

研究要旨

β-ガラクトシダーゼ酵素が欠損するとガングリオシドG_{M1}が蓄積し，β-ガラクトシドーシスという疾患を引き起こす．この酵素の欠損の原因として一残基変異が多数判明しており，変異の種類によって発症時期が異なる．しかしながら多くの一残基変異体タンパク質について分子的な解析はほとんどなされていない．本研究は，そのような多種の変異体に対して立体構造予測と分子シミュレーションを用いた代謝系の変化を解析する手法の確立を目的とする．その第一歩として解析目標をタンパク質のミカエリス･メンテン定数（K_m）の予測に設定した．ヒトβ-ガラクトシダーゼ（HBG）の立体構造を予測した結果，TIMバレルが保存されていた．さらに，HBGとラクトースの複合体の生成に成功し，K_m値を予測した．

A.　研究目的

β-ガラクトシダーゼはライソゾーム内で加水分解により糖鎖の末端にあるガラクトースを遊離させる酵素である．この酵素が欠損するとガングリオシドG_{M1}が蓄積し，β-ガラクトシドーシスという疾患を引き起こす．この酵素の欠損の原因として一残基変異が多数判明しており，変異の種類によって発症時期が異なる．

本研究は，そのような多種の変異体に対して立体構造予測と分子シミュレーションを用いた代謝系の変化を解析する手法の確立を目的とする．その第一歩として解析目標をタンパク質のミカエリス･メンテン定数（K_m）の予測に設定した．K_m値を始めとする速度定数が計算できれば代謝系のシミュレーションが可能であり，ガングリオシドG_{M1}の蓄積過程を追跡できる．

B.　研究方法

K_m値はタンパク質と基質が複合体を形成するときの自由エネルギー変化ΔGから算出できる．ΔGは分子シミュレーションによって計算する．そのためにはタンパク質，基質，複合体，それぞれの構造情報が必要である．ヒトβ-ガラクトシダーゼ（HBG）の構造は未知であるので，ホモロジーモデリングにより配列から立体構造を予測する．基質（問題簡単化のためラクトースを使用）の構造は既知である．次に，複合体の構造は基質をタンパク質の活性部位に配置し，分子シミュレーションにより生成する．

しかし，分子シミュレーションはシミュレーション条件により結果が大きく異なることがあるため，その条件の決定が重要である．そこでガラクトースとの複合体の構造が知られており，構造予測の際にテンプレートに用いたアオカビβ-ガラクトシダーゼ（PBG）のK_m値予測を行った．予測したK_m値と実験値と比較することで最適な条件を探った．

次に，決定した条件で野生型HBGおよび疾患の原因となるR201C，Y83H変異型HBGのK_m値を予測した．

C.　研究結果と考察

まず初めに，HBGの立体構造の予測を行った．糖質分解酵素に多く見られるTIMバレルが再現されており，妥当な結果と言える．

PBGを用いた実験ではラクトースとの複合体の生成に成功した．この際，ガラクトースに結合している糖がβ型でないと立体障害が大きく，複合体を形成しにくいことを確認した．K_m値の予測は，条件を変え幾度か計算したが，実験値と

一致させることはできなかった（表1）．原因としてシミュレーションの不備やΔGの計算誤差などが考えられるが，その解決は今後の課題である．

次に，HBGを用いた実験では野生型およびR201C変異型HBGとラクトースの複合体の生成に成功した．HBGでは，立体構造が未知であり，当然ラクトースとの複合体の構造も未知であるのでこの分子的結果は大きな成果である．一方，Y83H変異体はラクトースが活性部位にとどまらず，複合体を生成しなかった．複合体を生成できた野生型とR201C変異型HBGのK_m値の予測結果を表1に示す．R201C，Y83H変異型HBGは野生型HBGより複合体を形成しにくいことを確認した．疾患との関連が示唆される．

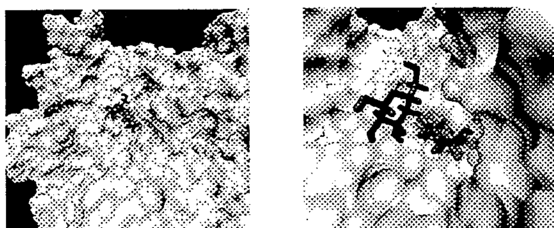

図1 野生型HBGとラクトースの複合体

表1 K_mの予測結果

	ΔG [kcal]	K_m [M]
PBG（予測値）	-22.52	4.145×10^{-14}
PBG（実験値）	-2.69	1.11×10^{-2}
HBG WT	-29.41	4.031×10^{-19}
HBG R201C	-15.77	3.372×10^{-9}

D. 結論

HBGの立体構造を予測した結果，TIMバレルが保存されていた．PBGおよびHBGとラクトースの複合体の生成に成功し，K_m値を予測した．

今後は基質をガングリオシドG_{M1}にすること，代謝系のシミュレーションに発展させるため，K_m値の改善とそれ以外の速度定数も精度よく予測することが必要となる．

E. 研究発表

なし

F. 知的財産権の出願・登録状況

なし

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研究成果の刊行に関する一覧表

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研究成果による特許権等の知的財産権の出願・登録状況

なし

健康危険情報

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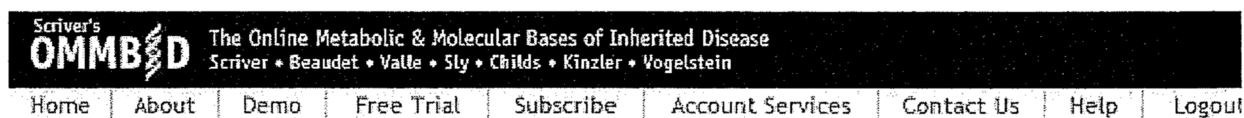
研究成果の刊行物・別刷



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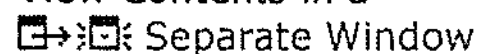

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Part 16: LYSOSOMAL DISORDERS
Chapter 151:

β-Galactosidase Deficiency (β-Galactosidosis) G_{M1} Gangliosidosis and Morquio B Disease

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1. Hereditary deficiency of lysosomal acid β-galactosidase (β-galactosidosis) is expressed clinically as two different diseases, G_{M1} gangliosidosis and Morquio B disease. The mode of inheritance is autosomal recessive. G_{M1} gangliosidosis is a neurosomatic disease occurring mainly in early infancy (infantile form; type 1). Developm arrest is observed a few months after birth, followed by progressive neurologic deterioration and generalized rigospasticity with sensorimotor and psychointellectual dysfunctions. Macular cherry-re spots, facial dysmorphism, hepatosplenomegaly, and generalized skeletal dysplasia are usually present in infantile cases. Cases of lat onset have been described as late infantile/juvenile form (type 2) or adult/chronic form (type 3). They are observed as progressive neurologic diseases in childhood or in young adults. Dysmorphic changes are less prominent or absent in these clinical forms, althou vertebral dysplasia is often detected by radiographic studies. No spe neurologic manifestations are known for late infantile/juvenile patie with G_{M1} gangliosidosis. Extrapyramidal signs of protracted course, mainly presenting as dystonia, are the major neurologic manifestati in adults with G_{M1} gangliosidosis.

2. Morquio B disease is clinically a mild phenotype of Morquio A diseas is expressed as generalized skeletal dysplasia with corneal clouding, resulting in short stature, pectus carinatum (sternal protrusion), platyspondylia, odontoid hypoplasia, kyphoscoliosis, and genu valgu There is no central nervous system involvement, although spinal co compression may occur at the late stage of the disease. Intelligence normal, and hepatosplenomegaly is not present. X-ray changes are pathognomonic significance.

3. There is diffuse atrophy of the brain in patients with early onset G_{M1} gangliosidosis. Neurons are filled with numerous membranous cytoplasmic bodies (MCB), and inclusions of other types are observe glial cells: pleomorphic lipid bodies, membranovesicular bodies, or l compact oval deposits. There are histiocytes with distended cytopla in visceral organs. Cytoplasmic inclusions observed under electron microscopy are different from MCB in neurons. They are vacuoles fil with fine granular, tubular, or amorphous osmiophilic material. Thes changes are less prominent in cases of mild phenotypic expression.

4. Glycoconjugates with terminal β-galactose are increased in tissues a urine from patients with G_{M1} gangliosidosis and Morquio B disease. Ganglioside G_{M1} and its asialo derivative G_{A1} accumulate in the G_{M1} gangliosidosis brain. High amounts of oligosaccharides derived from keratan sulfate or glycoproteins have been reported in visceral orga and urine from G_{M1} gangliosidosis or Morquio B disease patients.

Undersulfated keratan sulfate has also been described.

5. Two lysosomal enzymes are known for hydrolysis of terminal β-linke galactose at acidic pH in various glycoconjugates. One is an enzyme usually called β-galactosidase (EC 3.2.1.23), catabolizing gangliosid G_{M1}, galactose-containing oligosaccharides, keratan sulfate, and oth β-galactose-containing glycoconjugates (G_{M1} β-galactosidase). The enzyme activity is markedly reduced or almost completely deficient cells and body fluids from patients with β-galactosidosis. Heterogene kinetic or physicochemical properties have been found in the mutant enzymes. The degree of substrate storage and residual enzyme acti is correlated with the severity of each clinical phenotype; infantile G gangliosidosis shows the highest substrate storage and the lowest residual enzyme activity as compared with other milder phenotypes. The second genetically different β-galactosidase is galactosylceramic (galactocerebrosidase; EC 3.2.1.46), catabolizing galactosylceramid galactosylsphingosine, and other lipid compounds. Genetic deficienc this enzyme results in globoid cell leukodystrophy, which is another neurometabolic disease.

6. The human β-galactosidase gene has been mapped on chromosome (3p21.33). The cDNA codes for a protein of 677 amino acids, includi putative signal sequence of 23 amino acids and 7 potential asparagi linked glycosylation sites. The gene spans more than 60 kb, and contains 16 exons. The promoter has the characteristics of a housekeeping gene, with GC-rich stretches and 5 SP1 transcription elements on the two strands. Molecular genetic analysis revealed heterogeneous gene mutations in all clinical forms of β-galactosidosi such as missense/nonsense mutation, insertion/duplication, and insertion causing splicing defect. Neither the type nor location of mutation in the gene is correlated to the clinical phenotype. Five common mutations have been known: R482H in Italian patients witl infantile G_{M1} gangliosidosis; R208C in American patients with infant G_{M1} gangliosidosis, R201C in Japanese patients with juvenile G_{M1} gangliosidosis; I51T in Japanese patients with adult G_{M1} gangliosido and W273L in Caucasian patients with Morquio B disease. Restrictio analysis has been successfully performed for the diagnosis of the common mutations in new patients.

7. Morphologic, pharmacologic, and biochemical aberrations have been found in the brain of G_{M1} gangliosidosis patients and animals. Meganeurites and ectopic dendrogenesis are observed in G_{M1} gangliosidosis, and the extent of meganeurite development is relate the onset, severity, and clinical course of the disease. Various pharmacologic abnormalities have been observed in feline G_{M1} gangliosidosis, such as cholinergic dysfunction, neuroaxonal dystrop in GABAergic neurons, and alteration of phospholipase C and adenyl cyclase activities. These data suggest that morphologic and metabol effects occur in the presence of excessive storage of ganglioside G_M

8. G_{M1} gangliosidosis has been recorded in cats, dogs, sheep, and calv These animals showed various central nervous system manifestatior β-Galactosidase is deficient, and storage of G_{M1} and oligosaccharide has been confirmed. Furthermore, mouse models have been genera by disruption of the β-galactosidase gene. The β-galactosidase-defici knockout mouse presented with progressive neurologic manifestatio few months after birth. Clinical, pathologic, and biochemical analysis indicated that this also is an authentic model of human G_{M1}

gangliosidosis. In addition, phenotype-specific model mice have bee produced by introducing human mutant genes, resulting in various clinical forms of β-galactosidosis (knockout-transgenic mice). These mice models are used for new therapeutic approaches to human β-galactosidosis patients.

9. The mouse model of juvenile G_{M1}-gangliosidosis expressing the R20 mutation was used for a new molecular therapy using a low-molecul weight compound, N-octyle-4-epi-β-valienamine (NOEV). Orally fed NOEV passed through the blood-brain barrier, enhanced the deficier galactosidase activity, and induced degradation of G_{M1} and G_{A1} in tl central nervous system. This new molecular therapy (chemical chaperone therapy) will be useful for certain patients with β-galactosidosis and potentially other lysosomal storage diseases with central nervous system involvement.

HISTORY

In 1959, Norman et al.[1] reported a patient with a specific form of amaurot idiocy-"Tay-Sachs disease with visceral involvement." Clinical and patholoç findings resembled those of Tay-Sachs disease, but lipid-laden histiocytes were observed in extraneural tissues. The stored material was ganglioside, not sphingomyelin. Craig et al.[2] also described an infant with clinical and radiologic features suggestive of Hurler disease-"an unusual storage diseas resembling the Hurler-Hunter disease." The "foam-cell" histiocytes that we found in viscera did not contain mucopolysaccharides.

Subsequently, after a preliminary study of four patients with "pseudo-Hurl disease,"[3] Landing et al.[4] established a new disease, called "familial neurovisceral lipidosis," as a clinicopathological entity. Their eight patients showed (a) clinical and radiologic findings suggesting those of Hurler disea (psychomotor deterioration with dysmorphism); (b) pathologic features resembling those of Niemann-Pick disease, but with certain distinctive features, including involvement of glomerular epithelium; and (c) histochemical properties of the stored material differing from those seen w previously defined lipidoses. Biochemical analysis revealed generalized accumulation of ganglioside G_{M1} in brain and viscera,[5] and the term *generalized gangliosidosis* was proposed as a new inborn error of metaboli Clinical signs and symptoms developed in early infancy in all patients in th reports. The same disease was described as a biochemically special form o infantile amaurotic idiocy,[6] Tay-Sachs disease with visceral involvement,[7] familial infantile amaurotic idiocy with visceral involvement,[8] Landing disea generalized gangliosidosis of Norman-Landing type,[10] and G_{M1} gangliosidosis.[11]

Later, cases were recognized of later onset ("late infantile systemic lipidosi without distinctive clinical or radiologic features.[12,13] Storage of G_{M1} was remarkable in brain but not in viscera. The patients with this G_{M1} storage disease were subsequently divided into two clinical forms on the basis of clinical and biochemical data.[14] Type 1 is characterized by the onset of neurologic deterioration and visceromegaly before 6 months of age, associ with dysmorphism and skeletal deformities, and type 2 by later onset (7 tc months) without specific physical findings.

β-Galactosidase deficiency was demonstrated first by Okada and O'Brien,[15] and then a widespread biochemical screening started. As a result, patients with later onset[16,17] and atypical cases in adults with more protracted clini

courses[18] were found. Extrapyramidal signs and symptoms were the majoı manifestations in adults, starting around 10 years of age and progressing ˈ slowly over 20 years.[18] Otherwise, there were no specific neurologic or somatic abnormalities except for slight vertebral deformities (flattening). Biochemical screening detected a specific deficiency of β-galactosidase in leukocytes and serum. This disease was classified as the adult form of G_{M1} gangliosidosis.[18]

On the other hand, spondyloepiphyseal dysplasia and somatic dysmorphisr were found in a patient with β-galactosidase deficiency.[19] Intelligence was normal, and no signs of central nervous system involvement were detecteı was described as a Morquio-like syndrome in another report,[20] and a conclusion was drawn for β-galactosidase deficiency in Morquio B disease a primary genetic defect due to allelic mutation of the enzyme gene.[21]

The molecular basis of these phenotypic variations became evident when a cDNA clone for human β-galactosidase was cloned and sequenced.[22] Varioı mutations of the β-galactosidase gene were found in both G_{M1} gangliosidos and Morquio B disease,[23,24] with some overlap between them,[25] and the tε *β-galactosidosis* was proposed on the basis of these molecular genetic observations.[25]

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β-Galactosidase deficiency: An approach to chaperone therapy

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Summary We propose a new molecular therapeutic approach to lysosomal diseases with severe neurological manifestations. Some low-molecular-weight compounds, acting as competitive inhibitors of a lysosomal enzyme *in vitro*, were found to stabilize and restore catalytic activities of the enzyme molecule as a molecular chaperone. We started this trial first in Fabry disease (generalized vasculopathy) using galactose and 1-deoxygalactonojirimycin, and then in β-galactosidase deficiency disorders (β-galactosidosis) with generalized neurosomatic and/or systemic skeletal manifestations (GM_1-gangliosidosis and Morquio B disease), using a newly developed chemical compound *N*-octyl-4-epi-β-valienamine (NOEV). Administration of this chaperone compound resulted in elevation of intracellular enzyme activity in cultured fibroblasts from patients and genetically engineered model mice. In addition, substrate storage was improved after NOEV had been transported into the brain tissue via the blood–brain barrier. We hope this new approach (chemical chaperone therapy) will be useful for certain patients with β-galactosidosis and potentially other lysosomal storage diseases with central nervous system involvement.

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Introduction

GM_1-gangliosidosis (OMIM 230500) is a neurogenetic disease caused by mutations of the gene *GLB1* (3p21.33) that codes for lysosomal β-galactosidase (EC 3.2.1.23) with clinical onset at various ages (Suzuki et al 2001). The forms are classified as infantile, juvenile and adult forms. Another rare systemic bone disease, Morquio B disease, is also known also to be caused by different mutations of the same gene, resulting in β-galactosidase deficiency. Glycoconjugates with terminal β-galactose residues accumulate in tissues and body fluids from patients with these clinical phenotypes. Ganglioside GM_1 and its asialo derivative GA_1 accumulate in the GM_1-gangliosidosis brain. High amounts of oligosaccharides derived from keratan sulphate or glycoproteins are detected in visceral organs and urine from GM_1-gangliosidosis and Morquio B disease patients.

At present only symptomatic therapy is available for human β-galactosidosis patients. Allogeneic bone marrow transplantation did not modify subsequent clinical course or cerebral enzyme activity in a Portuguese water dog affected with GM_1-gangliosidosis (O'Brien et al 1990). Amniotic tissue transplantation was not effective in a patient with Morquio B disease (Tylki Szymanska et al 1985). Enzyme replacement therapy conducted for Gaucher disease and other lysosomal storage diseases is not available at present for β-galactosidosis. An experiment to inhibit GM_1 synthesis resulted in reduction of the GM_1 content in the mouse brain, but not of GA_1 (Kasperzyk et al 2004, 2005). More evaluation is necessary for the therapeutic trial of this type.

We tried to develop a new therapeutic approach to lysosomal storage diseases, particularly with the central nervous involvement. A molecular analysis revealed that some mutant proteins expressed in culture cells from Fabry patients

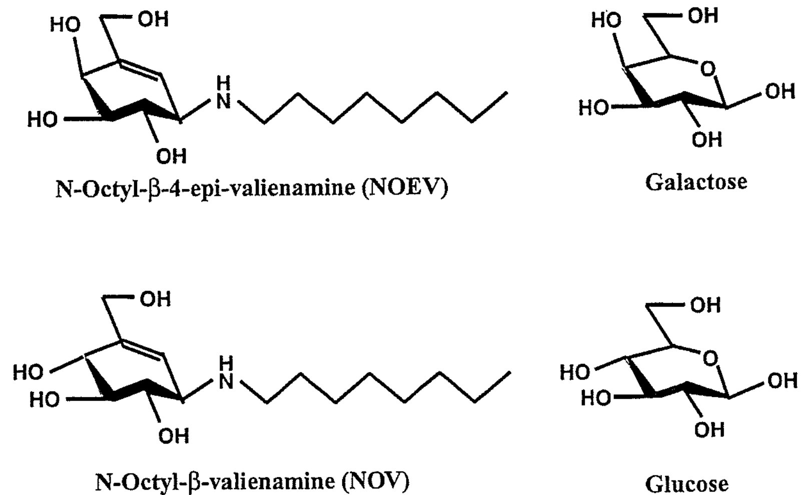

Fig. 1 Structure of NOEV and NOV. The compounds are analogues of galactose and glucose, respectively

do not exhibit the catalytic activity because of molecular instability of the molecule (Okumiya et al 1995a). Subsequently the unstable protein was found to have a defect in molecular folding and rapid degradation after biosynthesis (Ishii et al 1996). Zhang and colleagues (2000) reported the same result in a study of the mutant enzyme in an infantile GM_1-gangliosidosis patient. The R148S β-galactosidase mutation resulted in a major conformational change of the protein molecule with normal catalytic activity, and failed to reach the lysosome.

Simultaneously, trials to stabilize the mutant protein revealed that galactose in the culture medium was able to induce a high expression of the mutant α-galactosidase A gene in cultured lymphoblasts in both classical and atypical (cardiac) form of Fabry disease (Okumiya et al 1995b). This result prompted us to search for more potent inducers of mutant gene expression among commercially available chemical compounds structurally similar to galactose. 1-Deoxygalactonojirimycin (DGJ) was found to be the best candidate for a possible new molecular therapy of Fabry disease in cultured lymphoblasts and transgenic mice (Fan et al 1999).

Concept of chaperone therapy

There are three possible types of mutant gene expression in somatic cells.

1. No biosynthesis of the mutant protein.
2. Extremely low or completely deficient activity of the expressed mutant protein.
3. Expression of unstable mutant protein with normal or near-normal catalytic activity.

We tested these possibilities in Fabry disease, and found a surprisingly high percentage of the third possibility in Fabry disease and β-galactosidosis, although the rate of effectiveness depends on the definition of therapeutic effect in culture cell experiments (Iwasaki et al unpublished data). These mutant proteins are unstable at neutral pH in the endoplasmic reticulum/Golgi apparatus, and are rapidly degraded without appropriate molecular folding (Ishii et al 1996; Okumiya et al 1995a).

After galactose we found a commercially available compound, DGJ, for induction of enhanced mutant gene expression and enzyme activity of α-galactosidase A (Fabry disease; Fan et al 1999). Next, new chemically synthesized compounds were tried for this new approach: *N*-octyl-4-epi-β-valienamine (NOEV) for β-galactosidase (GM_1-gangliosidosis and Morquio B disease; Matsuda et al 2003) and *N*-octyl-β-valienamine (NOV) for β-glucosidase (Gaucher disease; Lin et al 2004) (Fig. 1).

Exogenous compounds that inhibit enzyme activity *in vitro* bind to the mutant enzyme intracellularly around the endoplasmic reticulum/Golgi apparatus, resulting in formation at neutral pH of a complex consisting of the mutant protein and chaperone compound. The catalytically active mutant gene is now stabilized, and the protein–chaperone complex is safely transported to the lysosome. The complex dissociates under the acidic conditions in the lysosome, and the mutant enzyme remains stabilized and its catalytic function is expressed (Fig. 2).

This strategy depends on the biological activity of the chaperone compound available for the study. In a previous study, we had to add a high dose of galactose (up to 200 mmol/L) in the culture medium of Fabry cells (Okumiya et al 1995b). This is obviously unnatural and deleterious to the function of somatic cells for long-term treatment, although a short-term human experiment demonstrated a positive therapeutic effect after high-dose intravenous galactose in a Fabry patient (Frustaci et al 2001).

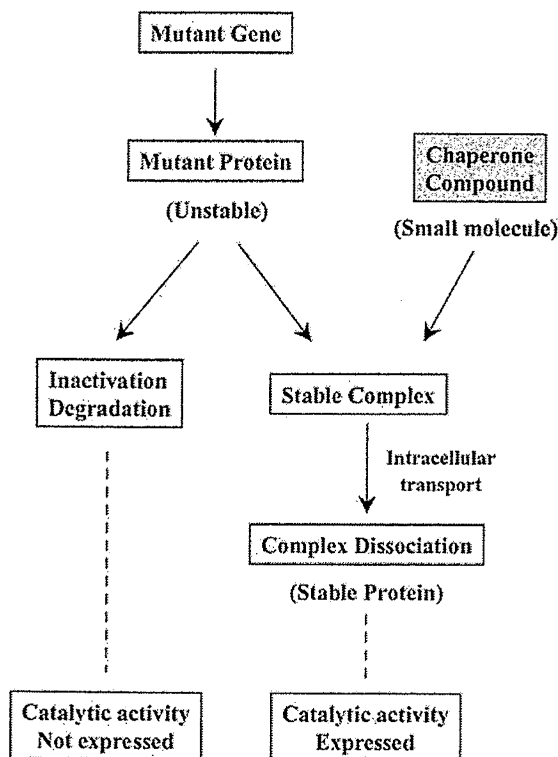

Fig. 2 Intracellular events induced by exogenous chemical chaperones supplied to the cell

NOEV was more efficient than DGJ for expression of β-galactosidase activity in GM_1-gangliosidosis as compared to α-galactosidase A activity in Fabry disease (Matsuda et al 2003; Tominaga et al 2001). Our calculation indicates that at least 10% normal enzyme activity is necessary for washout of the storage substrate in lysosomal diseases. The age of onset in patients expressing the enzyme activity above this level will be theoretically beyond the human lifespan (unpublished data).

Determination of intracellular chaperone concentration is technically not possible at present. We anticipate NOEV concentrations in human cells and animal tissues being much lower than the IC_{50} of NOEV *in vitro*. In fact, the NOEV concentration in the tissue culture medium was approximately equal to the IC_{50} in our cell culture experiments (Matsuda et al 2003).

NOV and NOEV: Chemical synthesis and characterization

Fortunately we found a commercially available compound, DGJ, for possible chaperone therapy of Fabry disease (Fan et al 1999). However, an extensive search for other galactose derivatives and analogous compounds did not reveal any material significantly active for β-linked galactose substrates. I happened to come across an inhibitor originally synthesized for chemical analysis of enzyme reactions catalysed by glucocerebrosidase (β-glucosidase)—NOV (Ogawa et al 1994, 1996, 1998; Tsunoda et al 1995). Gaucher disease is caused by deficiency of this enzyme. We then tried to develop chemical compounds related to this glucose derivative.

First, NOV was synthesized by chemical modification of the original glucocerebrosidase inhibitor, followed by replacing the ceramide moiety with simple aliphatic chains (Ogawa et al 1996, 1998). Subsequently, NOEV was synthesized by multistep epimerization of NOV at C4 (Ogawa et al 2002, 2004). Both NOV and NOEV (Fig. 1) were tested simultaneously, but characterization and evaluation of NOEV were quicker than for NOV simply because we had more experience in β-galactosidase and collected more clinical samples from patients with β-galactosidase deficiency.

NOEV is a potent inhibitor of lysosomal β-galactosidase *in vitro*. Addition of NOEV in the culture medium restored mutant enzyme activity in cultured human or murine fibroblasts at low intracellular concentrations, resulting in a marked decrease of intracellular substrate storage (see below). Its structure was assigned by a combination of COSY, TOCSY and HSQC NMR spectroscopy (Matsuda et al 2003). NOEV is stable at room temperature, and a is strong inhibitor of human β-galactosidase *in vitro*. It is freely soluble in methanol or dimethyl sulfoxide, and soluble in water up to 3–5 mmol/L at room temperature. Its molecular weight is 287.40. The IC_{50} is 0.2 μmol/L towards human β-galactosidase.

Effect of NOEV on cultured human and mouse fibroblasts expressing mutant human genes

In human fibroblast experiments, cells derived from juvenile and infantile GM_1-gangliosidosis patients expressed an increase of β-galactosidase activity after NOEV treatment (Iwasaki et al, unpublished data). Under the conditions of our study, we found two different types of response among the cells for analysis. Some cells responded to NOEV maximally at 0.2 μmol/L, such as R457Q, and others at 2 μmol/L, such as R201C and R201H. This result indicates that the molecular interaction between the chaperone compound and mutant protein is mutation-specific.

Mouse tissues expressing mutant human β-galactosidase showed essentially the same results (Matsuda et al 2003) (Fig. 3). However, the degree of enhancement was different in some mutations between the human and mouse cells. A 5- to 10-fold increase was observed for the R427Q mutation at 0.2 μmol/L of NOEV in the culture medium. A higher concentration (2 μmol/L) was necessary to reach the same degree with the R201C or R201H mutations (Iwasaki et al unpublished data). About one-third of the cells from patients with GM_1-gangliosidosis responded to this treatment; almost all patients with juvenile GM_1-gangliosidosis, and some of the patients with infantile GM_1-gangliosidosis, responded

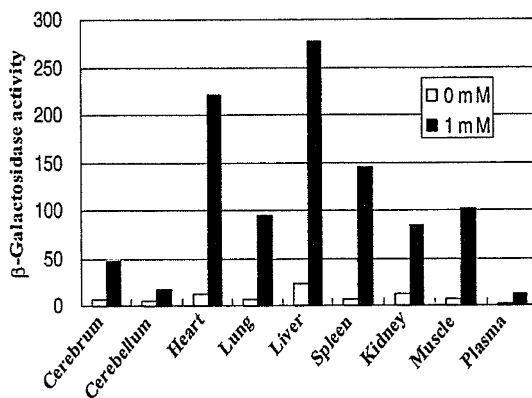

Fig. 3 Effect of NOEV on β-galactosidase activity in mouse tissues. Oral administration (*ad libitum*) of 1. mmol/L NOEV to 5-week old R201C mice for 5 weeks; each value is the mean of two experiments. The enzyme activity increased remarkably in all tissues examined, including cerebrum and cerebellum. Courtesy of Dr Junichiro Matsuda (National Institute of Infectious Diseases, Tokyo; currently National Institute of Biomedical Innovation, Osaka).

significantly. Almost the same or greater restorative effect was achieved with NOEV at 50-fold lower concentration than that with DGJ or *N*-butyldeoxygalactonojirimycin (Tominaga et al 2001).

After adding the mixture of gangliosides to the culture medium, intracellular GM_1 increased remarkably in R201C cells, but only slightly in the cells expressing the normal human gene. Incubation with NOEV significantly reduced GM_1 storage in the cells expressing the mutation R201C causing juvenile GM_1-gangliosidosis (Matsuda et al 2003).

Chaperone therapy on genetically engineered GM_1-gangliosidosis model mice

The R201C mice, expressing the human R201C-mutant β-galactosidase but lacking the endogenous mouse β-galactosidase (Matsuda et al 1997, 2003), had very low β-galactosidase activity in the brain (about 4% of the wild-type activity). They exhibited an apparently normal clinical course for the first 6 months after birth, followed by slowly progressive neurological deterioration, such as tremor and gait disturbance, during the next 9 months. Death occurred around 15 months of age due to malnutrition and emaciation.

Neuropathology revealed vacuolated or ballooned neurons, less abundant than in the knockout mouse brain described in our previous reports (Itoh et al 2001; Matsuda et al 1997). Cytoplasmic storage materials were present in pyramidal neurons and brainstem motor neurons, but not in neurons in the other areas of the brain.

Short-term oral administration of NOEV to the R201C model mouse (Matsuda et al 2003) resulted in significant enhancement of the enzyme activity in all the tissues examined, including the brain. Immunohistochemical staining revealed an increase in β-galactosidase activity and a decrease in GM_1 and GA_1 storage. However, mass biochemical analysis did not show the substrate reduction observed histochemically in these limited areas in the brain, probably because of the brief duration of treatment and only localized substrate accumulation at the early stage of the disease in this experiment. NOEV was found in significant amount in the central nervous system by mass spectrometric analysis, at the 30% level in the liver tissue in mice treated by oral administration of NOEV solution for 8 weeks (Kubo et al, unpublished data).

The experimental data to date are summarized in Table 1.

Neurological examination of genetically engineered GM_1-gangliosidosis model mice

We are currently trying to establish a system of neurological examination in the GM_1-gangliosidosis model mice that we prepared for the chaperone therapy experiments. This is essentially an application of the child neurology technique to the mouse species, using clinical observation, video monitoring, manual manipulation, and apparatus-assisted examination developed for neurological evaluation of mice and rats. We evaluate spontaneous movements, body and limb postures, behavioural patterns in an open field, primitive reflexes, postural reflexes, and equilibrium reactions. Data are being collected for normal (wild-type), transgenic and knockout mice, with or without NOEV administration. It is hoped that this systematic approach will be useful for monitoring the clinical course of a large number of genetically engineered model mouse strains for evaluating physiological roles of individual genes. Improvements of the posture and movements have been observed in some mice after NOEV

Table 1 Experimental data on biological activity of NOEV

1. NOEV inhibits β-galactosidase activity *in vitro* at high concentrations.
2. NOEV induces expression of mutant β-galactosidase activity *in situ* at low concentrations.
3. The biological activity of NOEV is mutation-specific.
4. NOEV is delivered through the blood–brain barrier by oral administration to disease model mice.
5. Oral administration of NOEV induces expression of mutant β-galactosidase activity in the mouse brain.
6. Oral administration of NOEV results in degradation of storage substrates.
7. Oral administration of NOEV improves or prevents the central nervous system manifestations (preliminary data).
8. Short-term oral administration of NOEV does not cause significant adverse effects to disease model mice.

administration for a few weeks in a preliminary experiment (Ichinomiya et al, in preparation).

Prospects

Chaperone therapy has two major advantages over enzyme replacement therapy: oral administration and accessibility to the brain. NOEV is a good candidate compound for this new therapeutic approach, particularly for the central nervous system pathology, as it is a small molecule that passes through the blood–brain barrier from the bloodstream, stabilizes mutant protein in neurons, and induces expression of enzyme activity. Clinical evaluation has not yet been completed, but we have some evidence that this compound could partially improve the disease progress in some mice after even a few months of low-dose administration in the early stage of the disease (Suzuki et al, unpublished data). We need long-term experiments to establish an optimal dose for prevention of clinical manifestations, accompanied by reduction of substrate storage, in these model mice. Possible adverse or toxic effects should be carefully evaluated before human clinical experiments are started.

We are aware that this new molecular approach is not justified for all patients with a single lysosomal enzyme deficiency disorder. Biosynthesis of a catalytically active enzyme is prerequisite in chemical chaperone therapy. Our initial survey indicates that 20–40% of β-galactosidosis (mainly GM_1-gangliosidosis) patients express unstable but catalytically active protein and respond to NOEV treatment in cultured fibroblasts. Patients of this type will be reasonable candidates for chemical chaperone therapy.

This strategy is in principle applicable to all lysosomal storage diseases if a specific compound is available for each enzyme in question. Special drug design technology may be needed to screen appropriate inhibitors. Bioinformatics analysis will develop a new aspect of molecular pathology in lysosomal storage diseases (Durand et al 2000; Fabrega et al 2000).

This study started with a lysosomal disease, and a number of related diseases became the target of this approach. Theoretically this principle can be applied to all other lysosomal diseases. At present our study is focused on diseases with storage of compounds with α- or β-linked glucose or galactose residues at the terminal end of the carbohydrate chain in the substrate molecule: α-glucosidase deficiency (glycogenosis II), β-glucosidase deficiency (Gaucher disease), α-galactosidase deficiency (Fabry disease), and β-galactosidase deficiency (β-galactosidosis; GM_1-gangliosidosis and Morquio B disease). We hope to extend this approach to the other lysosomal diseases in future if a specific chaperone compound is found for each disease.

Further, there may be other categories of diseases that are good targets of this approach if the molecular pathology in somatic cells has been studied and well understood in detail regarding mutant gene expression, mutant protein structure, intracellular transport of the protein, mechanism of functional expression, etc. It is hoped that studies in this direction will in future reveal new aspects of molecular therapy for inherited metabolic diseases with central nervous system involvement.

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