プラダー・ウイリー症候群では難聴がある場合もあり、口頭での指示に従えない場合は、難聴も疑う必要があります。

3）時間や場所の変化の概念が弱い？

プラダー・ウイリー症候群のある子で見られる、「日常の小さな変化に混乱する」、「質問を何度も繰り返す」「同じことを反復する＝同一性の保持」、は、自閉症のある子でも見られます。自閉症のある子は「場所や時間の変化の概念」が薄く、同じ場所で異なった目的の行動をする時や、突然のスケジュールの変更に混乱します。

自閉症では、理由なく泣き叫ぶ、自傷行為、おもちゃを破壊するなどの行動があり、その背景には、コミュニケーションの障害、情緒不安定、理解力の不足、こだわり、我慢することができないなどがあると言われています。このような問題行動には、場面の構造化、安定したスケジュール、学習や作業の手順を具体的に示す、視覚的にわかりやすい指示を与える、決まった手順を作る、実際の作業の流れを身につけさせるなどの工夫が薦められています（自閉症ガイドブック　シリーズ２　学齢期編。社団法人「日本自閉症協会」pp14～16、2003）。これは自閉症の教育で使われるＴＥＡＣＣＨ法の「場所や時間の構造化」と言われる方法です。プラダー・ウイリー症候群でも、日常生活の時間的変化や場所的変化を視覚的にわかりやすくすることは、問題行動の対応として有効な場合があるのではないかと考えています。また日常のスケジュールの変更に混乱のある場合は、前もって注意してやることが必要です。

4）他人の心を読むのが苦手？

高機能自閉症のある子は、「ひとの心を読むこと」が苦手で、他人の考えていることの理解が困難です。この結果、グループゲームに参加が困難です。プラダー・ウイリー症候群のある子もグループゲームに参加することは苦手の場合もあるようですが、「人の心を読むこと」が苦手とは思えません。むしろ人の行動をジーと観察し、自分に好意的な人かそうでないかを敏感に見抜く力があるのではないかと思っています。しばらく前のことですが、外来に、一人の成人の方が、手紙を持って来たり、外来を受診したりしてくれました。にこにことして、身体を傾け、得意そうに歩いてきます。自分からはほとんど話をせず、にこにこと返事をするだけです。帰る時も同じような表情と格好で帰って行きます。数回来て、その後、ぱたりと受診しなくなりました。私には、何となく心当たりがありますが、彼女の期待に私が添えなかったのだと思います。言葉で何か嫌みを言った訳ではありません。彼女は敏感に察したのだと思っています。

乳幼児期、診察医の顔はジーと見るのに、人見知りをされて泣かれたことは記憶にありません。診察医の顔をジーと見るのが印象的です。プラダー・ウイリー症候群のある子は、自閉症のある子と違って、人の心を読むことは、むしろ鋭く、好き嫌いの感情は強いのではないかと思います。好きな人と一緒の時は落ち着き、嫌いな人に対しては反抗的問題行動を多く見せるのではないかと言う気がしています。このあたりは注意欠陥／多動性障害で問題行動を起こすようになった年長児に似ているかも知れません。

5．プラダー・ウイリー症候群の良いところ

プラダー・ウイリー症候群のある子は、自尊心が強く（強制されることに反発）、大人

の注意を引こうとし、他人の面倒を見たがり、人に評価されることを望んでいます。他人の面倒をみたり、人の注意を引く様な行動は、自閉症のある子には見られません。注意欠陥／多動性障害や自閉症のある子に似たところはそれぞれの障害で薦められている方法で接することが薦められますが、プラダー・ウイリー症候群の子は、自尊心が強く、人に評価されることを求めていることを理解して対応することが大切だと思います。良いことをした時はしっかり褒めてあげましょう。また褒美をあげても良いでしょう。

プラダー・ウイリー症候群のある子は、盗んだり、ひとの見ていないところで何かを壊したり、問題行動が多いことは確かです。乱暴な行動や攻撃的な言動をすることはありますが、攻撃的行動によって他人に危害を加えることは、その他の知的障害のある人と頻度は全く変わりません（Eifnfeld et al. 1999）。攻撃的言動や何かを壊すような問題行動は、暴力的な性格によるのではないと思います。不安や情緒不安定はあるものの、本来は優しい性格の子が多いと考えて良いと思います。

6．おわりに

プラダー・ウイリー症候群は異常な食欲、肥満、糖尿病によって身体的健康が障害されやすいだけでなく、精神的健康も障害されやすい病気であることが理解いただけたと思います。身体的・精神的健康が両方障害され、プラダー・ウイリー症候群のある成人の生活の質は著しく低いと思います。これまで述べてきた問題が全ての人に見られるわけではありませんが、このような問題がおこりやすい病気であること、そしてそれは育て方の問題ではないこと、をまず理解する必要があります。

現在、成長ホルモン療法が多くの子で行われ、小さいときから将来の不適応行動について聞かされ、現在成人の方と10年後に成人になる方の糖尿病の発生率、社会的適応能力は大きく変化する可能性があります。また、成長ホルモン療法をきっかけに、多くの小児科医がプラダー・ウイリー症候群を定期的に診ています。これからプラダー・ウイリー症候群の問題行動の背景について研究しようとする小児科医も増えてきて、将来、問題行動を少なくする育児法・教育法が見つけられる可能性も期待できます。

しかし、プラダー・ウイリー症候群は遺伝子の異常でおこり、その結果、食欲の亢進と二次的な身体的／精神的障害、さらに現在はまだ明らかに出来ていない背景による行動の問題がおこっている病気であることは間違いありません。これから、身体的健康／精神的健康が障害されるのを軽減出来る可能性はありますが、これらの障害を完全に治すことは今のところ不可能だと考えないといけません。

家族と関係者がこの病気を正しく理解し、肥満をコントロールし、問題行動の出現を少なくする努力が大切ですが、それだけではいけないと思います。まず第1に、世の中の人にも、プラダー・ウイリー症候群を理解してもらうことが必要です。プラダー・ウイリー症候群Q＆Aやプラダー・ウイリー症候群のカロリーブックのように、成人になったウイリープラダー・ウイリー症候群が共通しておこしやすい問題とその対応を、学校の先生や施設の指導員に読んでもらえる資料が必要です。第2には、プラダー・ウイリー症候群のある人も日本国民であり、憲法にある「健康で文化的な生活を営む権利」を有しており、「健康で文化的生活」を保障される必要があります。身体的／精神的健康の障害を防ぐ医学・教育学的方法の研究と開発、防ぐことが出来ない段階では、その障害に対する成人後の医療費補助と適切な福祉を求めていく必要があります。

MUTATION IN BRIEF

Novel *TSC2* Mutations and Decreased Expression of Tuberin in Cultured Tumor Cells with an Insertion Mutation

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Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by hamartomas in many organs. Two genes responsible for TSC, *TSC1* and *TSC2*, were recently identified. *TSC1* and *TSC2* encode the proteins hamartin and tuberin, respectively, and 337 different mutations have been reported in these genes thus far. Here, we report six novel *TSC2* mutations including one missense mutation, two nonsense point mutations, two frameshifts, and an insertion mutation. The insertion mutation is unique because of its location at an exon/intron boundary that results in triplication of a 34-bp sequence. Cultured tumor cells from the patient with this insertion mutation exhibited a decreased level of tuberin as revealed by Western blotting, suggesting that the mRNA of *TSC2* is not translated as efficiently or the translated protein exhibits reduced stability. Five novel polymorphisms of *TSC2* were also identified. As previously reported, the missense mutations were located in the GTPase activating protein-related domain of *TSC2* encoded in exons 34-38. No *TSC1* mutations were identified in the present subjects. © 2004 Wiley-Liss, Inc.

KEY WORDS: giant cell astrocytoma; Japanese; mutation; *TSC2*; tuberin; tuberous sclerosis complex

INTRODUCTION

Tuberous sclerosis complex (TSC) (MIM# 191100) is an autosomal dominant disorder characterized by the development of hamartomatous growth in many different organs, most commonly in the brain, heart, kidney and skin (Gomez et al., 1999). Involvement of the brain is associated with the most problematic clinical manifestations

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of TSC, including intellectual handicap, epilepsy and abnormal behavior (Cheadle et al., 2000). Approximately two-thirds of the cases are sporadic, without family history, reflecting a high spontaneous mutation rate in the underlying genes (Osborne et al., 1991, Sampson et al., 1994).

Two TSC-related genes were previously identified by positional cloning. *TSC2* (MIM# 191092) is located on chromosome 16p13.3 and consists of 41 exons, whereas *TSC1* (MIM# 191100) is located on 9q34 and consists of 23 exons (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; van Slegtenhorst et al., 1997). *TSC2* encodes the 200-kDa protein tuberin that contains a GTPase activating protein (GAP)-related domain (The European Chromosome 16 Tuberous Sclerosis Consortium 1993). Hamartin, the 130-kDa predicted product of *TSC1*, is a novel protein that is predicted to form a complex with tuberin (van Slegtenhorst et al., 1997). Loss of heterozygosity (LOH) of either *TSC1* or *TSC2* in affected tissues indicates that each acts as a tumor suppressor (Green et al., 1994; Henske et al., 1995; Sepp et al., 1996).

At least, 131 and 343 different disease causing mutations have been reported in *TSC1* and *TSC2*, respectively (Cheadle et al., 2000; The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). Here, we used single-strand conformational polymorphism (SSCP) analysis of genomic DNA to identify six novel mutations in Japanese TSC patients. Tuberin expression was decreased in cultured tumor cells from a patient with a unique insertion mutation in *TSC2*.

MATERIALS AND METHODS

Patients

Clinical information for all patients is summarized in Table 1. Patient 1 was a 9-year-old female. Her early developmental milestones were normal. She suffered from febrile convulsions at age 2, and at age 5 was diagnosed with TSC due to sebacea and brain calcifications revealed by computed tomography (CT). At 6 years of age, she presented with afebrile convulsions, and electroencephalography (EEG) revealed right parietal focal spikes and waves. Her mental milestones indicated mild retardation.

Table 1. Summary of the Clinical Information of the Patients

Patient	Age	Gender	Skin	Neurological Findings	Brain Radiological Findings	Others
Patient 1 (s)	9	F	+	seizures, mild MR	calcification	
Patient 2 (nd)	20	M	+	seizures, severe MR	?	bilateral renal tumor
Patient 3 (f)	15	F	+	seizures, MR	+ (no detailed information)	bilateral renal tumor, lung lymphangima
Patient 4 (nd)	16	F	?	(normal intelligence)	calcification	left renal tumor
Patient 5 (s)	1	M	+	West, mild developmental delay	PN, CD, heterotopia	cardiac tumor
Patient 6 (s)	2	F	+	West	PN, CD, heterotopia	cardiac tumor
Patient 7 (f)	15	F	+	West to Lennox, severe MR	PN, tubers	cardiac tumor
Patient 8 (f)	24	F	+	West syndrome, moderate MR	PN, calcification	brain tumor
Patient 9 (s)	2	F	+	seizures	calcification	
Patient 10 (s)	7	F	+	West syndrome	PN, tubers	

skin, skin involvement; s, sporadic case; f, familial case; nd, not detected; F, female; M, male; ?, unknown

MR, mental retardation; PN, periventricular nodules; CD, cortical dysplasia

Patient 2 was a 20-year-old male. At 10 months of age, he suffered febrile convulsions and was diagnosed with a developmental delay. At 17 months of age, a left renal tumor was surgically removed, the pathological diagnosis of which was renal cell carcinoma. At age 17 years, right renal angiolipoma was identified. His mental state was one of severe retardation, and he displayed white skin patches.

Patient 3 was a 15-year-old girl. In infancy, she suffered from seizure attacks and subsequently exhibited delayed psychomotor development. Periventricular nodules were noted by radiological examination. White skin

patches were also noted. At 13 years of age, bilateral renal angiolipomas were identified and surgically removed. At age 14, she had a first incidence of spontaneous pneumothorax with recurrent episodes in subsequent years. A chest X-ray revealed bilateral lung cystic lesions that were suspected to be lymphangiomas.

Patient 4 was a 16-year-old girl. At age 3 months, a right renal tumor was surgically removed and found to be cystic dysplasia. Her first epileptic episode occurred at age 1 year. At present, she has normal intelligence in spite of brain calcification. She has no cystic lesions in her left kidney but has an angiomyolipoma in the liver.

Patient 5 was the second child of healthy parents, and was immediately diagnosed with multiple cardiac tumors just after delivery. At 1 month of age, he presented with white skin patches and developed tonic spasms. Brain CT showed periventricular nodules and MRI showed left fronto-parietal cortical dysplasia and heterotopia. Now, at 1 year of age, he exhibits mild developmental delay.

Patient 6 was a 2-year-old girl. At 4 months of age, she was afflicted with infantile spasms, and an EEG indicated hypsarhythmia. She had white skin patches and cardiac rhabdomyoma that was identified by echocardiography. Brain MRI showed small nodules in periventricular regions.

Patient 7 was a 15-year-old girl. Although her parents were healthy, her younger brother had retinal hamartomas. She had white patches, facial angiofibromas and ungual fibroma. Radiological findings suggested subependymal nodules and tubers in the brain and cardiac rhabdomyoma. In infancy, she had West syndrome that later developed into Lennox syndrome. Her present intelligence quotient (I.Q.) is below 20.

Patient 8 was a 24-year-old female. At age 11 months she suffered infantile spasms. Many white spots were present on her skin. Brain radiological examinations revealed a right anterior ventricular tumor and periventricular calcifications. When she was 8 months old, she had chronic left facial palsy that may have been related to a tumor, and her symptoms disappeared after resection of a tumor that was diagnosed as a giant cell astrocytoma (cells were cultured from the resected tumor tissues, and were used for Western blotting). Presently, she exhibits moderate mental retardation. Although her parents and an elder sister are healthy, her father has white macules.

Patient 9 was a 2-year-old girl. Following her first epileptic attack, a detailed investigation suggested TSC based on white macules and brain calcifications.

Patient 10 was a 7-year-old boy that displayed white macules. He developed West syndrome at 5 months of age. Radiological examination showed periventricular nodules and some tubers. Presently, he shows moderate mental retardation. His parents are healthy.

Molecular analysis

DNAs were extracted from peripheral lymphocytes using a standard method. Sixty-four normal control DNAs were also obtained from blood samples of healthy Japanese volunteers and used for a population study. Informed consent for genomic examinations was obtained from all patients and volunteers. The Caucasian Population Panel 100 was provided by the Coriell Institute for Medical Research (NJ, USA) and fifty DNAs samples were used for population study. Polymerase chain reaction (PCR) was used to amplify all exons of *TSC1* (GenBank accession number AF013168.1) and *TSC2* (GenBank accession number X75621.1) from genomic DNAs using standard methods with primers described elsewhere (Zhang et al., 1999; Pipo et al., 2000; Yamamoto et al., 2002). The PCR products were subjected to SSCP analysis using a minigel (10 cm X 10 cm). The samples were analyzed under four different electrophoresis conditions from a combination of two sets of gel mixtures (12% polyacrylamide gel with or without 5% (w/v) glycerol) and two temperatures (4°C or 22°C) (Zhang et al., 1999). DNA bands were visualized by silver staining. The PCR products that gave aberrant bands during the SSCP analysis were sequenced directly using the BigDye terminator cycle sequencing kit (Applied Biosystems, CA, USA) and the ABI PRISM 3100 genetic analyzer (Applied Biosystems). Each PCR product was sequenced in both directions using PCR primers.

Western blotting

Tumor cells of patient 8 were cultured from the resected tumor tissues, and harvested and homogenized by sonication in buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) supplemented with 1% (w/v) Triton X-100 and protease inhibitor cocktail (Boehringer Ingelheim, Ingelheim, Germany). The suspensions were centrifuged at 12,000 x g for 30 min at 4°C and the protein concentration of the supernatant was determined by the BCA protein assay (Bio-Rad, CA, USA). Protein (10 μg) was subjected to SDS-polyacrylamide gel

electrophoresis on a 10% gel, and the proteins were electrophoretically transferred to an Immobilon membrane (Millipore, Bedford, MA). Following a 1-h preincubation in 5% (w/v) skim milk, the membrane was incubated overnight at 4°C with the tuberin antibody, tuberin (C-20) (cat. # sc-893; Santa Cruz Biotechnology, Inc., USA; diluted 1:1000). Tuberin bands were detected using avidin-biotin-alkaline phosphatase (Vector ABC-AP kit).

RESULTS AND DISCUSSION

Table 2. Summary of the Disease-Causing *TSC2* Mutations

Patient	Location	Nucleotide Change*	Amino Acid Change	Type	Novel or Reported by
Patient 1 (s)	ex4	c.469G>T	p.E157X	nonsense	novel
Patient 2 (nd)	ex19	c.2163del G		frameshift	novel
Patient 3 (f)	ex24	c.2767_2768insC		frameshift	novel
Patient 4 (nd)	ex28	c.3355C>T	p.Q1119X	nonsense	novel
Patient 5 (s)	ex37	c.4952A>G	p.N1651S	missense	Maheshwar et al., 1997
Patient 6 (s)	ex37	c.4958C>T	p.S1653F	missense	novel
Patient 7 (f)	ex38	c.5024C>T	p.P1675L	missense	Maheshwar et al., 1997
Patient 8 (f)	IVS38	c.5068+20_5068+21ins34		splicing?	novel
Patient 9 (s)	ex40	c.5238_5255del	p.H1746_R1751del	deletion	Beauchamp et al., 1998
Patient 10 (s)	ex40	c.5238_5255del	p.H1746_R1751del	deletion	Beauchamp et al., 1998

s, sporadic case; c., complementary DNA No.; f, familial case; del, deletion; ins, insertion

*GenBank X75621.1. Nucleotide numbering, with A of the initiator ATG as +1

The mutation nomenclature according to the website (http://www.HGVS.org/mutnomen/).

Six novel and three previously known mutations of *TSC2* (Table 2) were identified. All of the patients exhibited mutations in *TSC2* only, and no *TSC1* mutations were identified despite the comprehensive screening of both genes. Two new nonsense mutations and two new frameshift mutations resulted from respective deletion and insertion of 1 bp would be definitely disease causing.

A novel missense mutation within exon 37, complementary DNA No. c.4958C>T (p.S1653F) in patient 6 was not found in healthy control subjects (64 Japanese and 50 Caucasians) suggesting that they are pathogenic for TSC. The known missense mutation, c.5024C>T (p.P1675L) within exon 38 in patient 7, was recurrent and thus represents a relatively common mutation (Beauchamp et al., 1998; Zhang et al., 1999). Interestingly, all three patients (patients 5, 6 and 7) with missense mutations presented with cardiorhabdomyoma. As reported elsewhere, these missense mutations were located in the GAP-related domain of *TSC2* encoded in exons 34-38 (Cheadle et al., 2000). None of the mutations were located in the sequence CpG, a dinucleotide sequence in which nucleotide alterations are prevalent.

Interestingly, both patient 2 and 3 having a novel frameshift mutation had renal cancers, and patient 3 also suffered from lung lesions. TSC with lung lesions is relatively rare and constitutes a distinct subset of the disease termed pulmonary TSC (Kalassian et al., 1997; Sullivan 1998). A common pulmonary lesion is a parenchymal cyst that is often associated with dyspnea or pneumothorax. Typically, patients with pulmonary TSC are women of childbearing age whose pulmonary lesions may be influenced by hormonal changes (Kalassian et al., 1997; Sullivan 1998).

An 18-bp deletion (c.5238_5255del) was identified within exon 40 in both sporadic patients 9 and 10. As this in-frame deletion has been frequently identified in TSC patients (Dabora et al., 2001), this region may constitute a hot spot. Another 18-bp in-frame deletion, c.5256_5273del (adjacent to 18-bp deletion at c. 5238_5255) was also identified frequently in four unrelated sporadic cases (Jones et al., 1999). These deletions occur in the sequence context of a direct repeat of eleven nucleotides with seven intervening nucleotides, and are likely the product of

slipped mispairing during replication (Cooper and Krawczak 1991). The recurrent 18-bp deletion in exon 40 of *TSC2* lies within the putative rabaptin-binding domain (Xiao et al., 1997).

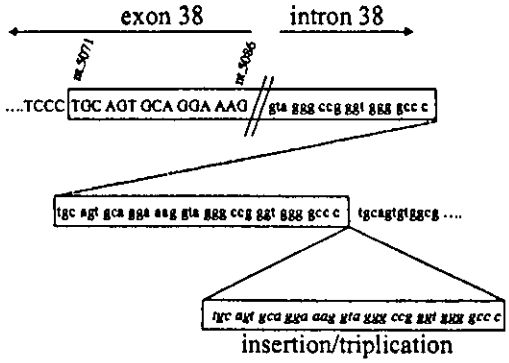

Figure 1. Schematic representation of the sequence around the site of the insertion detected in patient 8. Open boxes indicate the set of 34-bp sequences that is duplicated in the normal sequence and triplicated in patient 8. Uppercase and lowercase nucleotides indicate exonic and intronic sequences, respectively.

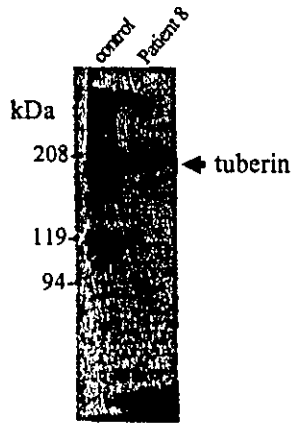

Figure 2. Western blot analysis. It was confirmed that the same amount of protein had been loaded in each lane and transferred by staining gel and unused portion of membrane with Coomassie brilliant blue (data not shown). The arrowhead indicates tuberin, the expression of which is decreased in the sample from patient 8 compared with that of control. The migration of molecular weight markers is shown to the left. C, control sample derived from an autopsied brain.

A 34-bp insertion (c.5068+20_5068+21ins34) was identified in patient 8. In the normal genomic sequence, this 34-bp sequence (TGC AGT GCA GGA AAG GTA GGG CCG GGT GGG GCC C) is duplicated across the boundary of exon 38 and intron 38 (Fig. 1). Patient 8 had three repeats of this 34-bp sequence and there was no other mutation in any of the exons of *TSC1* and *TSC2*. This patient was a familial case, but attempts to obtain samples from family members were unsuccessful. Thus, to exclude the possibility that the triplication represented benign polymorphisms, we analyzed this region in normal Japanese controls as well as in the Caucasian Population Panel 100. As expected, this duplication was not observed in any of the control samples. To test whether this insertion influences pre-mRNA splicing, cDNA was analyzed around this exon 38 using reverse-transcription PCR. However, no aberrant splicing was observed (data not shown). To estimate the impact of this genomic mutation, tuberin expression was assessed in primary-cultured cells from the patient's giant cell astrocytoma.

Tuberin expression was decreased compared with normal brain tissue (Fig. 3). This result is compatible with mutational loss of *TSC2* (Wienecke et al., 1997). However, we cannot exclude the possibility that secondary effects such as hamartin or rap1 expression may negatively influence tuberin expression.

Unlike many other symptoms that show age-dependant penetration, intellectual disability in TSC is almost invariably present from early childhood and rarely escapes detection. However, patient 4 (carrying a nonsense mutation) exhibited normal intellectual development. Patients 9 and 10 carried the same mutation, but their clinical features were different from one other. Thus, the extent of the protein truncation expected from mutations in *TSC2* does not necessarily correlate with the severity of the clinical symptoms. Therefore, the severity is likely dependent on other somatic mutations within the pathogenic lesions. Future determination of the pathogenesis of these genomic mutations will require the development of a functional assay for tuberin activity.

Five novel variations of *TSC2* were also identified (Table 3). The each variation was coincidentally identified in only one control sample which was used for population study. Thus, these variation would be very rare and not disease causing.

Table 3. Summary of the *TSC2* Polymorphisms

Location	Nucleotide change*	Amino Acid Change	Type	Novel or Reported by	Frequency#
ex14	c.1593C>T	p.I531	silent	Yamashita et al., 2000	
ex16	c.1819G>A	p.A607T	missense	novel	1/114
ex22	c.2585C>T	p.A862V	missense	novel	1/114
ex33	c.4285G>T	p.A1429S	minsense	novel	1/114
ex33	c.4349C>G	p.P1450R	missense	novel	1/114
IVS33	c.4493+17C>T			novel	1/114
IVS39	c.5161-9C>A			Jones et al., 1999	
ex40	c.5202T>C	p.D1734	silent	Au et al., 1997	
ex41 (3' non-coding region)	c.5424+55_5424+58delTAAA			Kumar et al., 1995	

c., complementary DNA No.; *GenBank X75621.1. Nucleotide numbering, with A of the initiator ATG as +1

#Frequency of each variation was described as 1/114, because the each variation was detected in only one sample among 64 healthy Japanese volunteers and 50 samples of Caucasian DNA panel.

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N-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease

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Abstract

Gaucher disease (GD) is the most common form of sphingolipidosis and is caused by a defect of β-glucosidase (β-Glu). A carbohydrate mimic *N*-octyl-β-valienamine (NOV) is an inhibitor of β-Glu. When applied to cultured GD fibroblasts with F213I β-Glu mutation, NOV increased the protein level of the mutant enzyme and up-regulated cellular enzyme activity. The maximum effect of NOV was observed in F213I homozygous cells in which NOV treatment at 30 μM for 4 days caused a ~ 6-fold increase in the enzyme activity, up to ~ 80% of the activity in control cells. NOV was not effective in cells with other β-Glu mutations, N370S, L444P, 84CG and RecNciI. Immunofluorescence and cell fractionation showed localization of the F213I mutant enzyme in the lysosomes of NOV-treated cells. Consistent with this, NOV restored clearance of ^{14}C-labeled glucosylceramide in F213I homozygous cells. F213I mutant β-Glu rapidly lost its activity at neutral pH in vitro and this pH-dependent loss of activity was attenuated by NOV. These results suggest that NOV works as a chemical chaperone to accelerate transport and maturation of F213I mutant β-Glu and may suggest a therapeutic value of this compound for GD.

Keywords: Gaucher disease; β-glucosidase; Valienamine; Glucosylceramide; Chaperone

1. Introduction

Gaucher disease (GD) is an inherited lipid storage disorder, characterized by lysosomal accumulation of glucocerebroside (glucosylceramide; GlcCer) in monocyte-macrophage cells [1]. It is caused by a defect of acid β-glucosidase (β-Glu; glucocerebrosidase EC 3.2.1.45). Patients with GD exhibit hepatosplenomegaly, anemia, bone lesions and respiratory failure, with or without progressive neurological symptoms. Patients without neurological symptoms are classified as type 1, whereas those with neurological symptoms are classified into type 2 (acute infantile form) and type 3 (juvenile form).

Abbreviations: α-Gal A, α-galactosidase A; α-Glu, α-glucosidase; β-Gal, β-galactosidase; β-Glu, β-glucosidase; β-Hex, β-hexosaminidase; NOEV, *N*-octyl-β-epi-valienamine; NOV, *N*-octyl-β-valienamine; DGJ, 1-deoxy-galactonojirimycin; ER, endoplasmic reticulum; GD, Gaucher disease; GlcCer, glucosylceramide; HPTLC, high performance thin layer chromatography; NN-DGJ, *N*-(*n*-nonyl)-deoxy-nojirimaycin

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Current therapeutic strategies for GD include enzyme replacement and substrate depletion. Enzyme replacement has been achieved by intravenous administration of macrophage-targeted recombinant β-Glu [2] and it has been proven to be quite effective for visceral, hematologic and skeletal abnormalities [3,4]. Unfortunately, the efficacy to neurological manifestations of this therapy is, if any, limited [5–7]. A high cost as well as necessity to continue the infusion every 2 weeks is not negligible, when indication of enzyme replacement is considered in practice [4]. Substrate depletion has been achieved by oral administration of *N*-butyl-deoxynojirimycin, which inhibits glucosyltransferase and decreases substrate biosynthesis. This therapy has been reported to be beneficial for non-neuropathic GD [8,9].

We have proposed a novel therapeutic strategy for glycolipid storage disorders to accelerate transport and maturation of mutant enzymes by using enzyme inhibitors as a chemical chaperone. This strategy was first applied to Fabry disease (α-galactosidase A [α-Gal A] deficiency) and we found that 1-deoxy-galactonojirimycin (DGJ), an inhibitor of α-Gal A, markedly enhanced activity of mutant enzymes in lymphoblasts from Fabry patients [10]. Although up-regulation of enzyme activity by an inhibitor appeared paradoxical, evidence was presented that DGJ prevented pH-dependent degradation of mutant α-Gal A at the site of its synthesis [i.e., the endoplasmic reticulum (ER)]. With the aid of DGJ, mutant α-Gal A escaped the ER quality control system and was transported to the lysosome where it is stabilized because of the acidic condition and restored cellular enzyme activity. This strategy was then tested in GM1-galactosidosis [β-galactosidase (β-Gal) deficiency] and we found that DGJ as well as another derivative *N*-(*n*-butyl)-deoxy-galactonojirimycin could up-regulate activity of mutant human β-Gal expressed in fibroblasts from β-Gal knockout mice [11]. In pursuit of the same therapeutic strategy, Sawkar et al. [12] reported that an inhibitor of β-Glu, *N*-(*n*-nonyl)-deoxynojirimaycin (NN-DNJ) up-regulated activity of N370S mutant β-Glu in GD fibroblasts. Although it is yet to be tested whether NN-DNJ can correct GlcCer accumulation in N370S GD cells, their findings suggested that this strategy might be extended to GD.

Valienamine is a synthetic carbohydrate mimic and we have prepared various *N*-alkyl and *N*,*N*-dialkyl-β-valienamines in continuation of a chemical modification program [13]. Among these substances, *N*-octyl-β-epivalienamine (NOEV) is an inhibitor of β-Gal and we have provided evidence that NOEV worked as a chemical chaperone to up-regulate mutant β-Gal activity both in cultured cells and in mice [14]. *N*-octyl-β-valienamine (NOV) is an isomer of NOEV and exerted the strongest inhibition of β-Glu activity in the mouse liver [15]. The purpose of the current studies was to test a possibility that NOV could up-regulate mutant β-Glu activity in cultured human cells. Preliminary findings of the current studies have been reported [16].

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS) and dialyzed serum were obtained from GibcoBRL. NOV was synthesized in our laboratory (Central Research Laboratories, Seikagaku). Stock solution of NOV was prepared in H_2O at 3 mM and stored at −20 °C. A mouse monoclonal antibody against human β-Glu (clone 8E4, Ref. [17]) was a kind gift from Dr. Barranger JA. Rabbit polyclonal anti-hexosaminidase A (HexA) has been described [18]. Rabbit polyclonal anti-calnexin was from Calbiochem. [1-^{14}C]Serine (1.85 GBq/mmol) was from American Radiolabeled Chemicals (St. Louis, MO).

2.2. Cell culture

Human skin fibroblasts were cultured in DMEM/10% FCS at 37 °C in 5% CO_2. We used two lines of control cells (H11, H34) and five lines of GD cells with β-Glu mutations of 754A(F213I)/754A(F213I), 754A(F213I)/1448C(L444P), 1126G(N370S)/84GG, 1448C(L444P)/RecNciI, and 1448C(L444P)/1448C(L444P) [19]. 84GG causes premature termination of the encoded protein and RecNciI causes amino acid substitutions L444P and A456P [1]. Culture medium was replaced every 2 days with fresh media supplemented with or without NOV at the concentrations indicated.

2.3. Enzyme assays

Lysosomal enzyme activities in cell lysates were determined as described [14,18,20,21]. Briefly, cells were scraped into ice-cold H_2O (10^6/ml) and lysed by sonication. Insoluble materials were removed by centrifugation at 12,000 × *g* for 10 min at 4 °C and protein concentrations were determined with a BCA microprotein assay kit (Pierce). Ten microliters of the lysates was incubated at 37 °C with 20 μl of the substrate solution in 0.1 M citrate buffer, pH 4.5. The substrates were 4-methylumbelliferone-conjugated β-D-galactopyranoside (for β-Gal, Ref. [14]), β-D-glucopyranoside (for β-Glu, Ref. [20]), α-D-glucoside (for α-Glu, Ref. [21]) and *N*-acetyl-β-D-glucosaminide (for β-Hex, Ref. [18]). The reaction was terminated by adding 1.0 ml of 0.2 M glycine sodium hydroxide buffer (pH 10.7). One unit of enzyme activity was defined as nanomoles of 4-methyl-umbelliferone released per hour.

2.4. Western blotting

Cell lysates (20 μg protein) were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane.

The blots were probed with antibodies against β-Glu (1:500) or HexA (1:1000) and developed with an ECL kit (Amersham Pharmacia). Densitometry was performed by using an NIH image software.

2.5. Immunofluorescence

We used staining procedures described previously [22]. Briefly, cells grown on cover glasses were fixed with 4% paraformaldehyde and permiabilized with 1% Triton X-100. Cells were incubated with anti-β-Glu (1:100), followed by Alexa488-conjugated anti-mouse IgG. Fluorescent images were collected by using a Bio-Rad MRC1024 confocal laser microscope. For localization of lysosomes, cells were exposed to Lysotracker Red (5 μg/ml; Molecular Probe) for 1 h prior to fixation.

2.6. Subcellular fractionation

Cell homogenates were fractionated by using Opti-prep (Axis-Shield plc., Dundee, UK) as described [23]. Briefly, cells were homogenized with a potter homogenizer in ice-cold buffer [HEPES 10 mM pH 7.0, 1 mM EDTA, 1 mM EGTA supplemented with a protease inhibitor cocktail (Boehringer)]. Homogenates were overlaid on Opti-prep and centrifuged at 100,000 × *g* for 16 h at 4 °C. Twelve fractions were recovered from the top and numbered accordingly.

2.7. Metabolic labeling of GlcCer

Cellular glycolipids were labeled with [^{14}C]serine as described [24]. Briefly, cells were cultured for 1 week in dialyzed serum supplemented with essential amino acids except for serine, and incubated with [^{14}C]serine (1 μCi/ml) for 3 days. The labeled cells were cultured in fresh DMEM/10% FCS for 5 days with or without NOV. We analyzed labeled lipids by high-performance thin layer chromatography (HPTLC) as described [25]. In brief, cells were harvested at the time indicated, and lipids were extracted with chloroform/methanol (2:1 v/v) and purified by an alkaline treatment. Neutral glycolipids were purified by C18 affinity chromatography. HPTLC was performed with chloroform/methanol/water (55:25:4) as a developing solvent. Labeled lipids were visualized by autoradiography (Fuji-BAS 2500: Fuji, Tokyo, Japan) and densitometry was performed using an NIH image software.

2.8. pH-dependent stability of β-Glu in vitro

Cell lysates were incubated in 0.1 M citrate-phosphate buffer at pH 5, 6 or 7 at 37 °C for the time indicated. Incubation was terminated by the addition of 3 volumes of 0.2 M citrate-phosphate buffer (pH 4.5), immediately followed by chilling on ice. The enzyme assay was done at pH 4.5 as described above.

3. Results

3.1. Inhibition of human β-Glu by NOV in vitro

Chemical structures of NOV and its isomer NOEV are shown in Fig. 1a. NOV inhibited β-Glu activity in mouse liver extracts with an IC_{50} value of 0.03 μM [15]. To test whether it also works on the human enzyme, we determined β-Glu activity in lysates from control human fibroblasts in the absence or presence of NOV. NOV caused dose-dependent inhibition of β-Glu activity with an IC_{50} value of 3 μM (Fig. 1b), indicating that it also works on human β-Glu. NOV contains a C8 fatty acid acyl moiety chain (Fig. 1a). A related compound with a C6

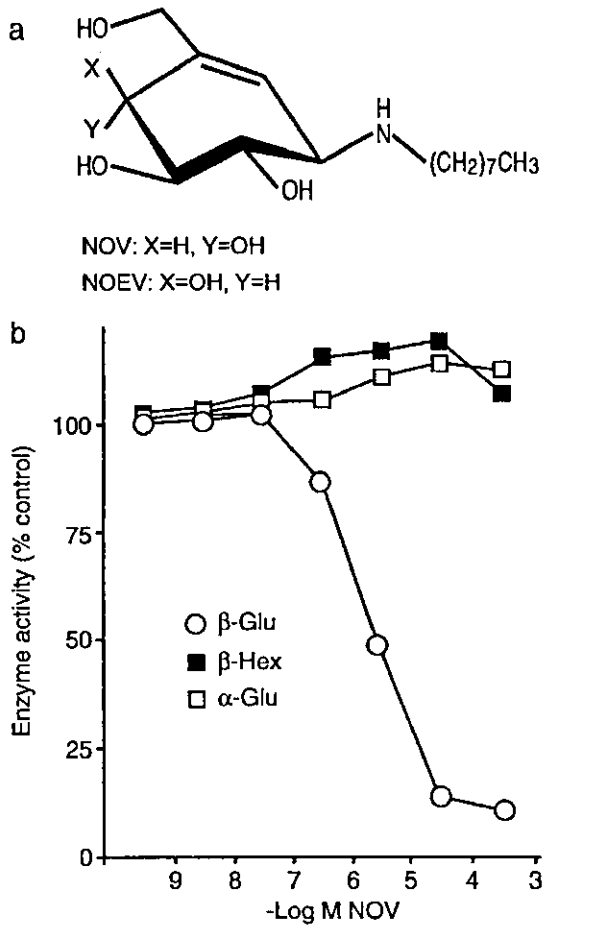

Fig. 1. Effects of NOV on lysosomal enzyme activities in lysates from control human fibroblasts. (a) Chemical structures of NOV and its isomer NOEV. (b) Dose dependence. Enzyme activity in H11 cell lysates was determined in the absence or presence of increasing concentrations of NOV. Each point represents means of triplicate determinations obtained in a single experiment. Values were expressed as relative to activity in the absence of NOV (100%). Values in the absence of NOV in this experiment were β-Glu 142, α-Glu 94 and β-Hex 5089 (units/mg protein). Similar results were obtained in two other experiments.

fatty acid acyl moiety chain inhibited human β-Glu with an IC_{50} value of 30 μM (data not shown), suggesting that the inhibitory activity can be regulated by the length of this chain. NOV caused no inhibition of other lysosomal enzymes α-Glu and β-Hex in the same cell lysates, suggesting a specificity of NOV as an inhibitor of β-Glu (Fig. 1b).

3.2. Up-regulation of F213I mutant β-Glu activity in GD cells treated with NOV

To explore an effect of NOV on mutant β-Glu activity, GD cells with five different genotypes were cultured for 4 days with increasing concentrations of NOV, and β-Glu activity in cell lysates was determined (Fig. 2a, right). NOV

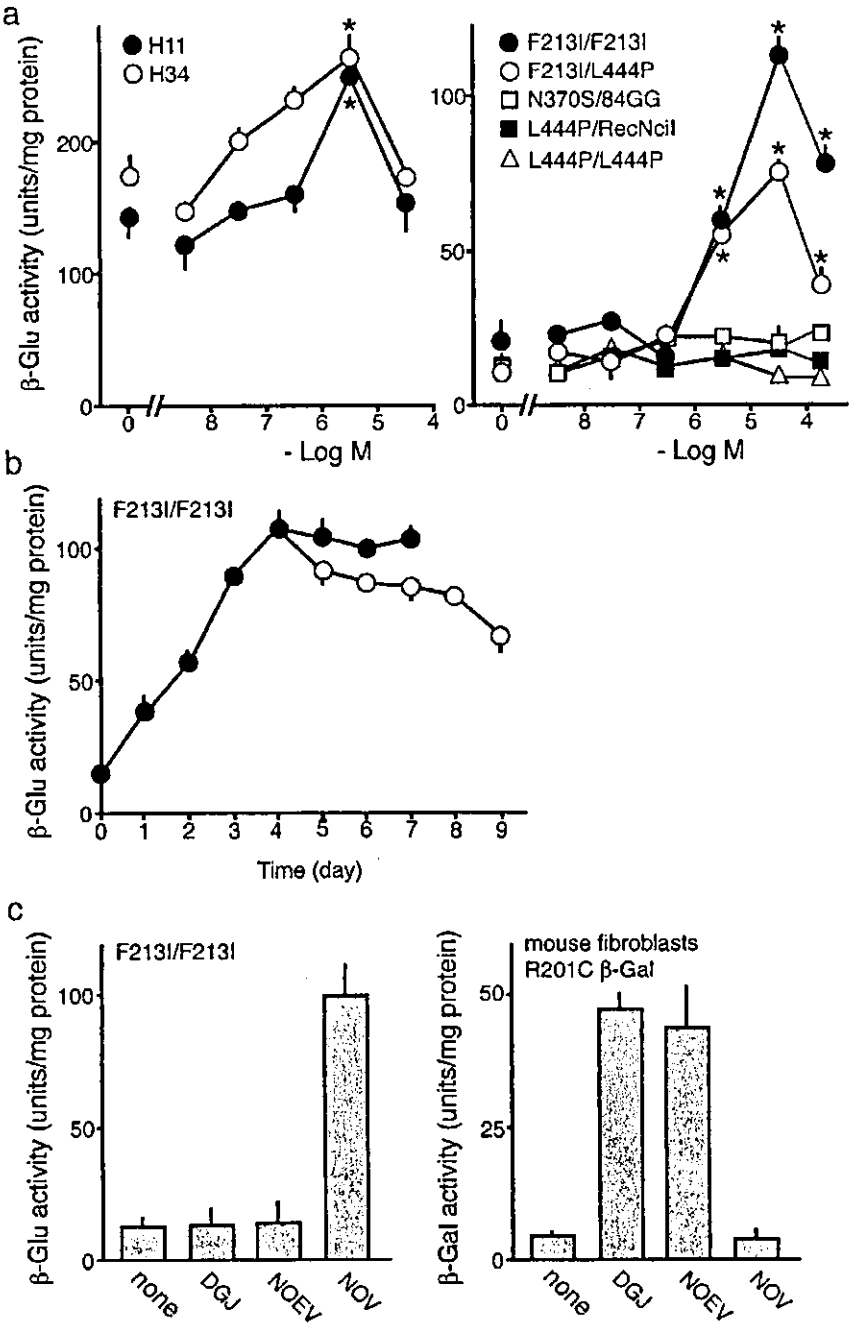

Fig. 2. Effects of NOV on cellular β-Glu activity. (a) Dose dependence. Two lines of control cells (left) and five lines of GD cells (right) were cultured for 4 days in the absence or presence of increasing concentrations of NOV and β-Glu activity in cell lysates was determined. (b) Time course. F213I/F213I cells were cultured in the presence of 30 μM NOV up to 7 days (●). A subset of cells was cultured with NOV for 4 days, washed and further cultured without the drug for 5 days (○). At the time indicated, cells were harvested and β-Glu activity in cell lysates was determined. (c) Specificity of the effects of NOV. F213I/F213I cells were cultured in the presence of indicated drugs (all at 20 μM) for 4 days and β-Glu activity in cell lysates was determined (left). In separate experiments, the same drugs were applied to mouse fibroblasts that express human R201C β-Gal and β-Gal activity in cell lysates was determined (right). Each point or bar represents mean ± S.E. of three determinations each done in triplicate. $^*P < 0.05$, statistically different from the values in the absence of the drug (*t* test).

caused dose-dependent increases in β-Glu activity in two lines of GD cells, F213I/F213I and F213I/L444P. The maximum effect of NOV was observed in F213I homozygous cells in which treatment at 30 μM caused a ~ 6-fold increase in the enzyme activity, up to ~ 80% of the basal activity in control cells. NOV at the same concentration caused a ~ 3-fold increase in F213I/L444P cells. There appeared to be an optimal concentration of NOV, because it was less potent at a higher concentration (100 μM). NOV caused no substantial increase in the enzyme activity of GD cells with other mutations, indicating a specificity of this up-regulation for F213I mutant β-Glu. This effect of NOV, however, was also observed in wild-type β-Glu with a different dose dependence profile; NOV at 3 μM caused a ~ 1.5-fold increase in the two control cell lines but was not effective at 30 μM (Fig. 2a, left).

Time-course analysis using F213I/F213I cells showed that in the presence of NOV (30 μM), β-Glu activity increased in a time-dependent manner and reached a plateau on day 4. When cells were deprived of NOV on

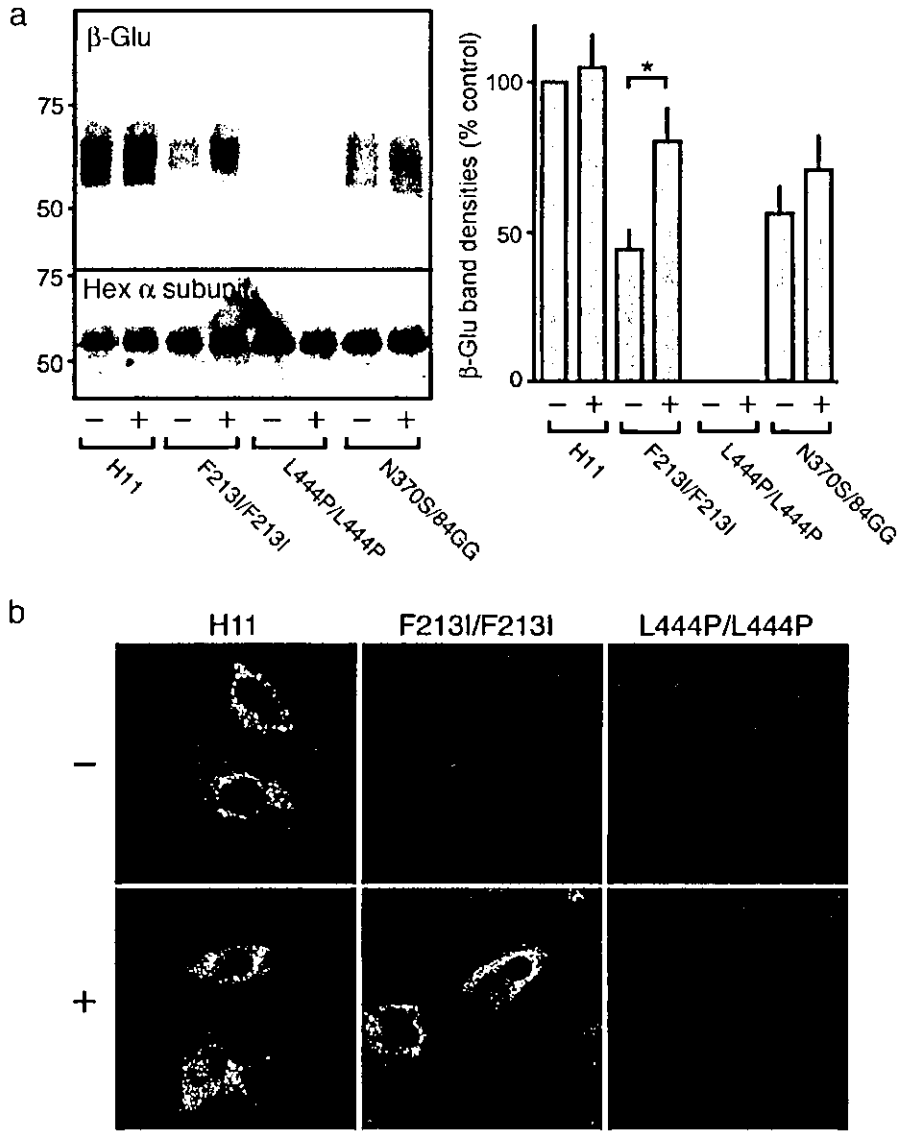

Fig. 3. Effects of NOV on the protein level and intracellular localization of mutant β-Glu. Cells were cultured with (+) or without (−) NOV at 30 μM for 4 days. (a) Western blotting. Cell lysates were analyzed by Western blotting with antibodies against β-Glu or Hex α subunit (left). Twenty micrograms of protein was loaded in each lane. Molecular weights are given on the left (kDa). Densities of β-Glu bands were quantified by densitometry (right). Each bar represents mean ± S.E. of 3 determinations. $^*P < 0.05$, statistically different from each other (t test). (b) Anti-β-Glu immunofluorescence. (c) Double labeling of F213I/F213I cells with anti-β-Glu and Lysotracker Red. In b and c, shown are the representative images obtained with a confocal microscope. All the images were obtained at the same laser intensity and window level. (d) Cell fractionation. Cells were cultured with (●) or without (○) NOV at 30 μM for 4 days. Cell homogenates were subjected to Opti-prep fractionation and each fraction was assessed for activity of β-Glu (upper) or β-Hex (lower). Fractions from F213I/F213I cells were also subjected to anti-calnexin Western blotting. Each point represents mean values of triplicate determinations obtained in a single experiment. Similar results were obtained in two other experiments.

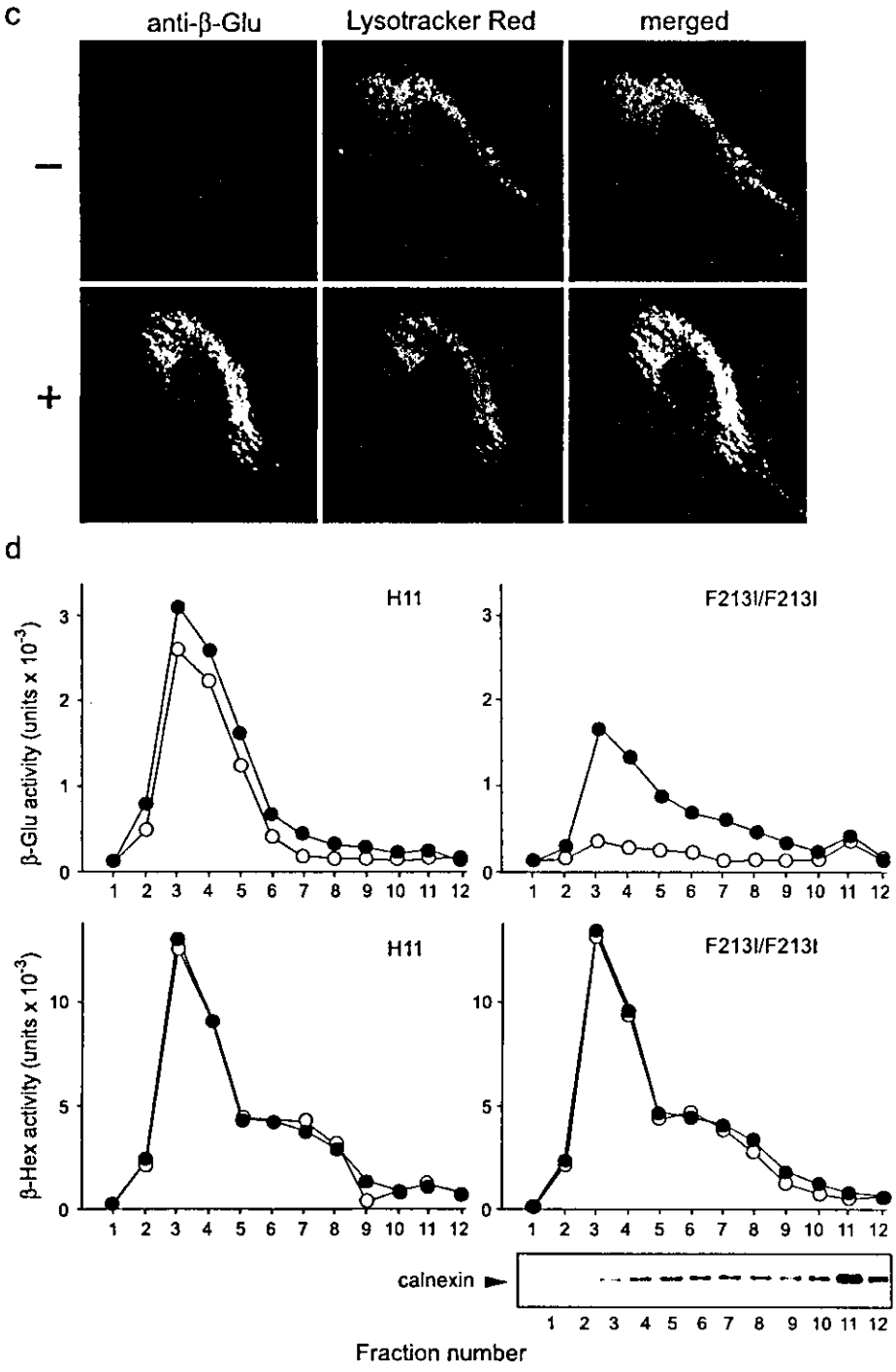

Fig. 3 (*continued*).

day 4, the activity gradually decreased thereafter but was still more than three times higher than the basal level on day 9 (Fig. 2b).

We have shown that both DGJ and NOEV could up-regulate the activity of R201C mutant β-Gal that was expressed in fibroblasts derived from β-Gal knockout mice [11,14]. To test specificity of NOV, first, we examined whether DGJ or NOEV could up-regulate F213I β-Glu activity, and found that neither of these substances caused any changes in the activity (Fig. 2e, left). Second, we examined whether NOV could up-regulate the activity of R201C mutant β-Gal, and found that unlike DGJ and NOEV, NOV had no effect on this mutant enzyme (Fig. 2e, right).

3.3. NOV increased the protein level of F213I mutant β-Glu and restored its localization in the lysosome

To examine the effect of NOV on the protein level, cell lysates were subjected to Western blotting with 8E4 monoclonal anti-β-Glu antibody and the protein levels were estimated by densitometry. This analysis showed that NOV treatment (30 μM for 4 days) of F213I/F213I cells caused a significant increase in the protein level of F213I mutant β-Glu. The same treatment did not increase the protein levels in N370S/84GG and control cells. As reported previously [26], the antibody did not recognize L444P mutant β-Glu. As a control, NOV treatment caused no changes in the protein levels of Hex α subunit (Fig. 3a).

Next, we examined intracellular localization of β-Glu by immunofluorescence and cell fractionation. Anti-β-Glu staining of control cells showed localization of β-Glu immunoreactivity in perinuclear punctate structures and this localization was not affected by NOV treatment. β-Glu immunoreactivity in F213I/F213I cells was lower than in control cells and there was no clear localization in perinuclear punctate structures. When these cells were treated with NOV, however, the immunoreactivity was clearly seen in these structures (Fig. 3b). Localization of F213I mutant β-Glu in the lysosome of NOV-treated cells was evidenced by co-localization of the immunoreactivity and a lysosome marker Lysotracker Red (Fig. 3c). β-Glu immunoreactivity was not detectable in L444P/L444P cells, indicating the specificity of this antibody staining (Fig. 3b).

When control cells were subjected to subcellular fractionation on Opti-prep, β-Glu activity was recovered in fractions #3–5. The same analysis of F213I/F213I cell fractions showed broad distribution of mutant β-Glu activity with peaks at #3 and #11. Both peaks were small but were consistently observed in three independent determinations. Anti-calnexin Western blotting showed that #11 contained a high amount of this ER marker protein [27]. NOV treatment of control cells caused marginal increases (~ 1.1-fold) in β-Glu activity recovered in #3–5. The same treatment of F213I/F213I cells caused ~ 4-fold increases in #3–5. As a control, we measured β-Hex activity in each fraction. Both in control and F213I/F213I cells, β-Hex activity was recovered in #3–4 and to a lesser degree, in #5–8. β-Hex activity in each fraction was not affected by NOV treatment in either cell line (Fig. 3d).

3.4. NOV restored clearance of ^{14}C-labeled GlcCer in F213I/F213I cells

NOV-induced increase of mutant β-Glu activity in the lysosome of F213I/F213I cells prompted us to examine whether NOV could correct abnormal catabolism of GlcCer in this cell line. By using conventional HPTLC analysis of cellular lipid extracts, accumulation of GlcCer was barely detectable in GD skin fibroblasts, most likely because of the low level of this lipid in these cells (data not shown). Therefore, we employed metabolic labeling of cellular glycolipids with [^{14}C]serine and assessed clearance of ^{14}C-labeled GlcCer. When control cells were chased for 5 days after the metabolic labeling, the content of ^{14}C-GlcCer decreased by ~ 50%. This clearance of ^{14}C-GlcCer was retarded in F213I/F213I cells in which there was only a ~ 10% decrease. Inclusion of NOV (30 μM) in the chase medium had no effect in control cells but accelerated the clearance in F213I/F213I cells. In the presence of NOV, the content of ^{14}C-GlcCer in F213I/F213I cells decreased by ~ 50% and reached a level that was comparable to that in control cells (Fig. 4b). The HPTLC analyses showed that besides ^{14}C-GlcCer, clearance of ^{14}C-LacCer was retarded in F213I/F213I cell and again it was accelerated by NOV (Fig. 4a). In addition, NOV caused decreases in the levels of ^{14}C-labeled lipids that corresponded to the positions of CTH, SM and

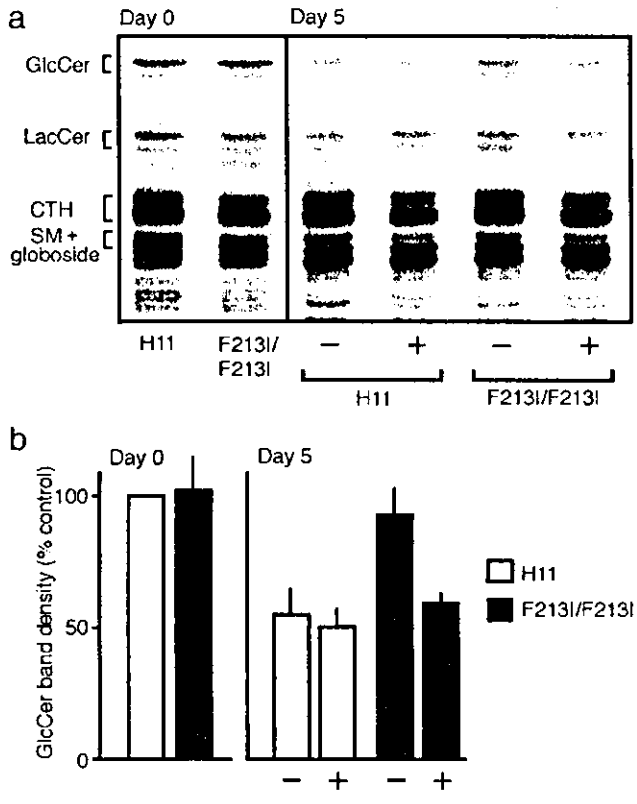

Fig. 4. Effects of NOV on cellular clearance of ^{14}C-labeled GlcCer. (a) HPTLC separation of ^{14}C-labeled cellular lipids. Cells were labeled with ^{14}C-serine for 3 days and then chased up to 5 days in the presence or absence of NOV (30 μM). At the beginning (day 0) and the end (day 5) of the chase period, cells were harvested and neutral glycolipid fraction was analyzed by HPTLC. Positions of standard lipids are given on the left (LacCer, lactosylceramide; CTH, ceramide trihexoside; SM, sphingomyelin). (b) Densitometry. Densities of ^{14}C-GlcCer bands on autoradiographs were quantified using an NIH image software. Values were expressed as relative to the band density of H11 cell extracts at the beginning of the chase period (day 0) as 100%. Each bar represents mean ± S.E. of three determinations.

globoside. The decreases, however, were observed both in control and F213I/F213I cells and the identities of these lipids were left unresolved.

3.5. NOV attenuated pH-dependent loss of F213I mutant β-Glu activity in vitro

Some mutations of lysosomal enzymes affect pH-dependent protein stability [1] and we reported that an α-Gal A inhibitor DGJ prevented in vitro degradation of mutant α-Gal A at neutral pH [10]. To examine whether a similar mechanism underlined NOV effects on F213I mutant β-Glu, we compared pH-dependent stability of wild-type and F213I mutant β-Glu and tested an effect of NOV. In these experiments, we used cell lysates prepared from untreated control cells and from F213I/F213I cells that had been treated with NOV at 30 μM for 4 days and further cultured without the drug for 1 day. When F213I/F213I cell lysates were incubated at pH 7, mutant β-Glu activity was rapidly lost and there remained less than 5% activity at 1 h. Mutant β-Glu activity also decreased in acidic conditions at pH 5 or 6, but ~ 60% activity retained at 1 h under these conditions. In contrast, there were only marginal decreases of wild-type β-Glu activity in control cell lysates and more than 80% activity retained after 1-h incubation at every pH (Fig. 5a). The decrease of F213I mutant β-Glu activity at neutral pH was attenuated by NOV in a dose-dependent manner (Fig. 5b).

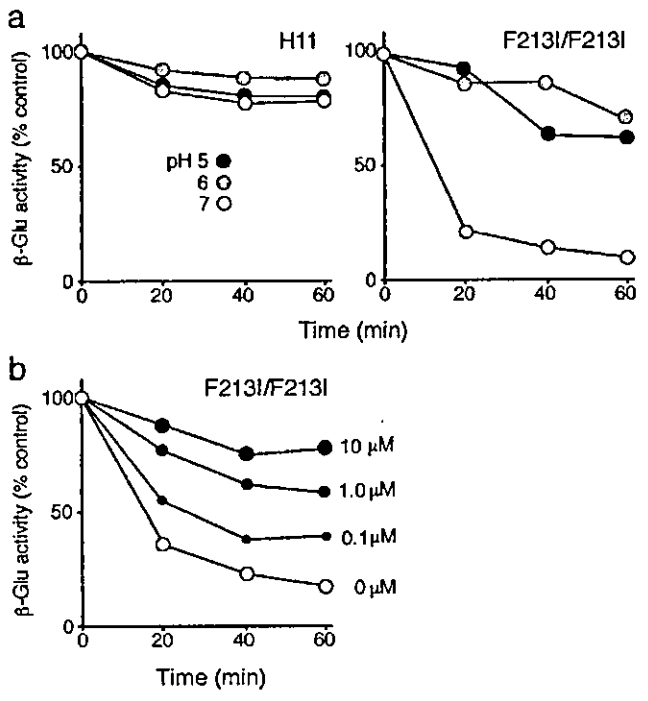

Fig. 5. Effects of NOV on pH-dependent loss of F213I mutant β-Glu activity in vitro. (a) Time course. Cell lysates were incubated at 37 °C in 0.1 M citrate-phosphate buffer at the indicated pH and the enzyme activity was determined at pH 4.5. (b) Effects of NOV. F213I/F213I cell lysates were incubated at pH 7 in the absence or presence of NOV at the concentrations indicated. Each point represents mean values of triplicate determinations obtained in a single experiment. Values were expressed as relative to the activity before the incubation (100%). Similar results were obtained in two other experiments.

4. Discussion

We found in the current study that, when applied to GD cells with F213I mutations, NOV up-regulated cellular β-Glu activity (Fig. 2) and accelerated cellular clearance of GlcCer (Fig. 4). NOV caused a modest but significant increase in the protein level of the mutant enzyme and increased its activity in the lysosome (Fig. 3). We also found pH-dependent loss of F213I mutant β-Glu activity in vitro and its prevention by NOV (Fig. 5). These findings are most likely explained by an activity of NOV as a chemical chaperone to accelerate transport and maturation of F213I mutant β-Glu. Although details are yet to be proven, we suppose that F213I mutant β-Glu is degraded in the ER because of its instability at neutral pH. With the aid of NOV, this mutant β-Glu is protected from degradation and is transported to the lysosome where it is stabilized because of the acidic condition and cellular enzyme activity is restored. Since effects of NOV on ER enzymes responsible for β-Glu degradation have not been examined, an alternative possibility remains to be excluded that the observed effects of NOV were secondary to inhibition of such enzyme(s). Although we have shown negative effects of NOV on some other lysosomal enzymes in vitro (Fig. 1b), potential effects of this compound on ER enzymes must be the subject of future studies.

Because NOV is an inhibitor of β-Glu (IC_{50} = 3 μM, Fig. 1b), it should inhibit β-Glu activity at the lysosome if it reaches to an appropriate concentration in this compartment. NOV was most effective in inducing F213I mutant β-Glu activity at 30 μM in the medium (Fig. 2a), which was 10 times higher than its IC_{50} value. NOV at this concentration, however, failed to inhibit ^{14}C-GlcCer clearance both in control and F213I/F213I cells (Fig. 4). One possible explanation for this apparent lack of inhibition is that at 30 μM in the medium, the concentration of NOV in the lysosome did not rise high enough to inhibit β-Glu activity (whereas the concentration in the ER did rise high enough to prevent mutant β-Glu degradation). If this is the case, the action of NOV as a β-Glu inhibitor may emerge at inappropriately high concentrations. Indeed, NOV was less effective at 100 μM in increasing F213I mutant β-Glu activity (Fig. 2a), although it is yet to be proven that it was due to this action of NOV. We noticed similar dose dependence for DGJ to increase mutant α-Gal A activity in Fabry lymphocytes [10]. Thus, it is apparent that there is an appropriate concentration range for an enzyme inhibitor to up-regulate cellular enzyme activities.

The effect of NOV on cellular enzyme activity was specifically observed in GD cells with F213I mutations

but not in cells with other point mutations N370S, L444P and RecNciI (L444P and A456P) (Fig. 2a). Although a precise molecular basis is left unknown, this selectivity might depend on the differences in the stability of individual enzymes, or alternatively, on the differences of NOV-binding capacities. In this context, the lack of NOV effects on N370S/84GG cells is in contrast to the reported effects of NN-DNJ that caused a twofold increase of β-Glu activity in N370S homozygous cells [12]. It should be clarified in future studies whether this difference in the effects of NOV and NN-DNJ is due simply to the cell lines employed, or to differential binding capacities of N370S mutant enzyme to these substances.

N370S is the most common mutation in GD patients and is associated only with type 1 non-neuronopathic GD [1]. F213I is one of the two prevalent mutations in Japanese GD patients, found in 15% of alleles [19]. Clinically, patients with F213I mutations have either non-neuronopathic or neuronopathic GD. NOV may be of particular therapeutic value in the latter group, since there is no established therapy against GD brain lesions. We have shown that NOEV, an isomer of NOV, could penetrate the blood–brain barrier in mice [14]. It must be determined in a future study whether NOV penetrates the blood–brain barrier and exerts its activity on mutant β-Glu in the brain.

Finally, besides F213I, other β-Glu mutant forms are a potential target of NOV or related compounds. In this context, it should be noted that in a report of a neuronopathic GD patient with G202R mutations, ultrastructural immunohistochemistry revealed the absence of the mutant protein in the lysosome, despite its abundant presence in the ER [28]. So far, nojirimycin derivatives have been an only known class of carbohydrate mimics with chemical chaperone activity for lysosomal enzymes [10–12]. Together with NOEV [14], NOV represents a novel class of carbohydrate mimics with a potential chemical chaperone activity. It is a subject of future studies to test whether NOV and related compounds work as a chemical chaperone for other β-Glu mutant forms, and further for other mutant lysosomal enzymes.

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