413. Suzuki K, Vanier MT, Hahn CN, Schroder M, Hara Y, d'Azzo A, del Pilar: Generalized CNS disease and massive G_{M1}-ganglioside accumulation in mice defective in lysosomal acid β-galactosidase. *Hum Mol Genet* **6**:205, 1997. PMID 9063740

414. Lindsey JR, Herndon RM, Farrell DF, Baker HJ, McKhann GM: Feline G_{M1}-gangliosidosis: Biochemical and ultrastructural comparisons with the disease in man. *J Neuropathol Exp Neurol* **32**:1, 1973. PMID 4119597

415. Lindsey JR, Baker HJ: Animal model: Feline G_{M1}-gangliosidosis. *Am J Pathol* **74**:649, 1974. PMID 4856037

416. Blakemore WF, Murray JA, Barnett KC: Ocular lesions in cats with G_{M1}-gangliosidosis with visceral involvement. *J Small Anim Prac* **18**:1, 1977. PMID 404469

417. O'Brien JS, Holmes EW: Feline G_{M1}-gangliosidosis: Characterization of the residual liver acid β-galactosidase. *Am J Hum Genet* **30**:505, 1978. PMID 83795

418. Baker HJ, Steiss JE, Braund KG, Cox NR, Wright JC: Profile of electrodiagnostic abnormalities in cats with G_{M1}-gangliosidosis. *Am J Vet Res* **58**:706, 1997. PMID 9215443

419. Thompson JN, Baker HJ, Nowakowski RW: Diagnosis of feline G_{M1}-gangliosidosis by enzyme assay of cultured conjunctival cells. *Invest Ophthalmol Vis Sci* **29**:487, 1988. PMID 3125124

420. Walkley SU, Karabelas AB: Altered patterns of evoked synaptic activity in cortical pyramidal neurons in feline ganglioside storage disease. *Brain Res* **339**:329, 1985. PMID 2992703

421. Baker HJ, Jope RS, Koenig ML, Lally KM: Reduced Ca^{2+} flux in synaptosomes from cats with G_{M1}-gangliosidosis. *Brain Res* **424**:169, 1987. PMID 3121133

422. Baker HJ, Shanker G: Phorbol ester receptors in cerebral cortex of cats with G_{M1}-gangliosidosis. *Neurochem Res* **16**:11, 1991. PMID 1904996

423. Zhou J, Cox NR, Ewald SJ, Morrison NE, Basker HJ: Evaluation of G_{M1}-ganglioside-mediated apoptosis in feline thymocytes. *Vet Immunol Immunopathol* **66**:25, 1998. PMID 9847018

424. Alroy J, DeGasperi R, Warren CD, Raghavan SS, Richard R, Orgad U, Knowles K, Thalhammer JG: Canine G_{M1}-gangliosidosis: A clinical, morphologic, histochemical, and biochemical comparison of two different models. *Am J Pathol* **140**:675, 1992. PMID 1546746

425. Kolodny EH, Pastores GM, Alroy J, Johnson GS, Wang ZH, Raghavan S, Shibuya H, Zeng B: Isolation and characterization of the normal canine β-galactosidase gene and its mutation in a dog model of G_{M1}-gangliosidosis. *J Inherit Metab Dis* **23**:593, 2000. PMID 11032334

426. Wood PA, Giger U, Carithers R, Saunders GK, Shell LG, Katherman A, Potthoff AL: Neuronal-visceral G_{M1}-gangliosidosis in Portuguese water dogs. *J Vet Intern Med* **3**:1, 1989. PMID 2494322

427. Muller G, Moritz A, Kustermann-Kuhn B, Sewell A, Baumgartner W: Biochemical findings in a breeding colony of Alaskan huskies suffering from G_{M1}-gangliosidosis. *J Inherit Metab Dis* **21**:430, 1998. PMID 9700604

428. Muller G, Moritz A, Sewell A, Baumgartner W, Alldinger S, Zurbriggen A, Kirchhof N: G_{M1}-gangliosidosis in Alaskan huskies: Clinical and pathologic findings. *Vet Pathol* **38**:281, 2001. PMID 11355658

429. Tajima M, Umemura T, Maede Y, Iijima M, Yamato O, Ochiai K, Masuoka Y, Hayashida E, Omae S: G_{M1}-gangliosidosis in shiba dogs. *Vet Rec* **146**:493, 2000. PMID 10887996

430. Hatakeyama A, Satoh H, Tajima M, Kobayashi A, Yamasaki M, Maede Y, Yamato O, Masuoka Y, Endoh D, Yonemura M: A novel mutation in the gene for canine acid β-galactosidase that causes G_{M1}-gangliosidosis in Shiba dogs. *J Inherit Metab Dis* **25**:525, 2002. PMID 12555949

431. Alroy J, Kaye EM, Rosenberg AE, Schelling SH, Knowles K: Retarded bone formation in G_{M1}-gangliosidosis: A study of the infantile form and comparison with two canine models. *Virchows Arch* **426**:141, 1995. PMID 7757284

432. Alroy J, Kaye EM, Zuniga G, Raghavan SS, Schwarting GA, Thalhammer JG, Adelman LS, Runge V, Gelblum D: Dysmyelinogenesis in animal model of G_{M1}-gangliosidosis. *Pediatr Neurol* **8**:255, 1992. PMID 1388413

433. Yamauchi T, Satoh H, Arai T, Asano T, Yamasaki M, Maede Y, Yamato O, Yonemura M, Hasegawa D, Orima H: Cerebrospinal fluid biomarkers showing neurodegeneration in dogs with G_{M1} gangliosidosis: Possible use for assessment of a therapeutic regimen. *Brain Res* **1133**:200, 2007. PMID 17196562

434. O'Brien JS, Hubert JJ: Dog and human acid β-d-galactosidases are structurally similar. *Biochem J* **213**:473, 1983. PMID 6412684

435. O'Brien JS, Kretz KA, Ahern-Rindell AJ: Comparison of the canine and human acid β-galactosidase gene. *Am J Med Genet* **63**:340, 1996. PMID 8725782

436. Alroy J, Warren CD, De Gasperi: Application of lectin histochemistry and carbohydrate analysis to the characterization of lysosomal storage diseases. *Carbohydr Res* **213**:229, 1991. PMID 1933939

437. Ahern-Rindell AJ, Prieur DJ, Murnane RD: Ultrastructural lesions of ovine G_{M1}-gangliosidosis. *Mod Pathol* **4**:755, 1991. PMID 1788267

438. Ahern-Rindell AJ, Prieur DJ, Murnane RD: Lectin histochemistry of an ovine lysosomal storage disease with deficiency of β-galactosidase and β-neuraminidase. *Am J Pathol* **135**:623, 1989. PMID 2508478

439. Ahern-Rindell AJ, Prieur DJ, Murnane RD: Interspecific genetic complementation analysis of human and sheep fibroblasts with β-galactosidase deficiency. *Somat Cell Mol Genet* **15**:525, 1989. PMID 2512653

440. Walkley SU, Ahern-Rindell AJ, Prieur DJ, McCluer RH, Daniel PF, Raghavan SS, Murnane RD, Parish SM: Inherited lysosomal storage disease associated with deficiencies of β-galactosidase and β-neuraminidase in sheep. *Am J Med Genet* **31**:39, 1988. PMID 3146925

441. Ahern-Rindell AJ, Prieur DJ, Murnane RD, Parish SM, Holler LD: Clinical and clinicopathologic characteristics of ovine G_{M1}-gangliosidosis. *J Vet Intern Med* **8**:221, 1994. PMID 8064659

442. Prieur DJ, Murnane RD, Ahern Rindell, Wright RW: Prenatal lesions in an ovine fetus with G_{M1}-gangliosidosis. *Am J Med Genet* **39**:106, 1991. PMID 1907804

443. Ryder SJ, Simmons MM: A lysosomal storage disease of Romney sheep that resembles human type 3 G_{M1} gangliosidosis. *Acta Neuropathol* **101**:225, 2001. PMID 11307621

444. Donnelly WJ, Sheahan BJ, Johnson AH: The glycosaminoglycan content of the liver in bovine G_{M1}-gangliosidosis. *Res Vet Sci* **22**:256, 1977. PMID 404680

445. Donnelly WJ, Sheahan BJ, Grimes TD: Ocular pathology of bovine G_{M1}-gangliosidosis. *Acta Neuropathol* **41**:91, 1978. PMID 416644

446. Itoh M, Oshima A, Suzuki Y, Matsuda J, Ogura A, Takashima S, Tai T, Suzuki O: Development of lysosomal storage in mice with targeted disruption of the β-galactosidase gene: A model of human G_{M1}-gangliosidosis. *Brain Dev* **23**:379, 2001. PMID 11578847

447. Tettamanti G, Bassi R, Riboni L, Caminiti A: The degradative pathway of gangliosides G_{M1} and G_{M2} in Neuro-2a cells by sialidase. *J Neurochem* **64**:451, 1995. PMID 7798945

448. Venn G, Mason RM: Absence of keratan sulphate from skeletal tissues of mouse and rat. *Biochem J* **228**:443, 1985. PMID 3160342

449. Heinegard D, Wikstrom B, Engfeldt B, Hjerpe A: Proteoglycans and glycosaminoglycans in cartilage from the brachymorphic (*bm/bm*) mouse. *Collagen Related Res* **5**:193, 1985.

450. O'Brien JS, Kishimoto Y, Storb R, Graham T, Morimoto S, Appelbaum F, Raff RF, Harding J, O'Brien SL, Ahern Rindell: Bone marrow transplantation in canine G_{M1}-gangliosidosis. *Clin Genet* **38**:274, 1990. PMID 2125250

451. Tylki-Szymanska A, Kidawa M, Macielko D, Jablonska-Budaj U: Amniotic tissue transplantation as a trial of treatment in some lysosomal storage diseases. *J Inherit Metab Dis* **8**:101, 1985. PMID 3939584

452. Ikeda S, Yanagisawa N, Ushiyama M, Hanyu N: A case of type III (adult) G_{M1}-gangliosidosis that improved markedly with trihexyphenidyl. *Clin Neurol* **26**:221, 1986.

453. Oshima A, Suzuki Y, Maeda M, Tanaka A, Yagi T, Nanba E, Yamano T, Takaura N: Attenuation of ganglioside G_{M1} accumulation in the brain of G_{M1}-gangliosidosis mice by neonatal intravenous gene transfer. *Gene Ther* **10**:1487, 2003. PMID 12900764

454. Suzuki Y, Ishii S, Kase R, Sakuraba H: Characterization of a mutant β-galactosidase gene product for the late-onset cardiac form of Fabry disease. *Biochem Biophys Res Commun* **197**:1585, 1993. PMID 7904161

455. Suzuki Y, Ishii S, Kase R, Sakuraba H, Okumiya T, Kamei S: β-Galactosidase gene mutations in Fabry disease: Heterogeneous expressions of mutant enzyme proteins. *Hum Genet* **95**:557, 1995. PMID 7759078

456. Suzuki Y, Ishii S, Kase R, Sakuraba H, Takenaka T, Okumiya T, Kamei S: Galactose stabilizes various missense mutants of β-galactosidase in Fabry disease. *Biochem Biophys Res Commun* **214**:1219, 1995. PMID 7575533

457. Suzuki Y, Ishii S, Asano N, Fan JQ: Accelerated transport and maturation of lysosomal β-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med* **5**:112, 1999. PMID 9883849

458. Desnick RJ, Ricci R, Frustaci A, Russo MA, Eng CM, Chimenti C, Natale L, Pieroni M: Improvement in cardiac function in the cardiac variant of Fabry's disease with galactose-infusion therapy. *N Engl J Med* **345**:25, 2001. PMID 11439944

459. Oshima A, Ohno K, Suzuki Y, Taniguchi M, Ogawa Y, Nanba E, Tominaga L: Galactonojirimycin derivatives restore mutant human β-galactosidase activities expressed in fibroblasts from enzyme-deficient knockout mouse. *Brain Dev* **23**:284, 2001. PMID 11504597

460. Eto Y, Lin H, Ohno K, Suzuki Y, Sugimoto Y, Ogawa S, Inoue T, Taniguchi M, Nanba E, Ida H, Oka A, Higaki K, Ninomiya H, Sawa M, Matsuzaki Y, Ohsaki Y: *N*-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: A potential chemical chaperone therapy for Gaucher disease. *Biochim Biophys Acta* **1689**:219, 2004. PMID 15276648

461. Suzuki Y, Ogawa S, Kanto M: Development and medical application of unsaturated carbaglycosylamine glycosidase inhibitors. *Mini Rev Med Chem* **7**:679, 2007. PMID 17627580

462. Suzuki Y: β-Galactosidase deficiency: An approach to chaperone therapy. *J Inherit Metab Dis* **29**:471, 2006. PMID 16763919

463. Suzuki Y: Chemical chaperone therapy for G_{M1}-gangliosidosis. *Cell Mol Life Sci* **10**:351, 2008.

464. Itoh M, Iwasaki H, Brady RO, Watanabe H, Ohno K, Suzuki Y, Ogawa S, Iida M, Nanba E, Higaki K, Noguchi Y, Takimoto K, Ohkubo M, Kubo T, Tabe M, Ichinomiya S, Kurosawa M, Matsuda : Chemical chaperone therapy: Clinical effect in murine G_{M1}-gangliosidosis. *Ann Neurol* **62**:671, 2007. PMID 17994547

465. Iwasaki H, Watanabe H, Suzuki Y, Matsuda J, Maruyama K, Toda H, Ichinomiya S, Kurosawa M: Neurological assessment of G_{M1}-gangliosidosis model mice. *Brain Dev* **29**:210, 2007. PMID 17027211

466. Eto Y, Suzuki M, Brady RO, Ohno K, Suzuki Y, Ogawa S, Inoue T, Iida M, Lei K, Kaneski C, Ida H, Ninomiya H, Sawa M: Enzyme enhancement activity of *N*-octyl-β-valienamine on β-glucosidase mutants associated with Gaucher disease. *Biochim Biophys Acta* **1772**:587, 2007. PMID 17363227

467. Parenti G, Andria G, Meroni G, Rossi M, Filocamo M, Zuppaldi A, Gabriela PM, Rosaria TM, Annunziata I, Porto C, Donaudy F, Rossi B, Donati A, Bembi BB: Pharmacological enhancement of mutated β-glucosidase activity in fibroblasts from patients with Pompe disease. *Mol Ther* **15**:508, 2007. PMID 17213836

468. Kroos MA, Reuser AJ, Takeuchi H, Okumiya T, van der, Vliet LV: Chemical chaperones improve transport and enhance stability of mutant β-glucosidases in glycogen storage disease type II. *Mol Genet Metab* **90**:49, 2007. PMID 17095274

469. Clarke JTR, Tropak M, Mahuran DJ, Kok F, Stockley T, Maegawa GHB, Butner J: Pyrimethamine as a potential pharmacological chaperone for late-onset forms of G_{M2}-gangliosidosis. *J Biol Chem* **282**:9150, 2007. PMID 17237499

ケミカルシャペロン療法

鈴木義之
[国際医療福祉大学大学院]

はじめに

神経遺伝病に対するケミカルシャペロン療法の概念・方法論・開発に関する研究は，われわれが日本国内で行った研究データをもとに発展させ，薬剤となる化合物を発見し，モデル動物での治療効果を確認し，ヒト患者診療を目標としている新しい独創的な治療アプローチである．

遺伝病治療の最終目標は原因遺伝子の修復である．しかし，成人で 10^{14}〜10^{15} 個と予想される体細胞すべてを治療することは，現在では技術的に不可能である．筆者の大まかな印象では，遺伝病の 2/3 は小児期に発生する神経病である．われわれは，過去 15 年あまり，神経遺伝病に発現する変異蛋白質の機能復元を目的として，低分子シャペロンによる治療実験を進めてきた．

"シャペロン"は元来フランス語"chaperon"であるが，イギリスで，貴族や貴婦人の頭巾・帽子(14〜16 世紀)，ガーター勲位装束(16 世紀)，棺を引く馬の飾り(17 世紀)から，社交界にデビューする若い未婚女性に付き添う既婚女性(18 世紀)の意味に使われた．現在の臨床医学では，医師が異なった性(特に女性)の患者を診察するとき医師に付き添う人(女性)という意味で使われる．現代英語(特に米語)では chaperone という女性形単語として使われることが多い．生物学では，他の蛋白質や蛋白質複合体の適正な折りたたみ(フォールディング)を助ける別の蛋白質を"分子シャペロン"とよぶ(熱ショック蛋白質など)．

リソゾーム病における酵素欠損の病態

リソゾームは，酸性の条件で高分子代謝産物を順序よく加水分解する数十の酵素をもつ細胞内消化器官である．酵素遺伝子の変異は酵素欠損，細胞機能障害による全身病の原因となる(リソゾーム病)．小児期の進行性中枢神経疾患としての病像，経過を示すことが多い[1]．われわれは特に脂質代謝異常(リピドーシス)を対象として，蓄積物，酵素機能，酵素分子，遺伝子変異などを系統的に調べた結果，最終的に臨床医学の目標である予防・治療に目を向けるに至った．なかでもガラクトースとグルコース分子を認識する酵素に関心をもち，β-ガラクトシダーゼ欠損症(GM_1-ガングリオシドーシス，MorquioB 病)，α-ガラクトシダーゼ A 欠損症(Fabry 病)，β-グルコシダーゼ欠損症(Gaucher 病)の分析を進めてきた．

研究の中心はβ-ガラクトシダーゼであり，1988 年に責任遺伝子構造を解明後，変異遺伝子解析，変異蛋白質の動態解析を行い，"酵素欠損"の分子病態が一様でないことを知った[1,2]．そして，①蛋白質分子の合成障害，②蛋白質分子の機能障害，③蛋白質分子の細胞内不安定性の三種に整理することができた．

上記の①，②の病態では正常な酵素蛋白質または遺伝子を補給しない限り，細胞機能の正常化は不可能である．しかし，③の場合，構造上，活性をもつべき酵素蛋白質の不完全なフォールディングのため速やかに分解されてしまう．この蛋白質に適切な細胞内環境を提供すれば，働くべき場所で活性を復元できるかもしれない．

この論理をまず Fabry 病に適用した．実際にガラクトース，1-デオキシガラクトノジリマイシンなどの分子が患者細胞の酵素活性復元に有効であることがわかった[3,4]．しかし筆者の関心は脳病変にあり，全身血管病としての Fabry 病からさらに，神経遺伝病を対象とした研究開発に移ることにした．それが GM_1-ガングリオシドーシスであり，モデル動物の脳の病態修復を試み，成功した[5,6]．

ケミカルシャペロン療法の原理

上記のように，変異蛋白質分子が細胞内で速やかに分解，不活化され，酵素活性が発現しないリソゾーム病患者が存在する．一般に酵素分子に親和性の高い基質類似化合物が試験管内に存在すれば，酵素の競合的阻害薬となる．ところが細胞内にこの化合物が低濃度に存在する場合，**図1**のような分子機構により，中性条件で変異分子とシャペロン分子が安定な複合体をつくり，リソゾームに輸送される．リソゾームの酸性条件で蛋白質分

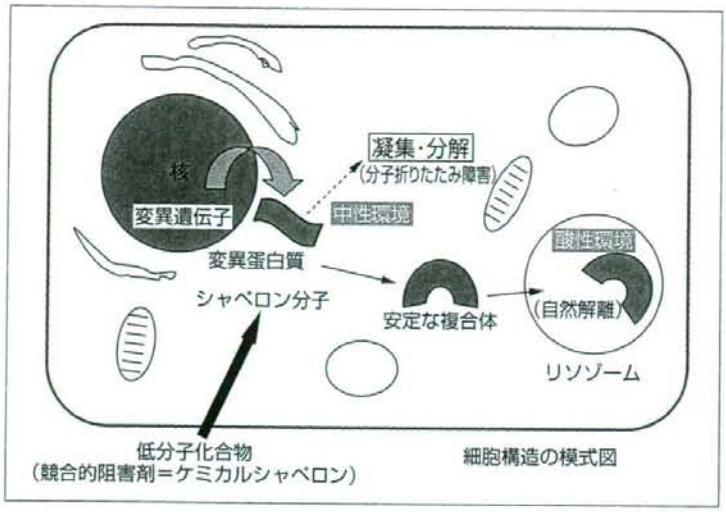

図1 リソゾーム病に対するケミカルシャペロン療法の原理

投与した低分子シャペロン化合物は経口投与後，血流に入り，血液脳関門を通過して中枢神経系でシャペロン効果を示す．細胞内小胞体・ゴルジ体の中性環境で変異蛋白質と結合してその立体構造を修復し，細胞内輸送システムにより無事にリソゾームに運ばれる．リソゾームの酸性環境と過剰の基質存在下で酵素分子とシャペロン分子の複合体は自動的に解離する．変異蛋白質は正常の構造を維持し，酵素としての活性を発現する．ただしシャペロン化合物が過剰に存在すると酵素阻害薬としての効果が出現する．

子は自動的に解離し，安定な酵素活性を発現する．この種の化合物は上記の分子シャペロンと本質的に同じ働きをもつので，ケミカルシャペロン(chemical chaperone)とよぶことにする．

シャペロン化合物の投与により，細胞内酵素の基質処理能力がある閾値以上になれば病気の発症を遅らせることができる．β-ガラクトシダーゼ欠損症の場合，活性が正常の8～10%になれば，計算上，発症年齢が無限大となる．発症と寿命との競争である．

新しい化合物のシャペロン効果

Fabry病細胞の酵素活性復元に有効な市販化合物ガラクトースと1-デオキシガラクトノジリマイシンをGM_1-ガングリオシドーシスにも試みたが，効果は不十分であった．そこで化合物の広範なスクリーニングを行い，β-ガラクトシダーゼ阻害薬NOEV (N-octyl-4-epi-β-valienamine)とβ-グルコシダーゼ阻害薬NOV (N-octyl-β-valienamine)を見出した[7]．

まず培養線維芽細胞へのNOEV投与実験で，β-ガラクトシダーゼ活性が著しく上昇するGM_1-ガングリオシドーシス患者細胞を発見した．若年型の変異R201Cに最も有効であり，乳児型にも有効な変異があった．検査細胞の35%が陽性反応を示した[8]．次いで，すでに確立した酵素欠損ノックアウト(KO)マウス[9](重症型GM_1-ガングリオシドーシス)の線維芽細胞にR201Cを導入したところ，ガングリオシド負荷により脂質が著しく蓄積したが，NOEVにより減少した[5]．

遺伝子組換えモデルマウスの作成とNOEV治療効果

動物個体実験のために，KOマウスにR201Cを導入したトランスジェニック(Tg)マウスを作成した[5]．このTgマウスが軽症型GM_1-ガングリオシドーシスのモデルであることを確認し，NOEV水溶液を1週間経口投与したところ，脳を含むすべての組織の酵素活性が上昇し，脳組織の脂質蓄積が消失した[5]．その後の長期投与実験で，NOEVは経口投与後，速やかに脳組織に入り，酵素活性を上昇させ(正常マウスの30%)，投与中止後速やかに組織から消失した[6]．これまでのところ，マウスに嗜好性や忌避はなく，体重，飲水量，血液生化学分析，検尿，病理観察データに異常を認めていない．

この実験結果から，NOEVが腸管で分解されずに吸収され，血液脳関門を通過して中枢神経系に到達し，酵素分子を安定化し，活性を発現させたとの結論を得た．

シャペロン治療の神経学的評価

NOEVの臨床効果を知るために，マウスの神経学的検査法を開発した．ヒト乳幼児の神経学的診察法をマウスに適用した．自発運動，個体各部位の姿勢肢位，原始反射，姿勢反射，平衡反応など，合計11項目をセットとしたシステムを確立した[10]．それぞれの検査項目を4段階にスコア化し，その合計スコアで総合評価を行った．加齢とともにWTマウス(野生型)，Tgマウス(軽症型)，

KOマウス(重症型)の重症度の差が明確になった[6,10]．そしてTgマウスに発症早期(生後2ヶ月)からNOEVを投与したところ，3ヶ月以内にスコア値の差が明らかになり，神経学的臨床効果を確認することができた[6]．病気進行の中期以後の投薬では効果が必ずしもはっきりしなかった．つまり，当然のことながら神経症状には早期治療が必須であるという結論となった．

他のリソソーム病・他の遺伝病への応用

現在の主要な研究対象はβ-ガラクトシダーゼ欠損症(GM_1-ガングリオシドーシス)であるが，この新しいアプローチはすべてのリソソーム病に適用できるはずである．NOEVはKrabbe病欠損酵素の強い競合的阻害薬でもある．しかし，欠損酵素の活性復元効果はまだ確認できていない．またNOVの有効性がGaucher病で確認されており[11,12]，現在モデル動物作成の試みが進行中である．

われわれはリソソーム病という，細胞内分子病態解析がかなり進んだ疾患群を対象とした研究を行ってきた．他のグループの遺伝病でも細胞内蛋白質動態が明らかになれば，ケミカルシャペロン療法が可能であるはずである．今後多くの種類の遺伝病についての研究が発展することを期待している．

謝　辞

本研究は文部科学省科学研究費(13680918，14207106)ならびに厚生労働省科学研究費(H10-脳-006，H14-こころ-017，H17-こころ-019)の補助金を受けた．ケミカルシャペロン療法の開発は，黒澤美枝子，大久保真人，岩崎博之，渡辺浩史，一ノ宮悟史(国際医療福祉大学)，松田潤一郎(医薬基盤研究所)，難波栄二，檜垣克美，大野耕策(鳥取大学)，滝本一広(国立感染症研究所)，伊藤雅之(国立精神・神経センター)，石井　達(帯広畜産大学)，小川誠一郎，榊原康文(慶應義塾大学)，飯田真己，久保孝利(生化学工業株式会社)，田部美穂(株式会社エスアールエル)の諸氏のほか，多くの共同研究者のご協力により進行中である．ここに記して感謝の意を捧げる．

文献

1) Suzuki Y, Nanba E, *et al.*: β-Galactosidase deficiency (β-galactosidosis): GM_1-gangliosidosis and Morquio B disease, In: Valle D, Beaudet AL, *et al.* (ed), McGraw-Hill, New York, 2008(http://www.ommbid.com/)
2) Suzuki Y: β-Galactosidase deficiency; an approach to chaperone therapy. *J Inherit Metab Dis* 29：471-476, 2006
3) Okumiya T, Ishii S, *et al.*: Galactose stabilizes various missense mutants of α-galactosidase in Fabry disease. *Biochem Biophys Res Commun* 214：1219-1224, 1995
4) Fan J, Ishii S, *et al.*: Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med* 5：112-115, 1999
5) Matsuda J, Suzuki O, *et al.*: Chemical chaperone therapy for brain pathology in GM_1-gangliosidosis. *Proc Natl Acad Sci USA* 100：15912-15917, 2003
6) Suzuki Y, Ichinomiya S, *et al.*: Chemical chaperone therapy: clinical effect in murine GM_1-gangliosidosis. *Ann Neurol* 62：671-675, 2007
7) Ogawa S, Matsunaga YK, *et al.*: Chemical modification of the β-glucocerebrosidase inhibitor N-octyl-β-valienamine: synthesis and biological evaluation of 4-epimeric and 4-O-(β-D-galactopyranosyl) derivatives. *Bioorg Med Chem* 10：1967-1972, 2002
8) Iwasaki H, Watanabe H, *et al.*: Fibroblast screening for chaperone therapy in β-galactosidosis. *Brain Dev* 28：482-486, 2006
9) Matsuda J, Suzuki O, *et al.*: β-galactosidase-deficient mouse as an animal model for GM_1-gangliosidosis. *Glycoconj J* 14：729-736, 1997
10) Ichinomiya S, Watanabe H, *et al.*: Neurological assessment of GM_1-gangliosidosis model mice. *Brain Dev* 29：210-216, 2006
11) Lin H, Sugimoto Y, *et al.*: N-Octyl-β-valienamine upregulates activity of F213I mutant β-glucosidase in cultured cells: A potential chemical chaperone therapy for Gaucher disease. *Biochim Biophys Acta* 1689：219-228, 2004
12) Lei K, Ninomiya H, *et al.*: Enzyme enhancement activity of N-octyl-β-valienamine on β-glucosidase mutants associated with Gaucher disease. *Biochim Biophys Acta* 1772：587-596, 2007

Cell. Mol. Life Sci. 65 (2008) 351 – 353
1420-682X/08/030351-3
DOI 10.1007/s00018-008-7470-2

Cellular and Molecular Life Sciences

Visions & Reflections (Minireview)

Chemical chaperone therapy for G_{M1}-gangliosidosis

Y. Suzuki

International University of Health and Welfare Graduate School, 2600-1 Kita-Kanemaru, Otawara 324-8501 (Japan), Fax: +81-287-24-3229, e-mail: SuzukiY@iuhw.ac.jp

Received 10 October 2007; received after revision 31 October 2007; accepted 6 November 2007
Online First 19 January 2008

Abstract. We have proposed a chemical chaperone therapy for lysosomal diseases, based on a paradoxical phenomenon that an exogenous competitive inhibitor of low molecular weight stabilizes the target mutant molecule and restores its catalytic activity as a molecular chaperone intracellularly. After Fabry disease experiments, we investigated a new synthetic chaperone compound *N*-octyl-4-epi-β-valienamine (NOEV) in a G_{M1}-gangliosidosis model mice. Orally administered NOEV entered the brain through the blood-brain barrier, enhanced β-galactosidase activity, reduced the substrate storage, and clinically improved neurological deterioration. We hope that chemical chaperone therapy will prove useful for some patients with G_{M1}-gangliosidosis and potentially other lysosomal storage diseases with central nervous system involvement.

Keywords. Chemical chaperone therapy, G_{M1}-gangliosidosis, β-galactosidase, *N*-octyl-4-epi-β-valienamine, neurogenetic disease.

Introduction

For more than 15 years we performed molecular analyses of two genetic diseases: G_{M1}-gangliosidosis (OMIM 230500) with generalized neurosomatic dysfunctions and Morquio B disease (OMIM 253010) with generalized skeletal dysplasias without neurological involvement [1]. Both are caused by allelic mutations of the gene *GLB1* (3p21.33) coding for lysosomal β-galactosidase (EC 3.2.1.23) catalyzing hydrolysis of ganglioside G_{M1} and related glycoconjugates. Ganglioside G_{M1} accumulates in the G_{M1}-gangliosidosis-affected brain. Oligosaccharides derived from keratan sulfate or glycoproteins are detected in visceral organs and urine from G_{M1}-gangliosidosis and Morquio B patients.

At present only symptomatic therapy is available for the brain lesion in human G_{M1}-gangliosidosis patients. Enzyme replacement therapy is not possible. In 2003, we proposed chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis, using *N*-Octyl-4-epi-β-valienamine (NOEV) as a potent stabilizer of mutant β-galactosidase [2].

Concept of chemical chaperone therapy

In general, molecular events in genetic diseases are affected by various molecular processes [3]. Three possible molecular abnormalities can be listed in genetic diseases: (1) biosynthetic defect; (2) deficient catalytic activity of a mutant protein; and (3) unstable mutant protein with normal or near-normal biological activity. We tested these possibilities first in Fabry disease, and found some mutant enzyme proteins were unstable at neutral pH in the endoplasmic reticulum (ER)/Golgi apparatus, and rapidly degraded because of inappropriate molecular folding [4]. Galactose surprisingly induced a high expression of α-galactosidase A activity in cultured fibroblasts from Fabry patients [5]. We then found a commercially available compound 1-deoxygalactonojirimycin (DGJ) for stabili-

zation of the enzyme [6]. After extensive molecular analysis we came to the following conclusion.
A competitive inhibitor, a substrate of transition state analog, binds to misfolded mutant protein as a molecular chaperone in the ER/Golgi compartment of the cell, resulting in formation of a stable molecular complex at neutral pH. The protein-chaperone complex is safely transported to the lysosome, where it dissociates under the acidic conditions, the mutant enzyme remains stabilized, and its catalytic function is expressed.

NOEV: Physicochemical and biological characteristics

After using galactose and DGJ for α-galactosidase A, I came across two synthetic compounds: β-galactosidase inhibitor *N*-octyl-4-epi-β-valienamine (NOEV) and β-glucosidase inhibitor *N*-octyl-β-valienamine (NOV). NOV was the first compound synthesized as a glucocerebrosidase inhibitor, and NOEV was subsequently synthesized by epimerization of NOV [7]. NOEV experiments moved faster than NOV simply because of more experimental data and materials for β-galactosidase and G_{M1}-gangliosidosis.
NOEV is a potent inhibitor of lysosomal β-galactosidase *in vitro*. It is stable and freely soluble in methanol or DMSO. The hydrochloride salt is freely soluble in water. Its molecular weight is 287.40, and it has an IC_{50} of 0.125 μM toward human β-galactosidase [2].
NOEV is 50-fold more efficient than DGJ for expression of mutant β-galactosidase activity in G_{M1}-gangliosidosis. Our calculations suggest that at least 10% of normal enzyme activity is necessary for catalytic digestion of the storage substrate below the pathological level in lysosomal diseases. Theoretically the disease will not occur in patients expressing enzyme activity above this level during his or her lifetime. We anticipate that the effective NOEV concentrations in human cells and animal tissues are much lower than the IC_{50} for this agent *in vitro*. In fact, NOEV is effective at the IC_{50} concentration in the culture medium for enhancement of mutant enzyme activity [8]. Under the current experimental conditions, the NOEV content in the lysosomal compartment must be much lower than that in the extracellular environment.

NOEV effect on human and mouse fibroblasts expressing mutant human enzyme

About one-third of cultured fibroblasts from G_{M1}-gangliosidosis patients, mainly with the juvenile and some of the infantile forms, responded to NOEV. The effect was mutation specific [8]. The R457Q mutant cells responded to NOEV maximally at 0.2 μM, and the R201C/R201H mutant cells at 2 μM. The knockout (KO) mouse fibroblasts expressing mutant human β-galactosidase [9] showed essentially the same results [2]. Addition of ganglioside mixture in the culture medium increased intracellular G_{M1} in the R201C cells causing juvenile G_{M1}-gangliosidosis. This storage was almost completely prevented by NOEV.

Chemical chaperone therapy on genetically engineered G_{M1}-gangliosidosis model mice

For animal studies, we developed a KO mouse strain with complete deficiency of β-galactosidase [10], and then a transgenic (Tg) strain based on KO, expressing the human R201C mutation (4% normal β-galactosidase activity in the brain). Both strains showed neurological deterioration that differed in severity. Life-span was 7–10 months for KO and 12–18 months for Tg. Neuropathology corresponded to the clinical severity [2]. Short-term oral NOEV administration resulted in significant enhancement of the enzyme activity in all the R201C mouse tissues examined, including the brain [2]. Immunohistochemistry revealed an increase in β-galactosidase activity and decrease in G_{M1} and G_{A1} storage.
An early NOEV treatment study with the R201C mouse showed an increase of the NOEV content in the brain after starting oral administration, its rapid disappearance after withdrawal, parallel increase of β-galactosidase activity, and decrease of G_{M1} storage [11]. In this study we tried a new scoring system for neurological assessment [12]. Treatment at the very early clinical stage (2 months) resulted in a positive clinical effect within a few months, although complete arrest or prevention of disease progression was not achieved under this experimental condition. The latency before a clinical effect was longer if the therapy was started in the late symptomatic stage (6 months). We concluded that NOEV treatment at the early stage of disease is mandatory for prevention of the brain damage.
This result indicated the following sequence of events in the brain (Fig. 1). After oral administration, NOEV goes directly into the bloodstream without intestinal breakdown, is delivered to the mouse brain through the blood-brain barrier, and enhances the mutant β-galactosidase activity, resulting in substrate digestion and clinical improvement. No specific adverse effects have been observed for at least 6 months of continuous oral administration.

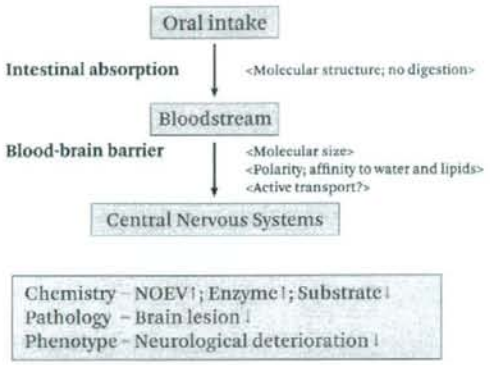

Figure 1. *N*-octyl-4-epi-β-valienamine (NOEV) goes directly from the gastrointestinal tract into the bloodstream without intestinal digestion, and into the brain through the blood-brain barrier. The catalytic activity of the mutant β-galactosidase is enhanced and the substrates are digested to improve phenotypic manifestations.

Future prospects

This new therapeutic strategy is in principle applicable to all lysosomal storage diseases if a specific compound is developed for each enzyme in question. We have already confirmed the effect in Fabry disease [5, 6], G_{M1}-gangliosidosis [2, 11], and Gaucher's disease [13, 14]. Our study started with a lysosomal disease, and is currently being expanded to a few related diseases. Further, there may be other genetic diseases that could be considered, provided that the molecular pathology in somatic cells has been studied and is understood in detail. I hope that studies in this direction will disclose a new aspect of molecular therapy for inherited metabolic diseases with central nervous system involvement in future.

Acknowledgments. This research was supported by grants from the Ministry of Education, Culture, Science, Sports, and Technology of Japan (13680918, 14207106, 16300141), and the Ministry of Health, Labor and Welfare of Japan (H10-No-006, H14-Kokoro-017, H17-Kokoro-019).

1 Suzuki, Y., Nanba, E., Matsuda, J. and Oshima, A. (2007) β-Galactosidase deficiency (β-galactosidosis): G_{M1}-gangliosidosis and Morquio B disease. In: The Online Metabolic and Molecular Bases of Inherited Disease, http://www.ommbid.com/, Valle, D. et al., (eds.), McGraw-Hill, New York.

2 Matsuda, J., Suzuki, O., Oshima, A., Yamamoto, Y., Noguchi, A., Takimoto, K., Itoh, M., Matsuzaki, Y., Yasuda, Y., Ogawa, S., Sakata, Y., Nanba, E., Higaki, K., Ogawa, Y., Tominaga, L., Ohno, K., Iwasaki, H., Watanabe, H., Brady, R. O. and Suzuki, Y. (2003) Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. Proc. Natl. Acad. Sci. USA 100, 15912–15917.

3 Suzuki, Y. (2006) β-Galactosidase deficiency: An approach to chaperone therapy. J. Inherit. Metab. Dis. 29, 471–476.

4 Ishii, S., Kase, R., Sakuraba, H. and Suzuki, Y. (1993) Characterization of a mutant α-galactosidase gene product for the late-onset cardiac form of Fabry disease. Biochem. Biophys. Res. Commun. 197, 1585–1589.

5 Okumiya, T., Ishii, S., Takenaka, T., Kase, R., Kamei, S., Sakuraba, H. and Suzuki, Y. (1995) Galactose stabilizes various missense mutants of α-galactosidase in Fabry disease. Biochem. Biophys. Res. Commun. 214, 1219–1224.

6 Fan, J. Q., Ishii, S., Asano, N. and Suzuki, Y. (1999) Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. Nat. Med. 5, 112–115.

7 Ogawa, S., Sakata, Y., Ito, N., Watanabe, M., Kabayama, K., Itoh, M. and Korenaga, T. (2004) Convenient synthesis and evaluation of glycosidase inhibitory activity of α- and β-galactose-type valienamines, and some *N*-alkyl derivatives. Bioorg. Med. Chem. 12, 995–1002.

8 Iwasaki, H., Watanabe, H., Iida, M., Ogawa, S., Tabe, M., Higaki, K., Nanba, E. and Suzuk, Y. (2006) Fibroblast screening for chaperone therapy in β-galactosidosis. Brain Dev. 28, 482–486.

9 Tominaga, L., Ogawa, Y., Taniguchi, M., Ohno, K., Matsuda, J., Oshima, A., Suzuki, Y. and Nanba, E. (2001) Galactonojirimycin derivatives restore mutant human β-galactosidase activities expressed in fibroblasts from enzyme-deficient knockout mouse. Brain Dev. 23, 284–287.

10 Matsuda, J., Suzuki, O., Oshima, A., Ogura, A., Noguchi, Y., Yamamoto, Y., Asano, T., Takimoto, K., Sukegawa, K., Suzuki, Y. and Naiki, M. (1997) β-Galactosidase-deficient mouse as an animal model for G_{M1}-gangliosidosis. Glycoconj. J. 14, 729–736.

11 Suzuki, Y., Ichinomiya, S., Kurosawa, M., Ohkubo, M., Watanabe, H., Iwasaki, H., Matsuda, J., Noguchi, Y., Takimoto, K., Itoh, M., Tabe, M., Iida, M., Kubo, T., Ogawa, S., Nanba, E., Higaki, K., Ohno, K. and Brady, R. O. (2007) Chemical chaperone therapy: Clinical effect in G_{M1}-gangliosidosis. Ann. Neurol. 62, 671–675.

12 Ichinomiya, S., Watanabe, H., Maruyama, K., Toda, H., Iwasaki, H., Kurosawa, M., Matsuda, J. and Suzuki, Y. (2006) Neurological assessment of G_{M1}-gangliosidosis model mice. Brain Dev. 29, 210–216.

13 Lin, H., Sugimoto, Y., Ohsaki, Y., Ninomiya, H., Oka, A., Taniguchi, M., Ida, H., Eto, Y., Ogawa, S., Matsuzaki, Y., Sawa, M., Inoue, T., Higaki, K., Nanba, E., Ohno, K. and Suzuki, Y. (2004) *N*-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: A potential chemical chaperone therapy for Gaucher disease. Biochim. Biophys. Acta 1689, 219–228.

14 Lei, K., Ninomiya, H., Suzuki, M., Inoue, T., Sawa, M., Iida, M., Ida, H., Eto, Y., Ogawa, S., Ohno, K., Kaneski, C., Brady, R. O. and Suzuki, Y. (2007) Enzyme enhancement activity of *N*-octyl-β-valienamine on β-glucosidase mutants associated with Gaucher disease. Biochim. Biophys. Acta 1772, 587–596.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 367 (2008) 616–622
BBRC
www.elsevier.com/locate/ybbrc

Enhanced autophagy and mitochondrial aberrations in murine G_{M1}-gangliosidosis

Ayumi Takamura [a,b], Katsumi Higaki [a,*], Kenya Kajimaki [a,c], Susumu Otsuka [a,d], Haruaki Ninomiya [e], Junichiro Matsuda [f], Kousaku Ohno [g], Yoshiyuki Suzuki [h], Eiji Nanba [a]

[a] Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, 86 Nishi-machi, Yonago 683-8503, Japan
[b] Department of Biosignaling, School of Life Sciences, Tottori University, 86 Nishi-machi, Yonago 683-8503, Japan
[c] Department of Molecular and Cellular Biology, School of Life Sciences, Tottori University, 86 Nishi-machi, Yonago 683-8503, Japan
[d] Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, Tottori University, 86 Nishi-machi, Yonago 683-8503, Japan
[e] Department of Biological Regulation, School of Health Science, Faculty of Medicine, Tottori University, 86 Nishi-machi, Yonago 683-8503, Japan
[f] National Institute of Biomedical Innovation, 7-6-8 Saito-asagi, Ibaraki, 567-0085, Japan
[g] Division of Child Neurology, Tottori University Faculty of Medicine, 36-1 Nishi-machi, Yonago 683-8504, Japan
[h] Graduate School, International University of Health and Welfare, 2600-1 Kita-Kanemaru, Otawara 324-8501, Japan

Received 28 December 2007
Available online 9 January 2008

Abstract

G_{M1}-gangliosidosis is an autosomal recessive lysosomal lipid storage disorder, caused by mutations of the lysosomal β-galactosidase (β-gal) and results in the accumulation of G_{M1}. The underlying mechanisms of neurodegeneration are poorly understood. Here we demonstrate increased autophagy in β-gal-deficient ($\beta\text{-gal}^{-/-}$) mouse brains as evidenced by elevation of LC3-II and beclin-1 levels. Activation of autophagy in the $\beta\text{-gal}^{-/-}$ brain was found to be accompanied with enhanced Akt–mTOR and Erk signaling. In addition, the mitochondrial cytochrome *c* oxidase activity was significantly decreased in brains and cultured astrocytes from $\beta\text{-gal}^{-/-}$ mouse. Mitochondria isolated from $\beta\text{-gal}^{-/-}$ astrocytes were morphologically abnormal and had a decreased membrane potential. These cells were more sensitive to oxidative stress than wild type cells and this sensitivity was suppressed by ATP, an autophagy inhibitor 3-methyladenine and a pan-caspase inhibitor z-VAD-fmk. These results suggest activation of autophagy leading to mitochondrial dysfunction in the brain of G_{M1}-gangliosidosis.

Keywords: G_{M1}-gangliosidosis; Lysosome; Autophagy; mTOR; Mitochondria; Astrocyte; Neurodegeneration

G_{M1}-gangliosidosis (OMIM 230500) is an autosomal recessive lysosomal lipid storage disorder with progressive central nervous system dysfunction, visceromegaly, and skeletal dysplasias. It is caused by deficiency of lysosomal acid β-galactosidase (β-gal) due to mutations in the *GLB1* gene [1]. Three clinical forms (infantile, juvenile, and adult/chronic) have been distinguished according to the age of onset and severity, mainly due to different residual activities of the mutant enzymes and hence different levels of the substrate accumulation in tissues, especially in the brain. Pathologically, typical lamellar inclusions or membranous cytoplasmic bodies are found in neurons of human, mouse, and other animal models of G_{M1}-gangliosidosis [2–4]. Neurons are the primary target of storage, but astrocytes may also appear abnormally vacuolated [5]. Recently, we have developed chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis [6,7]. However,

Abbreviations: LC3, microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3 kinase; LDH, lactate dehydrogenase; 3-MA, 3-methyladenine; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

* Corresponding author. Fax: +81 859 38 6470.
E-mail address: kh4060@grape.med.tottori-u.ac.jp (K. Higaki).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.12.187

underlying biological mechanisms responsible for neurodegeneration still remain uncertain [8].

Macroautophagy (hereafter referred to as autophagy) involves bulk degradation of complete regions of the cytosol [9]. The target regions are initially sequestered in multimembrane vacuoles, known as autophagosome which eventually fused with lysosomes for degradation. Autophagy plays a cytoprotective role in low-nutrient conditions and disease states by catabolizing intracellular substrates for energy supply and by removing failing mitochondria and other factors that trigger cell death [10]. Dysfunction of autophagy can disrupt neuronal function and ultimately lead to neurodegeneration [11].

In this study, we demonstrate enhanced autophagy and mitochondrial alterations in the G_{M1}-gangliosidosis mouse brain, which might lead to neurodegeneration in this disease.

Materials and methods

Antibodies and reagents. Monoclonal anti-G_{M1} (GMB16) was from Seikagaku Corp. (Tokyo, Japan), polyclonal anti-LC3 (PD014) was from MBL International Corp. (Woborn, MA, USA), polyclonal anti-beclin-1 (H-300) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and polyclonal anti-Atk, anti-phospho-Akt (Ser473), anti-mTOR, anti-phospho-mTOR (Ser2448), anti-S6 ribosomal protein (5G10), and anti-phospho-S6 ribosomal protein (Ser235/236) were from Cell Signaling Technology (Boston, MA, USA). Paraquat, ATP and chloroquine were purchased from Wako (Tokyo, Japan), 3-methyladenine (3-MA), and rapamycin were from Sigma (St. Louis, MO, USA) and z-VAD-fmk was from Promega (Madison, WI, USA).

Mice and tissue collection. A C57BL/6-based congenic mouse strain with β-gal-deficiency (β-gal$^{-/-}$) was established as reported previously [3,6]. All animal procedures were carried out following the protocols approved by the committee for animal experiments in Tottori University and β-gal$^{-/-}$ mice was obtained by cross bleeding. For tissue staining, mice were anesthesized and perfused with 4% paraformaldehyde (PFA) in sodium phosphate, pH 7.4. Brains were embedded in OTC compound (Sakura Finetechnical Co., Tokyo, Japan) and 8 μm sections were cut using a cryostat. For protein extractions, tissues were removed and frozen in liquid nitrogen.

Primary culture of astrocytes. For astrocyte preparation, brains from postnatal day four mice were removed under anesthesia. The cerebral cortex was dissociated and cells were seeded on plastic dishes in DMEM-F12 supplemented with 15% fetal bovine serum (FBS). They were cultured for 7 days, trypsinized, and seeded on dishes with DMEM-F12 with 10% FBS. They were confirmed to be GFAP-positive astrocytes at 3 weeks by immunostaining with polyclonal anti-GFAP (data not shown). Lactate dehydrogenase (LDH) cytotoxicity assay (Wako, Tokyo, Japan) was performed following the manufacturer's instruction.

Immunoblot analysis. Mouse brains were lysed by sonication in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA plus protease inhibitor cocktail (Roche). Protein was quantified using Color-Producing Solution (Wako). Samples were separated on 10% SDS–PAGE and transferred on a nylon membrane (Millipore) using a semi-dry transfer blotter (BioRad). Membranes were incubated in a polyclonal antibody followed by a horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham). Detection was performed using ECL (Amersham Pharmacia Biotech) and images were captured in X-ray film or a LAS-1000 plus imager (Fujifilm).

Immunofluorescence staining. Brain sections were permiabilized with 0.25% Triton X-100 in PBS for 15 min at room temperature, blocked with 1% BSA in PBS for 1 h at room temperature, and incubated with the first antibody at 4 °C overnight. Bind antibodies were detected with Alexa-fluor-conjugated secondary antibody for 1 h at room temperature. Fluorescence images were obtained using confocal microscopy (Leica, TCS-SP2; Wetzler, Germany).

Mitochondrial assay. Mitochondria were isolated from the mouse brain and cultured astrocytes using mitochondrial isolation kit (BioChain Ins. Hayward, CA, USA) and the enzyme activity of cytochrome *c* oxidase was determined using mitochondrial activity kit (BioChain Ins.) following the manufacturer's instruction. For mitochondrial labeling, cultured astrocytes were seeded on sterile cover slips or glass base dishes (Iwaki, Tokyo, Japan) and incubated in Hanks' balanced salt solution containing 100 nM MitoTracker Red CMXRos or 3 μM Mitotracker JC-1 (MolecularProbes Inc., Eugene, OR, USA) for 20 min at 37 °C. Cells were then washed with Hanks' balanced salt solution and fluorescent images were obtained using confocal microscopy.

Results

G_{M1} accumulation and sequestration of autophagosomes proteins in the β-gal$^{-/-}$ mice brain

Microtubule-associated protein 1 light chain 3 (LC3), a mammalian homolog of the yeast autophagic protein Atg8, has been used as an autophagosomal marker [9]. Cleavage of LC3 in its carboxy terminal gives rise to a cytosolic soluble form LC3-I which is further modified into LC3-II, a protein that associate with autophagosomes. Brain levels of LC3 were assessed by immunoblotting. Although levels of LC3-I and LC3-II in β-gal$^{-/-}$ mice did not significantly differ from those in wild type (WT) mice at 10-day-old, the level of LC3-II were significantly higher in mutant mice at 10 months of age (Fig. 1A). G_{M1} and LC3 double immunofluorescence showed co-localization of LC3-immunopositive-granules with G_{M1} in neurons of β-gal$^{-/-}$ mice at 10 months (Fig. 1B). Beclin-1 is the mammalian ortholog of yeast Atg6, and is a part of the Class III phosphatidylinositol 3 kinase (PI3K) complex that participate in autophagosome formation [9]. The level of beclin-1 was increased in brain lysates from 10-month-old β-gal$^{-/-}$ mice when compared to WT mice (Fig. 1C and D). The Akt/mammalian target of rapamycin (mTOR) and the extracellular signal kinase (Erk) are two major pathways that regulate autophagy [10,12]. Phsphorylation of Akt, Erk, and mTOR were increased, whereas no obvious alteration of S6 was detected in the brain lysates of β-gal$^{-/-}$ mice at 10 months (Fig. 2A and B).

Mitochondrial alterations in β-gal$^{-/-}$ mice

Autophagy is a highly regulated process that is involved in the turnover of long-lived proteins and whole organelles. It can specifically target distinct organelles, such as mitochondria in mitopathy and the endoplasmic reticulam in reticulopathy [9]. We next sought to examine whether sequestration in autophagic vacuoles affects mitochondrial function in this mouse model. The level of mitochondrial cytochrome *c* oxidase activity was significantly decreased in the brain of β-gal$^{-/-}$ mice than that of WT mice at 10 months (Fig. 3A). Similarly, cultured astrocytes from

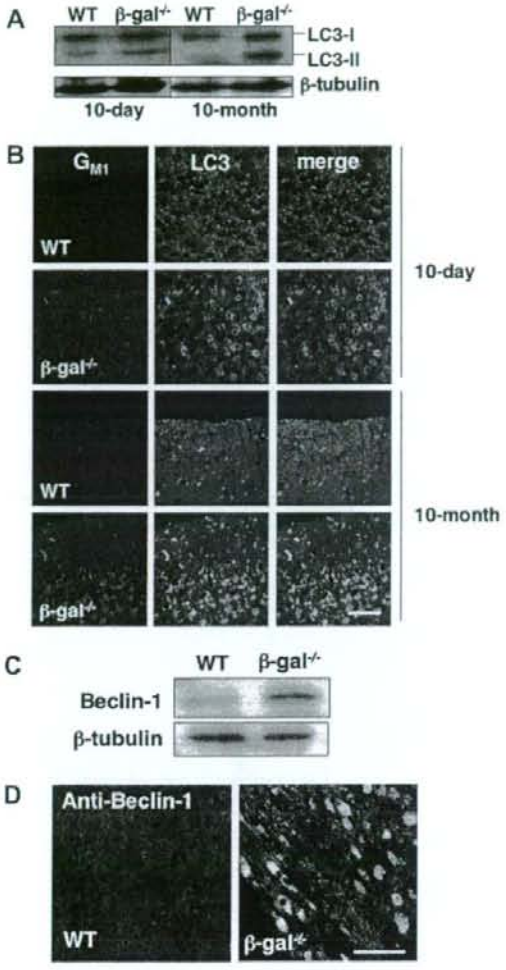

Fig. 1. Elevation of LC3-II and beclin-1 expression in β-gal$^{-/-}$ mouse brain. Cerebellar lysates from WT and β-gal$^{-/-}$ mice were subjected to Western blotting with anti-LC3 (A) or anti-beclin-1 (C). Immunofluorescence of cellular distribution of LC3 (B) and beclin-1 (D) proteins in the frontal cerebral cortex of WT and β-gal$^{-/-}$ mice. Scale bar = 80 μm.

β-gal$^{-/-}$ mice showed lysosomal accumulation of G_{M1} and elevated LC3-II and beclin-1 levels (data not shown), and it had a decreased cytochrome *c* oxidase activity (Fig. 3A). Next, the morphology and the membrane potential of mitochondria were examined in cultured astrocytes using confocal microscopy. There were obvious differences in mitochondrial morphology between WT and β-gal$^{-/-}$ astrocytes. In WT astrocytes, mitochondria were organized as extended tubular structures, whereas β-gal$^{-/-}$ astrocytes contained smaller, fragmented or circulated mitochondria (Fig. 3B and C). When cells were stained with Mitotracker JC-1, a marker of the mitochondrial membrane potential,

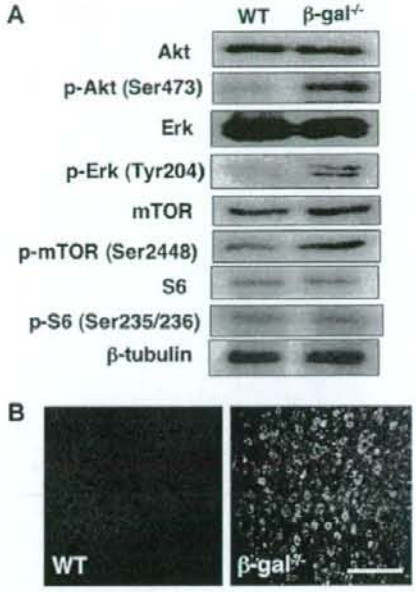

Fig. 2. Changes in Akt/mTOR and Erk signals in β-gal$^{-/-}$ mouse brain. (A) Cerebellar lysates from postnatal of 10-month-old WT and β-gal$^{-/-}$ mice were subjected to Western blotting with indicated antibodies. (B) Cellular distribution of p-mTOR (Ser2448) in the frontal cerebral cortex of WT and β-gal$^{-/-}$ mice at 10 months of age. Scale bar = 80 μm.

the intensity of red and green fluorescence was decreased in β-gal$^{-/-}$ astrocytes compared to the WT (Fig. 3D).

Dysfunction of autophagic-lysosomal pathways and mitochondria

To examine functional relevance of mitochondrial dysfunction to cell death, we treated cultured astrocytes with oxidative stress reagent paraquat. LDH release assay revealed a significant increase of the percentage of dead cells was noted in β-gal$^{-/-}$ astrocytes compared to that in WT cells (Fig. 4A). We also attempted to characterize the impairment in autophagy and mitochondria in β-gal$^{-/-}$ astrocytes. LDH release in paraquat (250 μM)-treated-β-gal$^{-/-}$ astrocytes was significantly suppressed by addition of 0.5 mM ATP in the medium for 24 h (Fig. 4B). We next examined effects of 3-MA and rapamycin, which inhibit or induce autophagy, respectively [13], 3-MA at 10 mM reduced paraquat-induced-LDH release in β-gal$^{-/-}$ astrocytes, whereas, rapamycin (2 μg/ml) had no effects on cell death. We also examined a cell-permeable pan-caspase inhibitor, z-VAD-fmk, since autophagic cell death was partly mediated by caspase activation [10]. z-VAD-fmk (100 μM) significantly decreased cell death in paraquat-treated-β-gal$^{-/-}$ astrocytes. Under these conditions, none of the drugs affected LDH release in

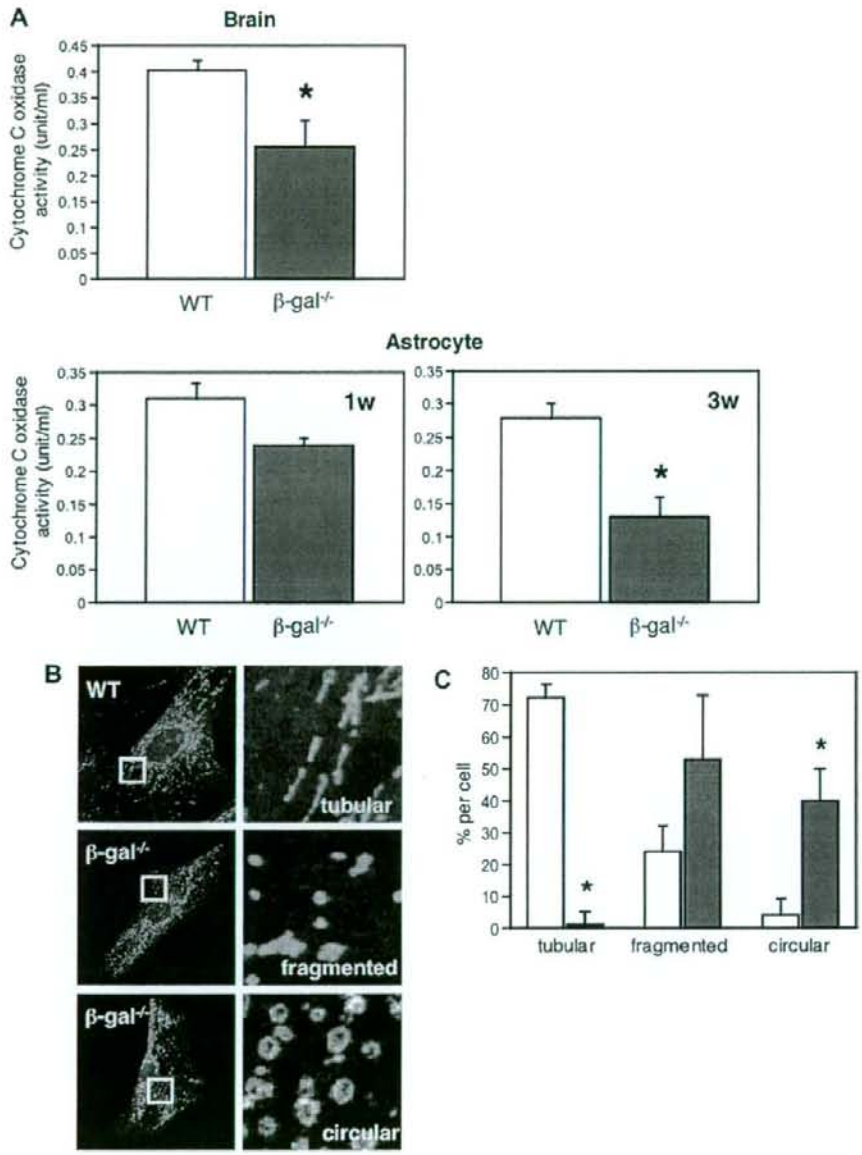

Fig. 3. Mitochondrial alteration in β-gal$^{-/-}$ mouse brain and astrocyte. (A) Levels of cytochrome *c* oxidase activities in extracts from the brain and primary astrocytes of WT and β-gal$^{-/-}$ mice. Values are means ± SEM from three independent experiments, $^{*}p < 0.01$ significantly differ from the value of WT cells. (B) Primary astrocytes from neonatal WT and β-gal$^{-/-}$ cortex were cultured for 3 weeks and labeled with MitoTracker Red. Morphological analysis of mitochondria was obtained using confocal microscopy. (C) The number of cells with each morphology of mitochondria was computed. Values for the percent of total cell number from three independent experiments. $n = 30$ cells and values are means ± SEM. (open bars: WT; dark bars: β-gal$^{-/-}$) (D) Primary-cultured astrocytes were labeled with JC-1. Shown are the representative images obtained by confocal microscopy using red and green channels. Scale bar = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

WT astrocytes. Chloroquine, an inhibitor of autophagosome–lysosome fusion, induced cell death in WT astrocytes after treatment with paraquat, and this cell death was suppressed by ATP, 3-MA and z-VAD-fmk (Fig. 4B).

Discussion

One of the most important functions of autophagy is to maintain cellular energy subjected to nutrient deprivation

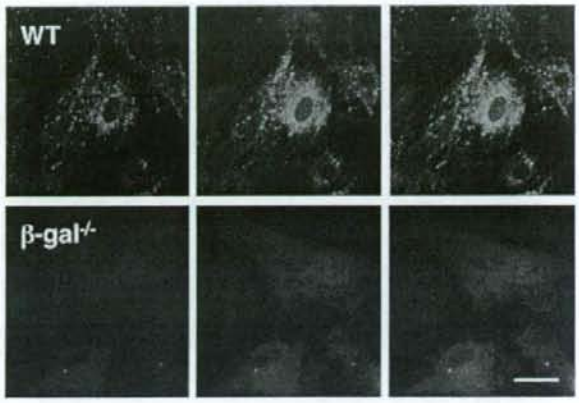

Fig. 3 (*continued*)

and potentially other forms of stress. Autophagy is a highly regulated process that is involved in the turnover of long-lived proteins and cytoplasmic constituents including mitochondria, endoplasmic reticulum, and ribosomes [10]. Molecular mechanisms that regulated autophagy in yeast and mammalian have recently been identified [9]. Knockout of autophagy genes causes abnormal accumulation in ubiquitinated inclusions and neurodegeneration in mice and that implicates in mechanisms of neurodegeneration [14,15].

In the present study, we showed increased levels of autophagic proteins in the β-gal$^{-/-}$ mice brain. Immunoblot analysis revealed an increase in levels of LC3-II, a widely used marker for autophagy, in all brain areas examined at 10 months of age. Levels were particularly high in cerebellum and brain stem, where severe neuronal death occurs in the β-gal-deficient human and mouse brain [1,3,4]. Increased autophagic stress was further confirmed by the presence of LC3-positive structures in cells with intracellular G_{M1} accumulations. This induction of autophagy was associated with increased expression of beclin-1. Beclin-1 is the mammalian ortholog of yeast Atg6, and is a part of the Class III PI3K machinery that participates in autophagosome formation [9].

Enhanced autophagy was recently reported in human skin fibroblasts and mice models of other types of lysosomal storage diseases, such as Danon disease [16], neuronal ceroid lipofuscinosis 2 [17], Pompe disease [18], mucolipidosis type IV [19] multiple sulfatase deficiency, mucopolysaccharidosis type IIA [20]. Induction of autophagy was also observed in the Niemann-Pick C1 (NPC1) mouse brain, which contained increased levels of beclin-1 [21].

The Akt–mTOR and Erk signaling pathways were also activated in β-gal$^{-/-}$ mice. Insulin signaling stimulates phosphorylation and activity of mTOR via Akt/PBK pathway and thereby represses autophagy in response to insulin-like and other growth factor signals [9]. Activation of these pathways is known to induce autophagy, although detailed mechanisms are still unknown. [12]. Previous studies have demonstrated localization of the active form of Erk in autophagosomes and mitochondria in degenerating brain [22], and that might happen in β-gal$^{-/-}$ brain.

Decrease in the cytochrome *c* oxidase activity, the morphological abnormality and high sensitivity to oxidative stress in the β-gal$^{-/-}$ astrocytes suggest mitochondrial abbreviations in this mouse. Inefficient autophagic-lysosomal fusion may cause accumulation of fragmented mitochondria. It is also possible that enhanced autophagy disrupted mitochondrial function. We showed that oxidative stress-induced cell death was suppressed by ATP, an autophagy inhibitor and a pan-caspase inhibitor in β-gal$^{-/-}$ astrocytes as well as in chloroquine-treated WT astrocytes, supporting the idea that enhanced autophagy induces mitochondrial dysfunction that leads to cell death. Mechanisms leading to cell death in astrocytes remain unclear, since functional relationship between autophagic cell death (also known as type II cell death) and apoptotic cell death (or type I cell death) is complex [10]. Autophagy and apoptosis may be triggered by common signals.

Autophagy has emerged as the major pathway involved in a number of neurodegenerative diseases, including Alzheimer disease [23], Parkinson disease [24], Huntington disease [25], and lysosomal storage diseases [16–21]. In each case, autophagic vacuoles accumulate in the affected neurons, indicating that activation of autophagy is a common feature of these diseases. However, the precise mechanisms leading to activation of autophagy remain elusive. Further investigation is warranted to clarify the mechanisms of enhanced autophagy in these disorders.

In summary, we provided evidence for abnormal activation of autophagy accompanied with mitochondrial alterations in the murine model of G_{M1}-gangliosidosis. Modulation of activity of autophagy and restoring mitochondrial functions may be of therapeutic benefit for this disease.

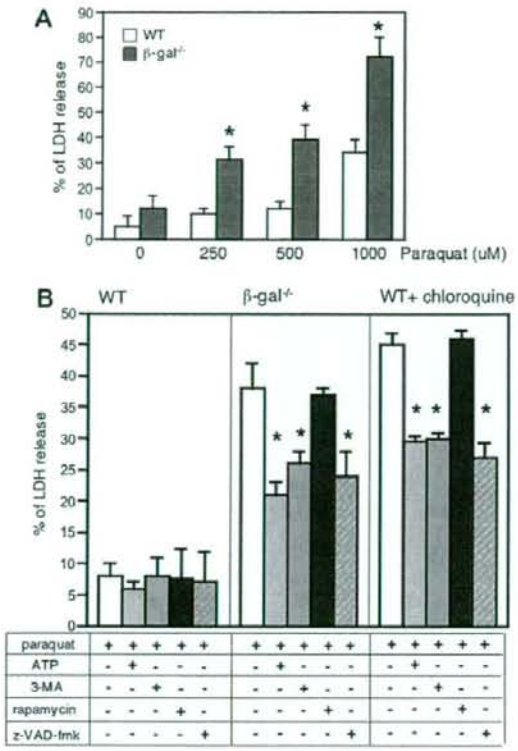

Fig. 4. Effects of ATP, 3-MA, rapamycin and a pan-caspase inhibitor on oxidative stress-induced cell death of astrocyte. (A) Lactate dehydrogenase (LDH) release assay. Astrocytes were cultured with or without paraquat for 24 h and the medium was collected for LDH release assay. Values were expressed as relative to the values from cells treated with 1% Tween 20. Each bar represents the mean (SEM) from three independent experiments. $^{*}p < 0.01$ significantly differ from the value of WT cells. (B) Both WT, β-gal$^{-/-}$ and chloroquine-treated WT astrocytes were treated with indicated drugs. LDH release assay were performed after 24 h treatment. Each bar represents the mean (SEM) from three independent experiments. $^{*}p < 0.01$ significantly differ from the value of paraquat-treated cells.

Acknowledgments

This work was supported by grants from Ministry of Education, Culture, Science, Sports, and Technology of Japan (13680918, 14207106, 16300141, 18390279, and 18790719) and Ministry of Health, Labour and Welfare of Japan (H10-No-006, H14-Kokoro-017, and H17-Kokoro-019).

References

[1] A. Oshima, E. Nanba, J. Matsuda, Y. Suzuki, β-Galactosidase deficiency (β-galactosidosis): GM1-gangliosidosis and Morquio B disease. In: D. Valle, A.L. Baudet, B. Vogelstein, et al., (Eds.), The Online Metabolic and Molecular Bases of Inherited Disease. 8th ed. McGraw-Hill, New York, 2007, Available from. <http://www.ommbid.com>.

[2] K. Suzuki, G.C. Chen, Morphological, histological and biochemical studies on a case of systemic late infantile lipidosis (generalized gangliosidosis), J. Neuropathol. Exp. Neurol. 27 (1968) 15–38.

[3] J. Matsuda, O. Suzuki, A. Oshima, A. Ogura, Y. Noguchi, Y. Yamamoto, T. Asano, K. Takimoto, K. Sukegawa, Y. Suzuki, M. Naiki, β-Galactosidase-deficient mouse as an animal model for G_{M1}-gangliosidosis, Glycoconj. J. 14 (1997) 729–736.

[4] C.N. Hahn, M. Martin, M. Schröder, M.T. Vanier, Y. Hara, K. Suzuki, K. Suzuki, A. d'Azzo, Generalized CNS disease and massive G_{M1}-ganglioside accumulation in mice defective in lysosomal acid β-galactosidase, Hum. Mol. Genet. 6 (1997) 205–211.

[5] J.E. Goldman, D. Katz, I. Rapin, D.P. Purpura, K. Suzuki, Chronic GM1 gangliosidosis presenting as dystonia: I. Clinical and pathological features, Ann. Neurol. 9 (1981) 465–475.

[6] J. Matsuda, O. Suzuki, A. Oshima, Y. Yamamoto, A. Noguchi, K. Takimoto, M. Itoh, Y. Matsuzaki, Y. Yasuda, S. Ogawa, Y. Sakata, E. Nanba, K. Higaki, Y. Ogawa, L. Tomonaga, K. Ohno, H. Iwasaki, H. Watanabe, R.O. Brady, Y. Suzuki, Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis, Proc. Natl. Acad. Sci. USA 100 (2003) 15912–15917.

[7] Y. Suzuki, S. Ichinomiya, M. Kurosawa, M. Ohkubo, H. Watanabe, H. Iwasaki, J. Matsuda, Y. Noguchi, K. Takimoto, M. Itoh, M. Tabe, M. Iida, T. Kubo, S. Ogawa, E. Nanba, K. Higaki, K. Ohno, R.O. Brady, Chemical chaperone therapy: clinical effect in murine G_{M1}-gangliosidosis, Ann. Neurol. 62 (2007) 671–675.

[8] M. Jeyakumar, R.A. Dwek, T.D. Butters, F.M. Platt, Storage solutions: treating lysosomal disorders of the brain, Nat. Rev. Neurosci. 6 (2005) 1–12.

[9] M.C. Maiuri, E. Zalckvar, A. Kimchi, G. Kroemer, Self-eating and self-killing: crosstalk between autophagy and apoptosis, Nat. Rev. Mol. Cell Biol. 8 (2007) 741–752.

[10] B. Levine, J. Yuan, Autophagy in cell death: an innocent convict? J. Clin. Invest. 115 (2005) 2679–2688.

[11] M. Martinez-Vicente, A.M. Cuervo, Autophagy and neurodegeneration: when the cleaning crew goes on strike, Lancet Neurol. 6 (2007) 352–361.

[12] E. Corcelle, M. Nebout, S. Bekri, N. Gauthier, P. Hofman, P. Poujeol, P. Fenichel, B. Mograbi, Disruption of autophagy at the maturation step by the carcinogen lindane is associated with the sustained mitogen-activated protein kinase/extracellular signal-regulated kinase activity, Cancer Res. 66 (2007) 6861–6870.

[13] R.C. Paraguison, K. Higaki, K. Yamamoto, H. Matsumoto, T. Sasaki, N. Kato, E. Nanba, Enhanced autophagic cell death in expanded polyhistidine variants of HOXA1 reduces PBX1-coupled transcriptional activity and inhibits neuronal differentiation, J. Neuroci. Res. 85 (2007) 479–487.

[14] M. Komatsu, S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami, K. Tanaka, Loss of autophagy in the central nervous system causes neurodegeneration in mice, Nature 441 (2007) 880–884.

[15] T. Hara, K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, M. Yokoyama, K. Mishima, I. Saito, H. Okano, N. Mizushima, Suppression of basal autophagy in neural cells causes neurogenerative disease mice, Nature 441 (2007) 885–889.

[16] Y. Tanaka, G. Guhde, A. Suter, E.L. Eskelinen, D. Hartmann, R. Lullmann-Rauch, P.M. Janssen, J. Blanz, K. von Figura, P. Saftig, Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice, Nature 406 (2000) 902–906.

[17] M. Koike, M. Shibata, S. Waguri, K. Yoshimura, I. Tanida, E. Kominami, T. Gotow, C. Peters, K. von Figura, N. Mizushima, P. Saftig, Y. Uchiyama, Participation of autophagy in storage of lysosomes in neurons from mouse models of neuronal ceroid-lipofuscinoses (Batten disease), Am. J. Pathol. 167 (2005) 1713–1728.

[18] T. Fukuda, L. Ewan, M. Bauer, R.J. Mattaliano, K. Zaal, E. Ralston, P.H. Plotz, N. Raben, Dysfunction of endocytic and autophagic pathways in a lysosomal storage disease, Ann. Neurol. 59 (2006) 700–708.

[19] J.J. Jennings Jr, J.H. Zhu, Y. Rbaibi, X. Luo, C.T. Chu, K. Kiselyov, Mitochondrial aberrations in mucolipidosis type IV, J. Biol. Chem. 281 (2006) 39041–39050.

[20] C. Settembre, A. Fraldi, L. Jahreiss, C. Spampanato, C. Venturi, D. Medina, R. Pablo, C. Tacchetti, D.C. Rubinsztein, A. Ballabio, A block of autophagy in lysosomal storage disorders, Hum. Mol. Genet. 17 (2008) 119–129.

[21] C.D. Pacheco, R. Kunkel, A.P. Lieberman, Autophagy in Niemann-Pick C disease is dependent upon beclin-1 and responsive to lipid trafficking defects, Hum. Mol. Genet. 16 (2007) 1495–1503.

[22] J.H. Zhu, F. Guo, J. Shelburne, S. Watkins, C.T. Chu, Localization of phosphorylated ERK/MAP kinases to mitochondria and autophagosomes in Lewy body diseases, Brain Pathol. 13 (2003) 473–481.

[23] P.I. Moreira, S.L. Siedlak, X. Wang, M.S. Santos, C.R. Oliveira, M. Tabaton, A. Nunomura, L.I. Szweda, G. Aliev, M.A. Smith, X. Zhu, G. Perry, Autophagocytosis of mitochondria is prominent in Alzheimer disease, J. Neuropathol. Exp. Neurol. 66 (2007) 525–532.

[24] J.H. Zhu, C. Horbinski, F. Guo, S. Watkins, Y. Uchiyama, C.T. Chu, Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death, Am. J. Pathol. 170 (2007) 75–86.

[25] A. Yamamoto, L.M. Cremona, J.E. Rothman, Autophagy-mediated clearance of huntingtin aggregates triggered by the insulin-signaling pathway, J. Cell Biol. 172 (2006) 719–731.

American Journal of Medical Genetics Part A 146A:1736–1740 (2008)

Clinical Report

Novel β-Galactosidase Gene Mutation p.W273R in a Woman With Mucopolysaccharidosis Type IVB (Morquio B) and Lack of Response to in vitro Chaperone Treatment of Her Skin Fibroblasts

Zoran S. Gucev,[1]* Velibor Tasic,[1] Aleksandra Jancevska,[1] Georgi Zafirovski,[1] Ivo Kremensky,[2] Ivanka Sinigerska,[2] Eiji Nanba,[3] Katsumi Higaki,[3] Filip Gucev,[1] and Yoshiyuki Suzuki[4]

[1]Medical Faculty Skopje, Skopje, Macedonia
[2]National Genetic Laboratory, Sofia, Bulgaria
[3]Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, Yonago, Japan
[4]International University of Health and Welfare Graduate School, Otawara, Japan

Received 1 August 2007; Accepted 19 February 2008

The patient is a 24-year-old woman who first came for consultation at age 10 years. Based on clinical phenotype and thin-layer chromatography of urinary oligosaccharides, peripheral leukocytes were sent for β-galactosidase assay. This testing showed a deficiency in enzyme activity, and gene mutation analysis identified a previously reported mutation p.H281Y (875C > T) and a novel mutation p.W273R (817T > C). Unlike previously reported patients, mutant enzymes in this patient's cultured skin fibroblasts did not respond to treatment with a chaperone compound, *N*-octyl-4-epi-β-valienamine. © 2008 Wiley-Liss, Inc.

Key words: Morquio B disease; β-galactosidase; gene mutation; compound heterozygote; Macedonian

How to cite this article: Gucev ZS, Tasic V, Jancevska A, Zafirovski G, Kremensky I, Sinigerska I, Nanba E, Higaki K, Gucev F, Suzuki Y. 2008. Novel β-galactosidase gene mutation p.W273R in a woman with mucopolysaccharidosis type IVB (Morquio B) and lack of response to in vitro chaperone treatment of her skin fibroblasts. Am J Med Genet Part A 146A:1736–1740.

INTRODUCTION

Mucopolysaccharidosis (MPS) IVB (OMIM# 253010) is a rare autosomal recessive disease [Suzuki et al., 2001] characterized by short-trunk dwarfism, and progressive and generalized skeletal dysplasia. Life threatening atlanto-axial subluxation may occur as a result of the instability of the odontoid process and ligamentous laxity. Genu valgus and kyphosis with short trunk and short neck are early signs of the disease. Fine corneal deposits, hepatomegaly, small teeth with thin enamel, frequent caries formation and cardiac valvular lesions are extra-skeletal abnormalities. Intelligence is preserved. The disease is caused by β-galactosidase deficiency, resulting in keratin sulphate and oligosaccharide storage in the skeletal system and connective tissue, with normal catalytic activity for ganglioside G_{M1}.

Several mutations of the β-galactosidase gene have been found in Morquio B patients. However, less than 30 Morquio B patients have been characterized for their respective DNA mutations [Santamaria et al., 2006].

CLINICAL REPORT

The patient is a 24-year-old Macedonian girl who first came for consultation for growth delay at the age of 10 years. Her intelligence was normal. Her final height was 138 cm. She progressively developed distinctive facial features with a broad mouth and a short nose with anteverted nares. Hearing was

Grant sponsor: Ministry of Education, Culture, Science, Sports, and Technology of Japan; Grant numbers: 13680918, 14207106; Grant sponsor: Ministry of Health, Labour and Welfare of Japan; Grant numbers: H10-No-006, H14-Kokoro-017, H17-Kokoro-019.

*Correspondence to: Zoran S. Gucev, Medical Faculty Skopje, 50 Divizija BB, 1000 Skopje, Macedonia. E-mail: gucevz@gmail.com

DOI 10.1002/ajmg.a.32318

normal. Both corneas were cloudy, with more prominent sight impairment in the left eye. Teeth were widely spaced, and enamel was brittle and greyish. She had a gait disturbance and walked slowly. Range of motion of her hips, knees and spine were limited, and movements were painful. In contrast, her wrists and small joints were lax and hyperextensible. She had a short neck and kyphoscoliosis, coxa valga and genu vera with medial spurs of the tibial metaphyses (Fig. 1).

Radiographs at age 24 years showed platyspondyly with ovoid vertebrae and anterior projection (Fig. 2a). Her sternum was bulging and her ribs were flared. Long bones were short with irregular tabulation, and metaphyses were widened. Her femoral head was flattened and her acetabulum was dysplastic and wave-like (Fig. 2b). Pseudocysts and arthrotic alterations were present, and her metacarpals had conical bases.

FIG. 1. Photo of the patient. Short neck and kyphosis.

Neither cardiac abnormalities nor hepatomegaly were observed. Her hip dysplasia was treated with Salter's osteotomy, and corrective surgical interventions were performed on both femurs at the age of 12 years. However, progressive bone dysplasia severely impaired her gait. At present, she is able to walk in the house and for short distances only with assistance.

LABORATORY ANALYSIS

Total excretion of mucopolysaccharides in her urine (DMB assay) [de Jong et al., 1989] was within normal range. Thin-layer chromatography of urinary oligosaccharides showed an abnormal pattern similar to that for Morquio B or adult G_{M1}-gangliosidosis [Humbel and Collart, 1975]. Galactose 6-sulphatase (deficient in Morquio A) in leukocytes [Van Diggelen et al., 1990] was within the range of the control values. However, leukocyte β-galactosidase activity was 9.8 nmol 4-methylumbelliferone/h/mg protein, which was 5.9% of the control mean enzyme activity (range 90–320; N = 270) [Sinigerska et al., 2006].

PCR amplification of the entire β-galactosidase coding sequence and their flanking intronic regions was carried out on genomic DNA. The PCR products were then subjected to denaturing high performance liquidchromatography using a WAVE DNA fragment analysis system (Transgenomic, Omaha, NE). Briefly, the PCR products were denatured at 95°C for 5 min, followed by gradual annealing to 50°C. Samples were automatically loaded on a DNAsep column (Transgenomic) and eluted with a linear acetonitrile gradient at the temperature for heteroduplex detection. The eluted DNA fragments were detected by an UV-C detector (Transgenomic). An abnormal heteroduplex pattern was detected on the amplification product spanning exon 8, which was re-amplified for direct sequencing (ABI3130xl, Applied Biosystems, CA). Two mutations were identified: a previously reported p.H281Y (c.841C > T) reported in G_{M1}-gangliosidosis [Paschke et al., 2001], and a novel mutation p.W273R (c.817T > C).

We performed a chaperone experiment for cultured skin fibroblasts, using the compound *N*-octyl-4-epi-β-valienamine (NOEV) [Iwasaki et al., 2006]. The cells were cultured for 4 days with 0.2 or 2 μM NOEV in the culture medium, and subjected to β-galactosidase assay. The residual enzyme activity in this patient's cells before treatment was 3% of the control mean. After culture with NOEV for 4 days, the enzyme activity did not change significantly under the experimental conditions in this study. As a control, a skin fibroblast culture with the homozygous R201C mutation for juvenile G_{M1}-gangliosidosis responded positively to NOEV, resulting in 4.5–5-fold enhancement of the enzyme activity, as reported in a previous study [Matsuda et al., 2003]. Immunostain of the enzyme with a β-galactosidase

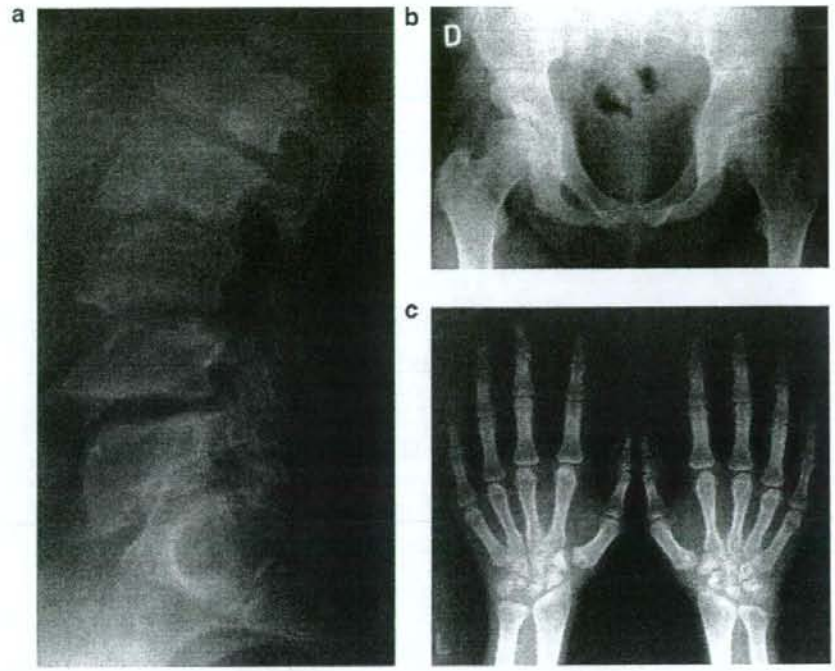

FIG. 2. Bone radiograph. a: Vertebra (lateral view)—platyspondyly with ovoid vertebrae and anterior projection; and end-plates have irregular and rough surfaces. b: Hip joints—coxa valga; and dysplastic and wave-like acetabula. c: Hand—hypoplastic scafoid and lunate bones; Madelung deformity of the radiocarpal articulation; and heads and bases of metacarpal bones spiky and mildly hypoplastic.

monoclonal antibody clone, H-80 (Santa Cruz Biotech, Santa Cruz, CA), did not show enzyme molecules either before or after the NOEV treatment in this patient's cells (data not shown).

DISCUSSION

This is a case report of a rare lysosomal disease, Morquio B, with phenotypic expression of systemic bone disease caused by β-galactosidase deficiency. The central nervous system was not involved. Gene analysis identified two abnormalities, a common disease-causing sequence variant, p.H281Y (c.841C > T), and a new mutation, p.W273R. The p.H281Y variant is a common mutation among Caucasian patients [Oshima et al., 1991], and it was found to have high frequency (79%) in unrelated European patients [Paschke et al., 2001].

Bagshaw et al. [2002] described three new mutations in patients with a mild form of Morquio B. p.N484K and p.T500A mutations were found in male and female twins, and p.G438E was found in the third case without identification of the second mutant allele. The p.T500A mutation had been described previously by Hinek et al. [2000] in a report on intracellular assembly with elastic fibers in G_{M1}-gangliosidosis and MorquioB. This mutation was also in a report on 2 more patients [Santamaria et al., 2006]. Paschke et al. [2001] described a novel missense mutation, p.Q408P, together with a known mutation p.T500A in a French patient, and heterozygous mutations p.Y270D and p.H281Y located in the vicinity of p.W273L in a German patient with neurological and skeletal abnormalities of early onset (possibly juvenile G_{M1}-gangliosidosis).

Analysis of five Spanish Morquio B patients [Santamaria et al., 2006] identified p.Y83C/p.D441N in one patient. However, an infantile G_{M1}-gangliosidosis patient was homozygous for p.D441N. The p.R201H mutation, originally reported by Ishii et al. [Santamaria et al., 2006] was homozygous in a Morquio B patient, and it was associated also with juvenile and adult G_{M1} phenotypes. The patient reported by Paschke et al. [2001] had been initially diagnosed as presenting with the Morquio phenotype with the p.T82M/p.Y270D genotype, but subsequently developed neurological manifestations. Caciotti et al. [2005] reported a juvenile GM1-gangliosidosis patient with p.R201H. Santamaria et al. [2006] concluded that a continuum is present