C 生活指導

多臓器にまたがる疾患であり，包括的な医療が必要となる．筋症状のある患者でも有酸素運動は筋力改善，維持に有効であるとされる．

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Original article

Long-term follow-up of tetrahydrobiopterin therapy in patients with tetrahydrobiopterin deficiency in Japan

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Abstract

Tetrahydrobiopterin (BH_4) deficiency is a rare, congenital and lethal condition resulting in phenylalanine build-up that can lead to mental retardation and developmental defects, unless properly treated. About 1 million newborn infants in Japan undergo neonatal PKU screening every year, of which about 1 in 2 million are diagnosed with the condition. In this post-marketing surveillance study, 19 patients with BH_4 deficiency in whom BH_4 supplementation with sapropterin dihydrochloride (Biopten®) (hereafter referred to as 'BH_4 therapy') was initiated before the age of 4 years, were followed up for ⩽28 years. Patients who screened positive for BH_4 deficiency were treated with supplemental BH_4 plus L-dopa and 5-hydroxytryptophan. Data on the patients' clinical courses were collected once yearly at 10 medical centers in Japan. Seventeen patients were diagnosed with 6-pyruvoyl tetrahydropterin synthase deficiency and two with dihydropteridine reductase deficiency at an average age of 3.6 months; the mean age at end of follow-up was 14.6 years. Average duration of BH_4 therapy (mean dose, 5 mg/kg per day) was 13.2 years. Serum phenylalanine was reduced from more than 10 mg/dL at the start of drug administration to less than 2 mg/dL at end of follow-up. No abnormalities in height or weight were observed in any patients, except for one female patient with familial obesity. No unwarranted side effects were reported throughout the long-term course of treatment, even during pregnancy. BH_4 therapy can effectively maintain serum phenylalanine levels within the normal range in patients with BH_4 deficiency, and demonstrated excellent long-term safety, with no side effects.

Keywords: Tetrahydrobiopterin deficiency; Biopten; Sapropterin; Hyperphenylalaninemia; PKU

1. Introduction

Phenylketonuria (PKU) and hyperphenylalaninemia (HPA) in infants are markers indicative of a congenital deficiency of tetrahydrobiopterin (BH_4), an essential cofactor in the enzymatic biosynthesis of nitric oxide and several neurotransmitters. High levels of phenylalanine in the brain due to untreated BH_4 deficiency are severely neurotoxic, and can lead to mental retardation and developmental defects [1,2]. About 1 million newborn infants in Japan undergo neonatal mass screening for PKU every year, since the program was started in 1977. The incidence of HPA in Japan is about 1 in 80,000, lower than in Europe [1,2]. However, BH_4 deficiency occurs in about 1 in 2 million Japanese people, in line with the global average frequency [3]. Thirty-two patients with BH_4 deficiency in Japan are currently being treated with sapropterin hydrochloride, an active form of BH_4 (R-BH_4), which was developed in Japan in 1981 (hereafter referred to as 'BH_4 therapy').

Sapropterin dihydrochloride granules 2.5%; Biopten® (Daiichi Sankyo, Japan) was approved in Japan 1992 to decrease serum phenylalanine values in HPA (atypical HPA) due to dihydrobiopterin synthase (DHBS) deficiency

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or dihydropteridine reductase (DHPR) deficiency. Since 2008, Biopten® has been further approved to decrease serum phenylalanine values in HPA due to BH_4-responsive hyperphenylalaninemia (BH_4-responsive HPA).

Postmarketing surveillance of Biopten® for BH_4 deficiency has been underway in Japan for the last 16 years. In this study, we assessed the efficacy and safety of this agent in 19 patients with BH_4 deficiency in whom treatment was initiated before the age of 4 years.

2. Patients and methods

Among 19 HPA patients detected by neonatal PKU screening in Japan (Table 1), 17 were diagnosed with 6-pyruvoyl tetrahydropterin synthase (PTPS) deficiency based on pteridine analysis of urine or serum, whereas two were diagnosed with DHPR deficiency, based on Guthrie test results. All 19 patients were diagnosed with BH_4 deficiency and received treatment with BH_4, L-dopa, and 5-hydroxytryptophan (5-HTP), initiated before the age of 4 years, between 1982 and 2008.

This study was performed as a post-marketing surveillance study at 10 medical centers in Japan, between April 1992 and December 2008. During this period, the doctors in charge reported annually on the patients' heights, weights, serum phenylalanine concentrations, BH_4 dosages, concomitant medications, and provided information on drug effectiveness and safety in accordance with regulated survey slips. Similar information was retrospectively collected from clinical records available between 1982 and 1991.

Serum phenylalanine concentrations were determined using an automated amino acid analyzer (L-8800; Hitachi, Tokyo, Japan). Serum pteridine was measured by high-performance liquid chromatography (LC-10; Shimazu, Kyoto, Japan) after iodine oxidation. DHPR activity was measured using Guthrie card specimens, as described previously [4].

Table 1
Baseline characteristics.

Parameter	*n* (%)
Type	
PTPS deficiency	17 (89.5)
DHPR deficiency	2 (10.5)
Sex (M/F)	10/9 (52.6/47.4)
Hospitalization	
Outpatients	11 (57.9)
In ↔ out	8 (42.1)
Age at initiation of drug administration (years)	
0	16 (84.2)
1	2 (10.5)
2	0 (0.0)
3	1 (5.3)
Age at end of follow-up (years)	
0	1 (5.6)
1– < 4	4 (22.2)
4– < 10	2 (11.1)
10– < 16	1 (5.6)
⩾16	10 (55.6)
BH_4 dosage at start of therapy (mg/kg/day)	
<5	8 (42.1)
5– < 10	7 (36.8)
⩾10	4 (21.1)
BH_4 dosage at end of follow-up (mg/kg/day)	
<5	7 (36.8)
5– < 10	4 (21.0)
⩾10	8 (42.1)
L-dopa use during follow-up	
Yes	19 (100.0)
No	0 (0.0)
5-HTP use during follow-up	
Yes	19 (100.0)
No	0 (0.0)
Phenylalanine-restricted diet during follow-up	
Yes	12 (63.2)
No	6 (31.6)
Unknown	1 (5.3)
Blood phenylalanine level at neonatal mass screening (mg/dL)*	
Mean	14.2
Range	6.0–48.9

* Data available for 13 patients.

3. Results

Patients' background clinical characteristics are shown in Table 1. Seventeen patients were diagnosed with PTPS deficiency and two with DHPR deficiency; the mean age at end of follow-up was 14.6 years, and the mean age at initiation of BH_4 supplementation was 3.6 months. The mean duration of BH_4 therapy was 13.2 years; more than half the patients received BH_4 continuously for more than 10 years. The longest treatment duration was 28 years ($n = 1$). The mean daily dosage was 5 mg/kg; eight patients received less than 5 mg/kg and 11 patients received more than 10 mg/kg.

Changes in BH_4 dosage with age are shown in Fig. 1. The mean dosage in patients with PTPS deficiency increased over the initial few years of treatment, then remained stable thereafter. As of 2008, the average daily dose in 15 patients with PTPS deficiency was 7.9 mg/kg (Fig. 1). Of the two patients with DHPR deficiency, one was controlled by a stable dose, while the other required a high dose to control their serum phenylalanine level (Fig. 2).

Serum phenylalanine values in 13 of the 19 cases for whom data were available at the time of newborn screening are shown in Table 1. Changes in serum phenylalanine levels in patients with PTPS deficiency are shown in Fig. 2a. Serum phenylalanine was high (10 mg/dL) at the start of drug administration, but decreased to less than 2 mg/dL following Biopten® administration, with good phenylalaninemic control being maintained thereafter (Fig. 2a).

However, one patient with deficiency of DHPR, an enzyme responsible for BH_4 recycling, struggled to con-

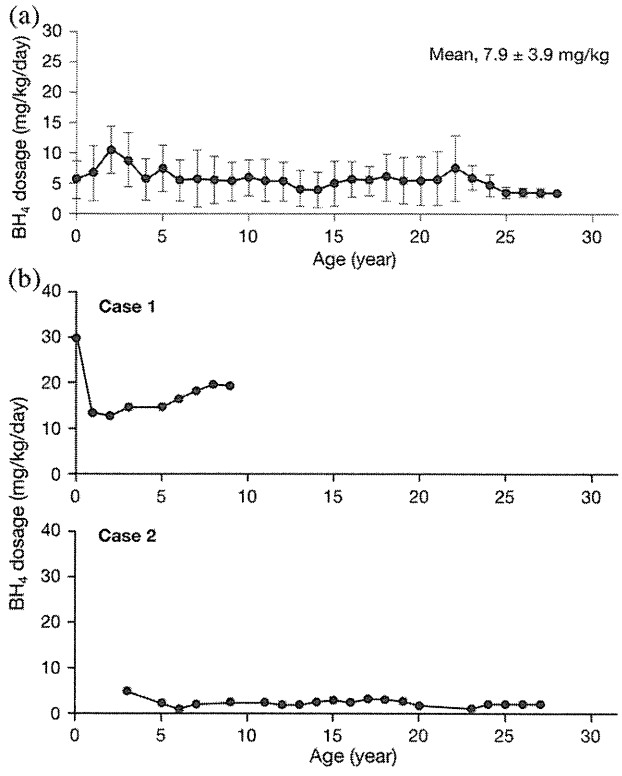

Fig. 1. Time courses of BH_4 dosage in patients with (a) PTPS deficiency ($n = 17$) and (b) DHPR deficiency (cases 1 and 2).

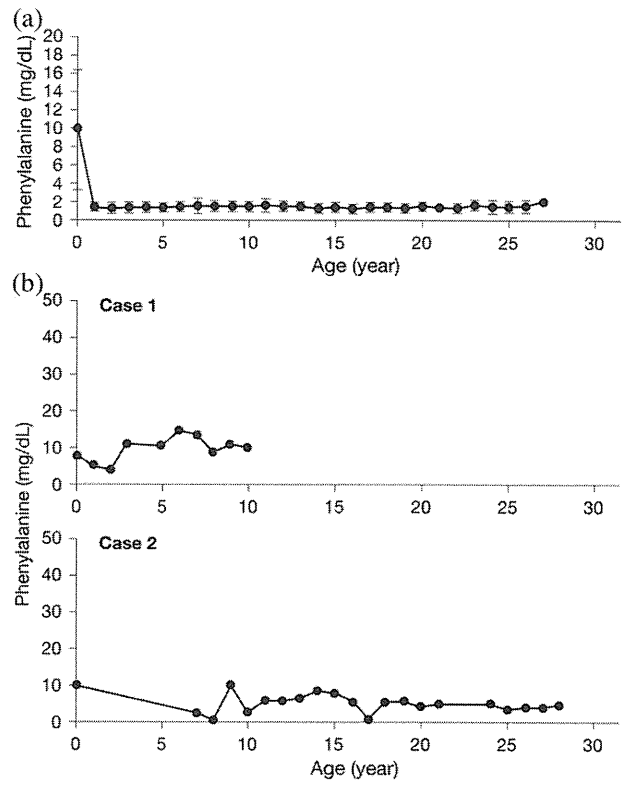

Fig. 2. Changes in serum phenylalanine levels in patients with (a) PTPS deficiency ($n = 17$) and (b) DHPR deficiency (cases 1 and 2).

trol serum phenylalanine levels despite the fact that the value at the start of treatment was slightly lower (9.1 mg/dL) than that in patients with PTPS deficiency. Serum phenylalanine levels fluctuated in this individual from the time of treatment initiation until the start-of-school age (Fig. 2b; case 1). However, another patient with DHPR deficiency exhibited stable long-term phenylalanine values (4.6 mg/dL at end of follow-up), within the age-specific reference range (Fig. 2b; case 2). The patients' heights and body weights by sex are shown in Fig. 3.

The mean height by age for male patients with PTPS deficiency ($n = 9$) was similar to that for the normal healthy population, but their body weight was lower by −1 standard deviation (SD), compared with healthy subjects (Fig. 3a). Female patients ($n = 8$), however, had greater mean height (+1 SD) and similar body weight to age-matched healthy females, except for one female patient with PTPS deficiency who had severe familial obesity (Fig. 3b). The two patients with DHPR deficiency (one male and one female) showed almost normal growth in terms of both height and body weight.

3.1. Combination therapy

Seventeen patients had evaluable data at the end of the survey period. L-dopa was used together with BH_4 in all patients, and 5-HTP was used in 13 patients. The doses of both L-dopa and 5-HTP tended to increase with age, probably consistent with normal weight gain, because the dosages/body weight remained roughly the same, at approximately 10 mg/kg per day of L-dopa (Fig. 4a) and 3–5 mg/kg per day of 5-HTP (Fig. 4b) throughout the study period. All patients with PTPS deficiency and one patient with DHPR deficiency showed good control of serum phenylalanine levels with BH_4 alone, with no need for restrictive diet therapy, indicating that BH_4 therapy could improve the patient's quality of life. However, one patient with DHPR deficiency received a phenylalanine-restricted diet in combination with BH_4 therapy, because BH_4 alone was unable to maintain serum phenylalanine levels within the normal reference range.

3.2. Safety and efficacy

No patients failed to respond to BH_4 therapy. One patient with PTPS deficiency who started BH_4 therapy soon after birth died at the age of 3 years, while not receiving BH_4 therapy. BH_4 therapy was considered to be effective in decreasing serum phenylalanine levels in all the remaining 18 patients with BH_4 deficiency.

Nervous system disorders were reported in two patients (10.5%); one patient experienced seizure and

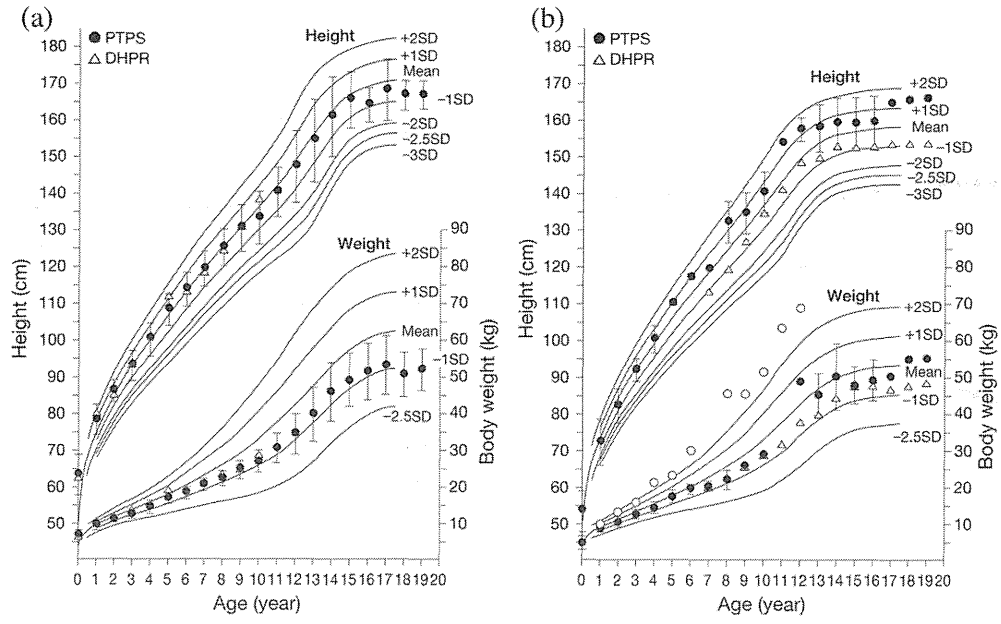

Fig. 3. Time courses of height and body weight changes in (a) male and (b) female patients. Although no undue abnormalities in height or body weight were noted in either sex, one female patient with PTPS deficiency had severe familial obesity (see text). ●, PTPS deficiency; △, DHPR deficiency; ○, outlier ($n = 1$).

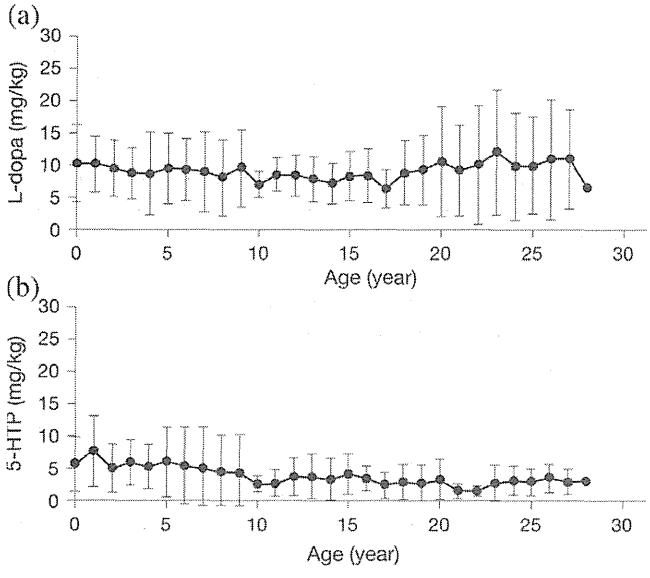

Fig. 4. Time courses of mean daily dosage of (a) L-dopa and (b) 5-hydroxytryptophan (5-HTP).

one developed increased muscle tone. These symptoms were deemed to have resulted from insufficient L-dopa doses, rather than being caused by BH_4. No other side effects were reported and no patient discontinued treatment because of side effects.

4. Discussion

BH_4 deficiency was first reported first by Smith et al. [5], while DHPR deficiency was the cause of BH_4 deficiency was recognized by Kaufman et al. [6]. Danks et al. [7] found no effect of oral BH_4 administration on DHPR deficiency, because of poor absorption from the intestinal tract. However, Schaub et al. [8] reported that oral administration of BH_4 was effective in a patient with DHBS deficiency, after which oral BH_4 was used to treat BH_4 deficiency. The form of BH_4 used initially was a mixture of the 6R and 6S isomers, because 6S form of BH_4 was inactive, but in 1982, Suntory Limited (Japan) succeeded in synthesizing 6R BH_4, after which 100% 6R BH_4, as Biopten® (sapropterin dihydrochloride), has been used in Japan. The first patient who was given Biopten® at that time now represents the longest-term usage of 28 years. Biopten® was approved for BH_4 deficiency in 1992, and over half the patients since then have been continuously administered BH_4 for more than 10 years. All 19 patients in this study also received treatment with L-dopa and 5-HTP, combined with BH_4, from before they were 4 years old, between 1982 and 2008. And, this study summarizes those patients, which represents a unique, long-term follow-up of a patient with BH_4 deficiency.

Control of serum phenylalanine levels by BH_4 appears to be easy in patients with PTPS deficiency [6], but more difficult in patients with DHPR deficiency, although levels can be still maintained within the range of age-specific reference values. Unlike the situation in PTPS deficiency, BH_4 cannot be recycled in DHPR deficiency. However, the sex-specific heights and body weights of patients with both PTPS and DHPR deficiencies were almost normal, or within 1SD. BH_4 administration in patients with BH_4 deficiency is intended to strictly limit serum phenylalanine

levels, though a phenylalanine-restricted diet, in combination with BH_4 therapy, may be necessary if BH_4 alone is unable to maintain levels within the normal range. The mental development of patients with BH_4 deficiency depends not only on the control of serum phenylalanine levels, but also on the treatment of neurotransmitter deficiencies, and combination therapy with a phenylalanine-restricted diet/BH_4, as well as supplementation with the neurotransmitter precursors, L-dopa and 5-HTP, is required. Because BH_4 does not pass the blood–brain barrier, L-dopa and 5-HTP are essential for the treatment of the central nervous system, and were used together with BH_4 in all patients. Therefore, although one patient started BH_4 administration after 3 years old, she developed normally because L-dopa and 5-HTP therapy with a phenylalanine-restricted diet had started already during infancy. The doses of both L-dopa and 5-HTP tended to increase with age, which was considered to be associated with weight gain. 5-HTP has not been approved as a drug in Japan, and although the 5-HTP sold as a supplement was used once, patients now buy 5-HTP as a supplement from overseas via the internet, because this represents a much cheaper option.

Nervous system side effects were reported in two patients (seizure and increased muscle tone, respectively), with an incidence of 10.5% (2/19). These symptoms were deemed to have occurred as a result of insufficient L-dopa, and were thought to be unlikely to be associated with BH_4. No other particular side effects were noted and no patients discontinued treatment because of side effects, suggesting that this drug showed a high degree of safety.

Women with PKU can have healthy children, as long as they maintain strict adherence to a low-phenylalanine diet throughout their pregnancy. One patient treated with sapropterin dihydrochloride for 28 years continued to take BH_4 during pregnancy to achieve adequate control of her serum phenylalanine. Apart from discontinuing BH_4 for a few days because of morning sickness, she recovered and resumed BH_4 therapy. She delivered a baby in October 2008, representing the first successful pregnancy of a patient while taking BH_4 in Japan. Both mother and baby were healthy, and the baby was confirmed as normal. The same patient is pregnant again, and continues to take BH_4 without morning sickness.

Sapropterin was originally found in Japan, and was approved for BH_4-responsive HPA in the US in 2007, and after a year EU approved for both BH_4-deficiency and BH_4-responsive HPA in 2008 (bland name in US and EU is Kuvan®). Then, more than 1000 patients of PKU were continuously administered sapropterin in each US and EU, and currently about 40 patients were taking in Japan as well. However, clinical trials were performed in patients older than 4 years in US and EU. The treatment of BH_4 deficiency requires lifelong administration of sapropterin, therefore it necessitates a high degree of safety. This was confirmed by the results of this survey.

5. Conclusions

BH_4 therapy is effective in controlling serum phenylalanine levels within the normal range in patients with BH_4 deficiency, with excellent long-term safety and no unwarranted side effects. Nineteen patients in Japan with BH_4 deficiency treated with BH_4 from before the age of 4 years have been followed for up to 28 years, representing a unique long-term follow-up of individuals with BH_4 deficiency. BH_4 deficiency requires lifelong drug therapy, and a high degree of drug safety is therefore required. The results of our survey suggest that BH_4 therapy is associated with a high level of safety in the setting of early treatment.

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＝総　説＝

小児神経伝達物質病

新宅治夫

要旨　小児神経伝達物質病は幼少期より発症するまれな遺伝性疾患としてその原因により個別に研究されてきたが，いずれも中枢神経系に症状があり代謝異常に起因する小児神経疾患として早期に適切な診断と治療が必要である．しかしながら，臨床症状による診断は困難であり適切に診断されず有効な治療を受けられていない可能性がある．これらの疾患について，小児神経伝達物質病として総合的に検討し，病態生理のさらなる理解，診断基準の確立，我が国における患者数，分布の把握が行われている．新生児マススクリーニングで発見できない希少疾患の早期診断と効果的な新しい治療法の確立は急務であるが，超希少疾患の場合に医療関係者への疾患概念の周知が重要である．

見出し語　小児神経伝達物質病，神経伝達物質，モノアミン，γアミノ酪酸，ビオプテリン

はじめに

小児神経伝達物質病はシナップスでの情報伝達を担う神経伝達物質の異常による疾患であるが，胎児期や乳幼児期のニューロンがシナプスを形成してネットワークを構成する際に神経伝達物質の異常が起こると，小児の神経発達に重大な異常が発生すると考えられる．この意味ですでにネットワークができあがった後の成人の神経伝達物質病であるParkinson病，うつ病などと病態が異なり，臨床症状や治療法が異なることが予測される．これまで神経伝達物質のうち，ドーパミン，セロトニン系の合成調節にはテトラヒドロビオプテリン（BH4）が補酵素として関与しており[1]，このBH4合成系の律速酵素の部分欠損により瀬川病[2]を来すことが見いだされている[3)~5)]．この他のBH4欠損症[1]やチロシン水酸化酵素欠損症[6]でも，不随意運動や精神症状を来すことが知られている．これまで瀬川病やBH4欠損症で髄液の化学診断，血球の酵素診断，遺伝子診断を行ってきた[7]が，神経伝達物質病に共通の診断基準は作成できていない．一方，芳香族アミノ酸脱炭酸酵素の欠損では低緊張や眼球回転発作（oculogyric crisis）などの症状を来すことが明らかになっている[8]．また，γ-アミノ酪酸（GABA）の異常では，コハク酸セミアルデヒド脱水素酵素欠損症などが知られている[9]．しかし，これらの諸疾患は幼少期より発症するまれな遺伝性疾患として個別に研究されてきており，神経伝達物質病として共通の診断基準は作成されていない．いずれも中枢神経系に症状があり小児神経疾患として取り扱われているが，病因は神経伝達物質の代謝異常症であり，早期に適切な診断と治療が必要である．しかし，臨床症状による診断は困難であり，患者が適切に診断されず，有効な治療を受けられていない可能性がある．これらの疾患について，小児神経伝達物質病として総合的に検討し，病態生理のさらなる理解，診断基準の確立，新しい治療法の開発が急務である．小児神経疾患の中で神経伝達物質の異常による疾患は極わずかで，そのため病態の解明や治療法の開発が進んでいないのが現状である．また，希少疾患のため正しく診断されない症例も認められ，治療の実態も不明な点が多い．これらの希少疾患を神経伝達物質病として集約し，共通の概念で病態を解明し診断基準を作成することで新しく患者を発見することが重要である．

表1　神経伝達物質の種類

- アミノ酸：グルタミン酸，グリシン，γ-アミノ酪酸（γ-amino butylic acid：GABA）
- モノアミン：ドーパミン，ノルアドレナリン，アドレナリン，セロトニン，アセチルコリン
- ペプチド：神経ペプチド
- その他：一酸化窒素

I　神経伝達物質と小児神経伝達物質病

小児神経伝達物質病は，表1のような神経伝達物質の代謝異常に関わる遺伝的疾患群のすべてが含まれる．具体的な疾患としては，表2のような疾患が神経伝達物質病と考えられており，伝達物質の種類やその異常の内容に基づき分類されている[10]．いずれの疾患も稀な疾患ではあるが，神経伝達物質の異常に基づく疾患として共通の要素をもつ一方で，診断の難しさのために確定診断できていない症例も多いと推測さ

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表2　神経伝達物質病

(1) モノアミン代謝の疾患
　1．モノアミン合成に関連する疾患
　　・チロシン水酸化酵素欠損症（Tyrosine hydroxylase deficiency: TH 欠損症）
　　・芳香族アミノ酸脱炭酸酵素欠損症（Aromatic L-amino acid decarboxylase deficiency: AADC 欠損症）
　2．BH4 合成に関連する疾患
　　・常染色体劣性 GTP cyclohydrolase I deficiency（GTPCH 欠損症）
　　・瀬川病（常染色体優性 GTP cyclohydrolase I deficiency: AD-GTPCH 欠損症）
　　・6-pyruvoyl-tetrahydropterin synthase deficiency（PTPS 欠損症）
　　・セピアプテリン還元酵素欠損症（Sepiapterin reductase deficiency: SR 欠損症）
　　・Dihydropteridine reductase deficiency（DHPR 欠損症）
　　・Pterin-4α-carbinolamine dehydratase deficiency（PCD 欠損症）
　3．モノアミン分解に関連する疾患
　　・Monoamine oxidase deficiency（MAO 欠損症）
　　・Dopamine β-hydroxylase deficiency（DBH 欠損症）
(2) グリシン代謝に関連する疾患
　4．Glycine encephalopathy（Nonketotic hyperglycinemia: NKH）
(3) γ-amino butyric acid（GABA）代謝に関連する疾患
　5．GABA 合成に関連する疾患
　　・Glutamic acid decarboxylase deficiency（GAD 欠損症）
　　・Pyridoxine and pyridoxal 5'-phosphate dependency
　6．GABA 分解に関連する疾患
　　・GABA-transaminase deficiency（GABA-T 欠損症）
　　・コハク酸セミアルデヒド脱水素酵素欠損症（Succinic semialdehyde dehydrogenase deficiency: SSADH 欠損症），
　　・Homocanosinosis

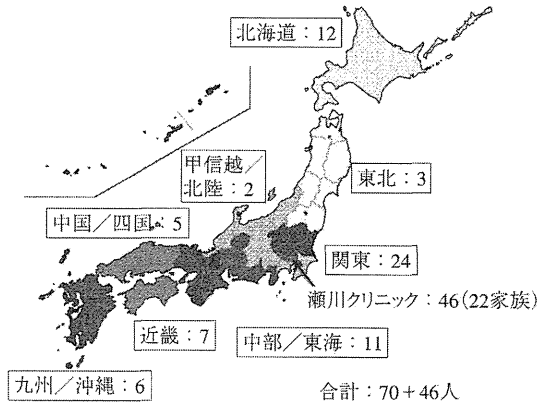
れる．

厚生労働省の難治性疾患克服研究事業では平成21年度から研究奨励分野が設けられ，小児神経伝達物質病の中から瀬川病（AD-GTPCH 欠損症），芳香族アミノ酸脱炭酸酵素欠損症（AADC 欠損症），コハク酸アルデヒド脱水素酵素欠損症（SSADH 欠損症），セピアプテリン還元酵素欠損症（SR 欠損症），チロシン水酸化酵素欠損症（TH 欠損症），の5つの疾患が研究対象として取り上げられた（表2）．これらの疾患は「小児神経伝達物質病の診断基準の作成と新しい治療法の開発に関する研究班」により初めての全国調査が行われ，その患者数と分布が明らかにされた．小児神経伝達物質病では原因不明の不随意運動（ジストニアや oculogyric crisis）などの特徴的症状をしばしば示すことから，このような症状をもとに神経伝達物質病を疑われる患者を調査の対象とし，さらに2次調査を行い診断基準と手順の確立が行われた．

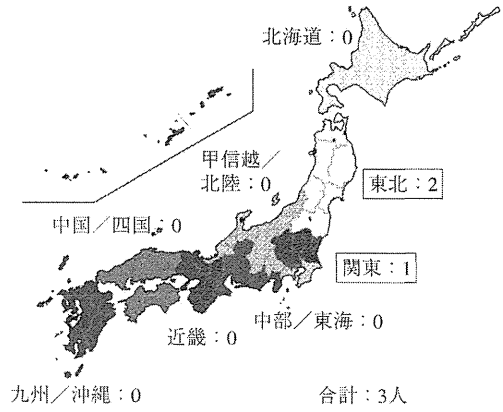
Ⅱ　小児神経伝達物質病の患者数とその分布（図1）

瀬川病は100人以上の患者が報告され，ほぼ全国的に均等に分布していた．しかし，東京都では瀬川クリニックに22家族46人の患者が報告されており他の地域に比べてきわめて多くの患者が発見されていた．このことは希少疾患の診断の難しさを示す結果であり，医療関係者へ疾患概念の周知をはかることが重要であると考えられた．AADC 欠損症は2家族

瀬川病



芳香族アミノ酸脱炭酸酵素(AADC)欠損症



コハク酸セミアルデヒド脱水素(SSADH)欠損症

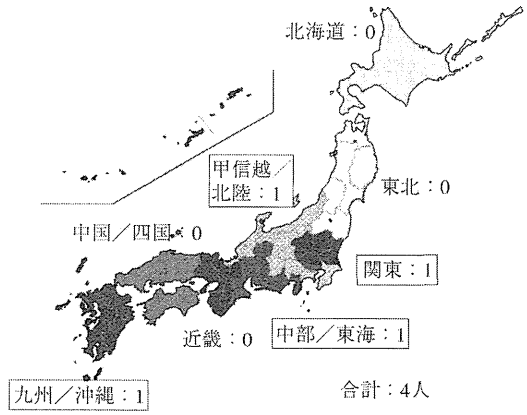


図1　小児神経伝達物質病の患者数とその分布

3人で，SSADH 欠損症は4人，SR 欠損症と TH 欠損症は報告がなく不明という結果であった．

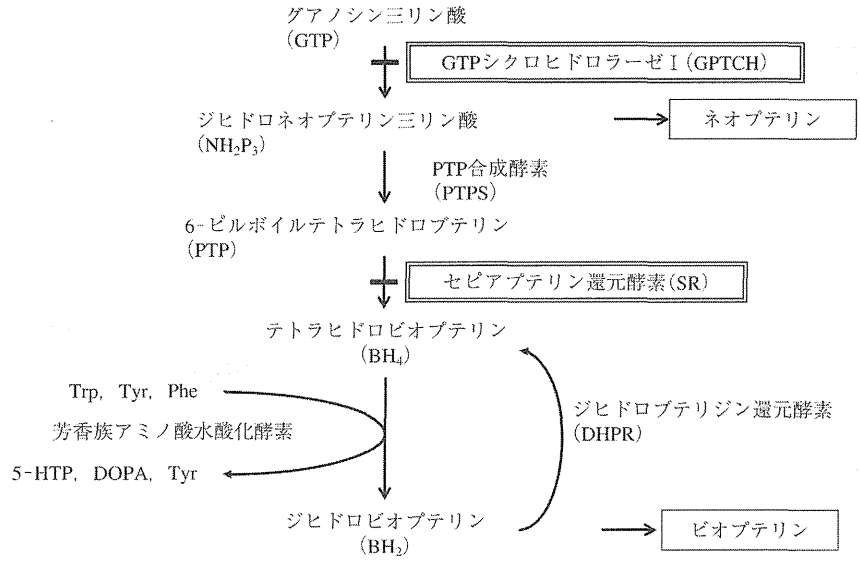


図 2　ビオプテリン代謝

Ⅲ　小児神経伝達物質病の概要

1. 瀬川病

概　要　瀬川病は 1971 年に瀬川[2]が，「著明な日内変動を呈する遺伝性進行性大脳基底核疾患」として世界ではじめて小児例を報告した．1990 年に藤田・新宅[3]は，瀬川病患者の髄液中プテリジン濃度の低下により，GTP シクロヒドラーゼⅠ（GTPCH）活性の低下を指摘したが，1994 年に一瀬ら[4]により瀬川病患者で GTPCH 遺伝子（*GCH1*）の異常が発見され，GTPCH の部分欠損によって起こることが報告された．典型例は 10 歳以下に発症，女性優位の性差を有する（男：女＝1：4）．ジストニア，特に小児期の姿勢ジストニアは著明な日内変動を呈する．

疫　学　平成 21 年度に施行された全国調査では 0.5～1.0/1,000,000 の発症頻度であった．遺伝形式は不完全な浸透率を呈する常染色体優性遺伝である．

原　因　14q22.1-22.2 に存在する GTP シクロヒドロラーゼ遺伝子異常に起因する常染色体優性遺伝性疾患で，黒質線条体ドーパミン神経系終末部のドーパミン欠乏による．

症　状　姿勢ジストニア型と動作ジストニア型の 2 型に分けられ，姿勢ジストニア型は，多くは 6 歳頃，一側下肢内反尖足で発症，15 歳頃までに全肢にひろがり，20 歳頃まで筋強剛が進行するが，その後，進行は緩やかになり，30 歳以後は定常状態となる．10 歳頃から姿勢振戦が認められる．動作ジストニア型は，これに加え，8 歳以後，上肢のジストニア運動，頸部後屈，眼球回転発作が発現，思春期以後，主に成人年齢で斜頸，書痙を併発する．この病型には運動誘発性ジストニア，むずむず足症候群を呈する症例もある．さらに，成人年齢で斜頸，書痙，または，Parkinson 病様症状で発症する症例がある．しかし，これは真性の Parkinson 病とは異なり，大脳基底核 GABA 系出力系の活性低下に起因する高活性型病態を有する．さらに，これらの症例にはすでに発達過程の終わった線条体へ投射するニューロン終末部ドーパミン低下に起因する全身性ジストニア姿勢はみられない．この年齢依存性の発現の機序と神経系の発達における意義も研究の 1 つとなる．

合併症　終末部のドーパミン欠乏症は，精神発達障害，精神運動障害，けいれんなど中枢神経症状を発現せず，ロコモーションも正常に保たれる．大脳の器質的病変も発現しない．しかし，動作ジストニア型では家系によりうつ病を合併することがある．また，早期，主に乳児期発症例ではセロトニン欠乏を発現，自閉傾向，うつ傾向，強迫神経症，頭痛を併発する例がある．また，筋緊張低下，ロコモーションの障害を来し，さらに，脚橋被蓋核活性低下を併発，ドーパミン欠乏も併発，思春期以後，Parkinson 病と同様の病状を呈することが知られている．

診　断　髄液中のネオプテリンとビオプテリンの低下を認めれば，*GCH1* 遺伝子解析を行う．

治療法　L-ドーパが著効を呈し，その効果は副作用なく永続する．しかし，動作ジストニア型では主病変が視床下核の D1 受容体の異常にあるため，D2 間接路に作用することにより，L-ドーパで十分な効果が得られない例がある．理論的には D1 作動薬が適剤となる．また，セロトニン欠乏を伴う症例には，早期からの 5 ハイドロキシ・トリプトファン，またはテトラヒドロビオプテリンの投与が望まれる．これらの治療法の開発も試みる予定である．

2. 芳香族アミノ酸脱炭酸酵素（AADC）欠損症

概　要　芳香族アミノ酸脱炭酸酵素は L-ドーパをドーパミンに，5-ヒドロキシトリプトファン（5HTP）をセロトニンに脱炭酸化する酵素であり，神経伝達物質であるドーパミン，

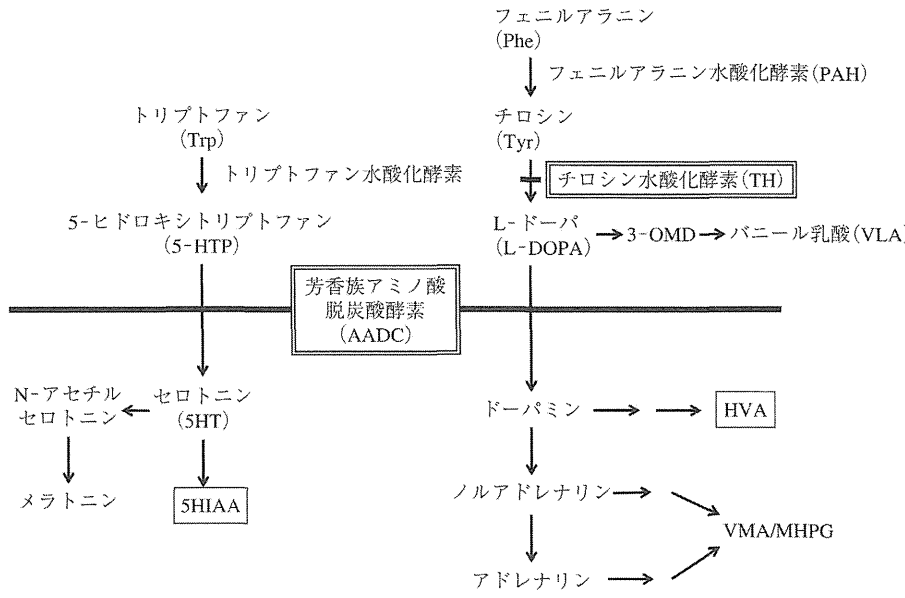


図3 モノアミン代謝

ノルエピネフリン，セロトニンの合成に必須の酵素である（図3）．その欠損症の典型例は，乳児期早期からの発達遅滞および間歇的な眼球回転発作など眼球運動異常と四肢ジストニアで発症し，髄液中の homovanillic acid（HVA）および 5-hydroxyindolacetic acid（5HIAA）の低値など特徴的な所見で診断される．ドーパミンアゴニストなどを用いた内服治療が試みられているが，予後は不良で多くは寝たきりで発語のない状態にとどまる．

疫 学 平成21年度に施行された全国調査では，患者として明らかになったのは2家族の3人であった．

原 因 7p12.1-p12.3 に存在する AADC 遺伝子（*DDC*）異常に起因する遺伝性疾患で常染色体劣性の遺伝形式を取る．AADC 活性の欠損は，①髄液検査，②血漿中酵素活性にて証明される．髄液検査では，AADC の基質（L-ドーパおよび5HTP）とその代謝産物である 3-o-methyldopa の髄液中濃度が上昇し，生成物のモノアミンとセロトニンの代謝産物である HVA，5HIAA は著減している．血漿中ドーパ脱炭酸活性は低下し，多くは測定感度以下となる．遺伝子変異は 30 数例の報告があり，多くはミスセンス変異であるが，台湾においては単一のフレームシフト変異の集積（IVS6+4 A>T）が報告されている．現在のところミスセンス変異の集積傾向はない．L-ドーパ反応性の軽症例で報告された基質結合部位でのアミノ酸置換を起こす G102S 変異や軽症例の S250F など特徴的な変異も見つかってきている．画像検査では，ドーパミン合成障害を反映して 18F-dopa PET 検査で線条体への取り込みが消失する．しかし，頭部 MRI 検査では異常は認めず，TRODAT-1 SPECT 検査では線条体への結合が確認できるなど，脳の構造，特に線条体のドーパミン神経終末の構造は保たれていると考えられている．

症 状 典型例では6カ月以内に，間歇的な眼球回転発作と四肢のジストニアで発症し，精神運動発達は遅滞する．その他に頻度の高い症状としては，随意運動の障害，易刺激性，眼球収束発作（ocular convergence spasm），口腔顔面ジストニア，ミオクローヌスなどがある．診察上は筋緊張は低下し，深部腱反射は亢進するが Babinski 反射は陰性である．多くは寝たきりで発語のない状態にとどまるが，一方で筋緊張低下と眼瞼下垂を主症状とし，独歩と会話が可能であった軽症例の報告もあり，症状の幅は広い．脳性麻痺との鑑別が困難な場合もあり，正しく診断を受けていない症例も多いと考えられる．この点については，診断基準作成など本研究の課題である．病態としては，AADC 欠損症例の FDG-PET 検査でドーパミン神経の投射が多い線条体と前頭前野での糖代謝低下の所見が報告されていることから，線条体の機能不全は AADC 欠損症の主な運動症状であるジストニアと随意運動の障害の原因となり，前頭前野の機能不全が精神遅滞症状を引き起こす原因の一つとなっていると考えることができる．

合併症 突発的な発汗，鼻閉，息止め，便秘や下痢，眼瞼下垂などの自律神経症状は，ほぼ全例で合併する．また，低血糖による意識障害やけいれんが起こることがある．これらの症状は末梢のカテコラミン不足を反映したものである．睡眠障害の合併も多く，睡眠ホルモンであるメラトニンはセロトニンから合成されるためにメラトニンの合成が障害されているためと推測される．てんかんの合併頻度は高くないが脳波異常も伴う症例もあり，その場合はジストニアの診断が遅れることもある．また，重症例においては，症状の進行とともに嚥下困難や呼吸障害が出現し，最重症例では乳幼児期に肺炎で死亡する場合がある．また，およそ半数に，哺乳障害，低体温，低血糖などの新生児期の異常の既往を認めることも

特徴の一つである．

診　断　髄液中のHVA，5HIAAの低下を認めれば，*DDC*遺伝子解析を行う．

治療法　ドーパミンアゴニスト，モノアミン酸化酵素阻害剤，補酵素であるビタミンB6などを用いた内服治療が行われているが，典型例に対してはわずかな効果しか期待できない．そのために現在は遺伝子治療に期待がかけられている．AADC欠損症では脳の構造がたもたれていること，さらにAADC遺伝子の導入はParkinson病の治療として研究されている手法が流用できることが有利な点である．適切な薬剤治療やリハビリテーションの知見を蓄積しながら，遺伝子治療の実現にむけた研究を進めて行くことが必要である．

3. コハク酸セミアルデヒド脱水素酵素（SSADH）欠損

概　要　γ-アミノ酪酸（γ-aminobutyric acid：GABA）の先天代謝異常症の一つで，SSADHの欠損でγ-ヒドロキシ酪酸尿症を来す（図4）．世界で150例程度の報告がある．

疫　学　平成21年度に施行された全国調査では，患者として明らかになったのは3名で，平成22年度に新たに発見された1名を加えて4名が報告されている．

原　因　責任遺伝子は6番染色体（6q23）のALDH5A1で，常染色体劣性遺伝形式をとる．SSADHの先天的な欠損により，GABAの代謝産物であるコハク酸セミアルデヒドがコハク酸に変換されないため，γ-ヒドロキシ酪酸が増加する．発達脳におけるGABAの増加，グルタミン酸の低下など神経伝達物質のバランスの崩壊が中枢神経障害を引き起こすと考えられる．しかし，γ-ヒドロキシ酪酸は，ヒトや動物にγ-ヒドロキシ酪酸を投与すると逆の作用が現れることがあるため，本疾患の病因におけるこの物質の役割は不明である．

症　状　臨床症状は，通常乳児期の初期に現れ始め，その症状には軽度から中等度の発達遅滞，精神遅滞，言語表出障害，著しい筋緊張低下，睡眠障害，不注意，多動，不安，腱反射低下，非進行性小脳失調，けいれんと多彩であり，通常は非進行性だが，まれに（10%）進行性の場合がある．運動失調は年齢とともに改善する場合がある．頭部MRIでは，典型的にはT2強調像で淡蒼球の対称性の高信号を認める．

合併症　眼球運動失行，舞踏病アテトーゼ，自閉症の特徴，攻撃行動などがある．

診　断　尿有機酸分析で4-ヒドロキシ酪酸の上昇を認めれば，培養リンパ芽球でSSADHの酵素診断を行う．

治療法　治療はほとんど効果が認められていないが，GABAトランスアミナーゼの阻害剤で抗けいれん薬のビガバトリン（vigabatrin：γ-ビニル-GABA）は，一部の患者で運動失調と精神状態をある程度改善する．タウリンの有効性が動物モデルや症例報告で報告されているが，その作用機序ははっきりしていない．抗てんかん薬のvalproate sodiumは禁忌とされる．

4. セピアプテリン還元酵素（SR）欠損症

概　要　2001年，Bonafeら[11]により，髄液中の5ヒドロキシ酢酸（5HIAA）とホモバニリン酸（HVA）の低値，ビオプテリンとジヒドロビオプテリンの高値を示す小児期発症の進行性精神・運動遅滞として初めて報告された．2005年，Nevilleら[12]によるマルタ島の家系7症例の検索から病像が明らかにされた．

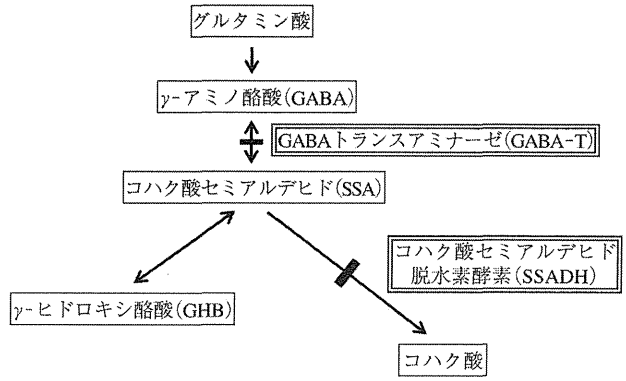


図4　γ-amino butyric acid（GABA）代謝

疫　学　主に地中海周辺で患者の報告が多く，地中海地域に患者血統が存在する可能性が示唆されている．本邦での報告例はまだなく，平成21年度に施行された全国調査でも患者は見いだされなかった．

原　因　培養皮膚線維芽細胞の分析により，セピアプテリン還元酵素（SR）の不活性化が明らかにされ，2p14-p12に位置するSR遺伝子（*SPR*）の異常が病因として解明された．遺伝形式は常染色体劣性遺伝を呈する．

症　状　Nevilleらの報告[12]では，全例で乳児期からの運動発達遅滞と言語発達遅滞を含む認知機能発達遅滞を示した．そのうち6例では日内変動を伴う運動障害や早期からの眼球回転発作を示し，5例には初期に低緊張を伴うジストニア，2例にParkinson様の振戦が認められた．乳児期には全例が躯幹の筋緊張低下を示した．乳児期後半から幼児期には舞踏運動や球麻痺症状を認めた症例もあった．睡眠により一部の運動障害の改善がみられ，眼球回転発作の消失をみた症例もあった．

合併症　症例により症状の強度が異なり，眼球回転発作，書痙が認められるが，これらは主病変の程度，ひろがり，また，年齢に起因するもので，合併症とはいえない．

診　断　髄液HVA，5HIAAの低下とビオプテリンとセピアプテリンの上昇でなされる．確定診断は*SPR*遺伝子解析による．

治療法　運動症状には脱炭酸酵素阻害剤を含むL-ドーパが著効を呈す．球症状，眼症状，振戦は完全に消失する．全例で歩行は可能となるが，歩行パターンは改善しない．これは，ロコモーションの障害の存在を示す．しかし，振戦，ジストニアは軽度であるが残り，また，症例によっては振戦が出現したり，治療前に振戦をみた症例ではL-ドーパ投与後に書痙を示した例がある．L-ドーパは認知機能を改善させない．ま

た，2歳に筋緊張低下，軽度認知機能の低下で発症，6歳で車いす使用となり，14歳でジストニアが発現した症例では，14歳時のL-ドーパと5-HTPが劇的効果を示したことが報告されている．SR欠損症ではドーパミンとともにセロトニンの低下が示唆され，また，運動症状からは瀬川病action typeと類似の病変，すなわち視床下核へ入力する黒質線条体終末部のドーパミン欠失が予想される．これらの解明が病態，治療法の解明につながる．

5．チロシン水酸化酵素（TH）欠損症

概　要　チロシン水酸化酵素（TH）はチロシンをドーパミンに水酸化する酵素であり，神経伝達物質であるドーパミンなどのカテコールアミンの合成に必須の酵素である．TH欠損症はドーパミン生成障害を主体とし，ドーパ反応性ジストニアの病像を呈する症例もあるが，ノルアドレナリン生成障害を併発，進行性の脳症を呈する例が主体を占める．

疫　学　過去に我が国にも症例報告が存在するが，平成21年度に施行された全国調査では患者は報告されなかった．

原　因　11p15.5に存在するチロシン水酸化酵素の遺伝子異常に起因する疾患で常染色体劣性の遺伝形式を取る．変異部位により，ドーパ反応性ジストニアの病型をとるものと，進行性脳症の病型をとるものとに分かれる原因の解明はできていない．前者は精神，知能に異常がなく，L-ドーパにより症状の寛解が得られるが，後者に治療法はない．この病態の相違，発現の病因の解明は病態解明の中核といえる．

症　状　発症は進行性脳症の症例で早く，生後3～6カ月に運動寡少，躯幹筋緊張低下，仮面様顔貌で発症し，これに腱反射亢進，錐体路徴候，注視発症，眼瞼下垂（交感神経作動点眼薬で改善），縮瞳を伴う．また，間歇的に嗜眠を伴う全身倦怠，被刺激性，発汗，流涎が発現，致命的となることもある．しかし，症例によってはこれらの症状を示さず，進行性の運動障害が前景となる．ドーパ反応性ジストニアを主症状とする症例は，初発症状はジストニアと筋強剛で，乳児期から幼児期に発現，ジストニアは下肢から全身にひろがる．また，乳児期早期に振戦が下肢に始まり，頭部，舌，上肢とひろがる．症例により，これらの運動症状は睡眠により改善を示す．筋強剛，ジストニアを主体とする症例は，知的発達は正常である．

合併症　症状は多彩であり，症例によりその強度が異なり多様性を示すが，特定の合併症はみられない．

診　断　髄液のHVA，3メトキシ-4ヒドロキシフェニルグリコールの減少，プテリン，チロシンおよび5-HTPが正常なことで可能である．確定診断は遺伝子解析による．

治療法　ジストニアを主体とする症例では，L-ドーパが著効を示し，その効果は永続する．ジスキネジアを併発することもあるが，用量を減じることで改善する．症例により多動，また，バリスムスの発現のため，L-ドーパを中止せざるを得ないことがある．しかし，再度，少量で開始，漸増することで効果が得られる．著明な躯幹筋筋緊張低下とバリスムスを伴った症例には少量L-ドーパとセレギリン・ヒドロクロライドの併用が有効であったことが報告されている．進行性脳症の症例には現時点では有効な治療法はない．

Ⅳ　小児神経伝達物質病の診断のポイント

診断のついていない不随意運動もしくは異常眼球運動を呈する重症心身障害児（者）の小児神経伝達物質病の鑑別診断について下記の点に注意が必要である．

1）臨床的にドーパ反応性のジストニアで，日内変動を認める場合，瀬川病が疑われるがL-ドーパに対する反応性は患者ごとに異なるためL-ドーパの効果が少しでもあると思われればプテリジン分析を行うことが勧められる．髄液・血液のプテリジン分析を行い，ネオプテリンとビオプテリンの両方の低下を認めた場合，瀬川病を疑い*GCH1*の遺伝子解析を行う[7]．

2）AADC欠損症ではドーパミンとセロトニンの2つの神経伝津物質の産生低下により多様な神経症状を来すが，効率的な診断スクリーニング法として，臨床症状からは眼球回転発作があげられる．低緊張型もしくは不随意運動を伴う原因不明の脳性麻痺で，眼球回転発作を来す症例では，髄液・血液のカテコールアミンとセロトニンの代謝産物の分析を行い，HVAと5HIAAの両方の低下を認めた場合，AADC欠損症を疑い遺伝子解析を行う[13]．最近では髄液採取の難しい重症児（者）からAADC欠損症をスクリーニングする方法として夜間唾液中メラトニン濃度測定が有効であると報告されている[14]．

3）原因不明の発達遅滞，発達障害の患者において積極的に尿中有機酸分析を行うことが脳内の神経伝達物質であるγ-アミノ酪酸（GABA）に関わる先天代謝異常での早期診断につながる．

4）不随意運動，知的障害を呈し，MRIで髄鞘化遅延，髄鞘形成不全・白質変性，脳梁菲薄化など大脳白質の異常を呈している場合は，神経伝達物質病を鑑別疾患に加えるべきである[15]．

5）BH4欠損症は，高フェニルアラニン血症と神経伝達物質の欠乏による神経症状を来す遺伝性疾患で，現在は新生児マススクリーニングで診断されるが，30歳以上の年長例では未診断例が存在すると推測される．このため日内変動を伴い低緊張型もしくは不随意運動を伴う原因不明の脳性麻痺例では，血中フェニルアラニン濃度の測定が勧められる．高フェニルアラニン血症を認めれば，BH4欠損症の鑑別のためプテリジン化合物分析と乾燥濾紙血のジヒドロプテリジン還元酵素（DHPR）活性の測定が必要である．

おわりに

厚生労働省の難治性疾患克服研究事業に平成21年度から研究奨励分野が設けられ，これまで臨床調査研究分野において組織的・体系的な研究が行われてなかった多くの希少疾患

について，広く医療関係者等の協力を求め，患者やその疾患の病態に関する実態把握を目的とした研究が行われるようになった．これらの研究班により小児神経伝達物質病の診断基準が作成されれば，診断が難しく見過ごされていた神経伝達物質の異常による種々の希少疾患が発見され，適切な診療が行われるようになるだけでなく，希少疾患のため患者情報が少ないことによる患者家族の不安についてもある程度緩和することができることが予測される．また，新しい治療法を開発することで，これまで対症療法のみを施されていた疾患も，患児の生活面への長期にわたる支障が飛躍的に改善される可能性がある．今回の研究奨励分野で取り上げられた難治性希少疾患の研究は，患者やその家族の不安を解消し，希望をあたえることで，国民の健康・福祉の向上につながるだけでなく医療に対する信頼が増し，行政および社会への貢献は計り知れないものがあると考えられる．

本稿における主な内容は，厚生労働科学研究費補助金（難治性疾患克服研究事業）により小児神経伝達物質病研究班（研究代表者：新宅治夫，分担研究者：瀬川昌也，加藤光広，齋藤伸治，浜野晋一郎，久保田雅也，遠山　潤，夏目　淳，服部英司，前垣義弘，松石豊次郎，井手秀平，藤岡弘季）で平成21年度から平成23年度に行われた調査研究に基づくものである．

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Pediatric Neurotransmitter Disease in Japan

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Pediatric neurotransmitter disease (PND) encompasses a range of rare genetic disorders that affect the metabolism of neurotransmitters in children. While these neurological disorders are often studied independently of each other, they all manifest central nervous system symptoms and require proper diagnosis and intervention at early stages. Since clinical symptoms of PND can be nonspecific, the conditions are often under-diagnosed, leaving patients without a chance to receive effective treatment. Envisioning PND as a whole, a comprehensive research effort is underway for a better understanding of pathophysiology and epidemiology in Japan, and toward the establishment of diagnostic criteria. The early diagnosis and development of new effective therapies are of urgent importance for these rare disorders that are not covered by newborn mass screening. For rarer forms of PND, at the same time, it is important to encourage recognition and understanding of the disease concept among healthcare professionals.

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T-cell receptor ligation causes Wiskott-Aldrich syndrome protein degradation and F-actin assembly downregulation

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Background: Wiskott-Aldrich syndrome protein (WASP) links T-cell receptor (TCR) signaling to the actin cytoskeleton. WASP is normally protected from degradation by the Ca^{++}-dependent protease calpain and by the proteasome because of its interaction with the WASP-interacting protein.
Objective: We investigated whether WASP is degraded after TCR ligation and whether its degradation downregulates F-actin assembly caused by TCR ligation.
Methods: Primary T cells, Jurkat T cells, and transfected 293T cells were used in immunoprecipitation experiments. Intracellular F-actin content was measured in splenic T cells from wild-type, WASP-deficient, and c–Casitas B-lineage lymphoma (Cbl)-b–deficient mice by using flow cytometry. Calpeptin and MG-132 were used to inhibit calpain and the proteasome, respectively.
Results: A fraction of WASP in T cells was degraded by calpain and by the ubiquitin-proteasome pathway after TCR ligation. The Cbl-b and c-Cbl E3 ubiquitin ligases associated with WASP after TCR signaling and caused its ubiquitination. Inhibition of calpain and lack of Cbl-b resulted in a significantly more sustained increase in F-actin content after TCR ligation in wild-type T cells but not in WASP-deficient T cells.
Conclusion: TCR ligation causes WASP to be degraded by calpain and to be ubiquitinated by Cbl family E3 ligases, which targets it for destruction by the proteasome. WASP degradation might provide a mechanism for regulating WASP-dependent TCR-driven assembly of F-actin. (J Allergy Clin Immunol 2013;132:648-55.)

Key words: *Wiskott-Aldrich syndrome, Wiskott-Aldrich syndrome protein, T-cell receptor, calpain, ubiquitination, proteasome, F-actin, Cbl family proteins*

Abbreviations used
Arp: Actin-related protein
Cbl: Casitas B-lineage lymphoma
EVH1: Ena-VASP homology domain 1
IS: Immune synapse
TCR: T-cell receptor
WAS: Wiskott-Aldrich syndrome
WASP: Wiskott-Aldrich syndrome protein
WIP: WASP-interacting protein
WT: Wild-type
ZAP-70: ζ Chain–associated protein kinase of 70 kDa

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by variable immunodeficiency, eczema, and thrombocytopenia.[1] The gene for Wiskott-Aldrich syndrome protein (WASP) is mutated in patients with WAS and X-linked thrombocytopenia. *WAS* is located on Xp11.22-p11.23 and encodes a protein of 502 amino acids and approximately 60 kDa in molecular weight.[2,3] WASP expression is restricted to hematopoietic cells.[3]

WASP has an N-terminal Ena/VASP homology domain 1 (EVH1) domain, a Cdc42/Rac GTPase-binding domain, a proline-rich domain, a G actin–binding verprolin homology (V) domain, a cofilin homology (C) domain, and a C-terminal acidic (A) segment.[1] The last 3 domains are located at the C-terminal end of WASP and are collectively referred to as the VCA domain. WASP interacts with WASP-interacting protein (WIP) through its EVH1 domain; with Cdc42-GTP through its GTPase-binding domain; with multiple SH3 domain–containing proteins, which include Nck, Grb2, and cortactin, through its proline-rich region; and with the actin-related protein (Arp) 2/3 complex that initiates actin polymerization through its VCA domain.[4-6]

WASP plays a critical role in T-cell activation and actin reorganization.[7-9] T cells from patients with WAS and $WASP^{-/-}$ mice are deficient in their ability to increase their F-actin content, secrete IL-2, and proliferate after T-cell receptor (TCR) ligation.[10-12] WASP exists in cells in a closed inactive conformation through intramolecular interactions that prevent the VCA domain from activating the Arp2/3 complex. Binding of Cdc42-GTP or of the SH3 domain of adaptor proteins, as well as phosphorylation of tyrosine (Y) at position 291, is thought to cause a conformational change in WASP, which allows the VCA domain to interact with and activate the Arp2/3 complex.[5,13-15] The WASP-interacting protein (WIP) is expressed at high levels in lymphoid tissues. Most of WASP is associated with WIP in T cells.[16] WIP binds through its C-terminal end to the EVH1 domain of WASP. WIP plays an important role in the recruitment of the WASP-WIP complex to ζ chain–associated protein kinase of 70 kDa (ZAP-70) and

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to the immunologic synapse after TCR ligation.[17] More importantly, WIP stabilizes WASP in T cells. This is evidenced by the finding that WASP levels are significantly reduced in T cells from $WIP^{-/-}$ mice and a WIP-deficient patient.[16,18] Furthermore, most missense mutations in WASP that result in diminished WASP levels are localized to the WIP-binding EVH1 domain of WASP and disrupt the WASP-WIP interaction.[19,20] Expression of the WASP-binding domain of WIP in these cells restores WASP levels close to normal.[21] Treatment with calpain and proteasome inhibitors increases WASP protein levels in T cells from $WIP^{-/-}$ mice and patients with WAS with missense mutations that disrupt WIP binding,[16] indicating that WASP can be subject to degradation by calpain and the ubiquitin-proteasome pathway.

Unregulated activation of WASP is detrimental to many cell types, especially cells of the myeloid lineage. Three different mutations of WASP, namely L270P, S272P, and I294T, destroy the autoinhibitory conformation of WASP, resulting in a constitutively active protein, and cause X-linked neutropenia.[22] The L270P and S272P mutations localize to the GTPase-binding domain,[23] whereas the I294T mutation is located close to the tyrosine residue Y291, which, when phosphorylated, results in the activation of WASP.[24] Knock-in mouse models mimicking the L270P and I294T mutations have been described.[25] T and B cells from these mice show a marked increase in F-actin levels but migrate normally in response to chemokines.

In this study we demonstrate that TCR ligation causes WASP to be degraded by calpain and by Casitas B-lineage lymphoma (Cbl)–mediated ubiquitination and proteasomal destruction. We demonstrate that WASP degradation provides a mechanism for downregulating TCR-driven assembly of F-actin.

METHODS

Cell lines and T cells

Jurkat T cells were obtained from the American Type Culture Collection and maintained in RPMI medium (Gibco, Carlsbad, Calif) supplemented with 10% FBS. 293T cells were a gift from Dr N. Ishii (Tohoku University, Sendai, Japan) and were maintained in Dulbecco modified Eagle medium (Gibco) supplemented with 10% FBS. Spleens from Cbl-b knockout (*Cbl-b*$^{-/-}$) mice and genetically matched wild-type (WT) shipping controls were a generous gift from Dr H. Gu, Columbia University. WASP-deficient mice were obtained from Dr Scott Snapper. Splenic T cells were isolated by using T-cell enrichment columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

Antibodies

Anti-WASP 5A5 mAb, which recognizes the epitope in the region corresponding to amino acids 146 to 265, was developed in our laboratory.[26] Anti-WASP rabbit polyclonal antibody K374 (a gift from Dr Ignacio Molina) is directed to the C-terminal 20 amino acids of WASP.[16] Anti–phospho-WASP antibody, which recognized WASP phosphorylated on Y291, was purchased from Abcam (Cambridge, United Kingdom). Anti-ubiquitin mAb P4D1, anti–c-Cbl mAb A-9, and anti–Cbl-b mAb G-1 were from Santa Cruz Biotechnology (Santa Cruz, Calif). Anti-actin mAb and anti-FLAG mAb were from Sigma (St Louis, Mo). Anti-HA mAb was from Cell signaling (Danvers, Mass). Control rabbit IgG was from Upstate (Billerica, Mass).

TCR stimulation, immunoprecipitation, and Western blotting

TCR ligation was performed, as described previously.[17] Briefly, T cells were incubated with 10 μg/mL anti-CD3 mAb UCHT1 (Calbiochem, San Diego, Calif) on ice for 20 minutes, followed by cross-linking with 15 μg/mL goat anti-mouse IgG (H+L; Caltag, Buckingham, United Kingdom) at 37°C for the indicated period. Cells were lysed in ice-cold lysis buffer containing 1% Triton X-100 and protease inhibitors. Cell lysates were clarified at 14,000*g* for 20 minutes at 4°C. For immunoprecipitation, cell lysates were precleared with protein G–Sepharose (GE Healthcare, Fairfield, Conn) for 2 hours and incubated overnight at 4°C with 4 μg of the indicated antibody preadsorbed onto protein G–Sepharose. Beads were washed 4 times with modified lysis buffer containing 0.2% Triton X-100. Bound proteins were eluted, run on 10% SDS-PAGE gels, and analyzed by means of Western blotting with the indicated antibodies followed by anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase and enhanced chemiluminescent detection (Amersham Life Sciences, Piscataway, NJ). Densitometry was performed with CS Analyzer version 2.08 software (ATTO Corporation, Tokyo, Japan) or ImageJ version 1.45 software.

Calpain and proteasome inhibition

The proteasome inhibitor MG132 and the calpain inhibitor calpeptin were purchased from Calbiochem. Cells were cultured with calpeptin (1 μmol/L) or MG132 (10 μmol/L) for 6 hours before anti-CD3 stimulation.

Expression vectors and transfection

Human pcDNA3.1-EGFP-hWASP-WT was a generous gift from Dr K. A. Siminovitch (University of Toronto, Toronto, Ontario, Canada). Control pAcGFP1-C1 vector was purchased from Clontech (Mountain View, Calif). Human pcDNA3.1-3xFLAG-c-Cbl-WT, pcDNA3.1-3xFLAG-Cbl-b-WT, and pcDNA3.1-HA-Ub were gifts from Drs N. Ishii and Y. Tanaka (Tohoku University).[27] Control p3xFLAG-CMV-14 vector was purchased from Sigma. 293T cells were transiently transfected with lipofectamine, as described previously,[27] and harvested 48 hours after transfection.

Determination of cellular F-actin content

Flow cytometric analysis of F-actin content was performed, as described previously.[16] Briefly, mouse T cells were purified by using negative selection with the Pan T Cell Isolation Kit (Miltenyi Biotec) and then incubated for 6 hours with 1 μmol/L calpeptin. Cells were then washed and incubated with 10 μg/mL rat anti-mouse CD3 mAb (Serotec, Oxford, United Kingdom) for 30 minutes on ice. Cells were stimulated for the indicated times by cross-linking with goat anti-rat IgG (H+L) secondary antibody (Jackson Immunoresearch, West Grove, Pa). Cells were fixed in 4% formaldehyde, washed, and permeabilized with the CytoFix/CytoPerm cell staining kit (BD Biosciences, San Jose, Calif). F-actin was stained with tetramethylrhodamine isothiocyanate–labeled phalloidin (Invitrogen, Carlsbad, Calif). F-actin content was analyzed by measuring the mean fluorescent intensity with FACS LSRFortessa (Becton Dickinson) and FlowJo (TreeStar, Ashland, Ore) software.

Statistical analysis

Statistical analysis was performed with the Student *t* test.

RESULTS

WASP is C-terminally truncated by calpain after TCR ligation

Purified peripheral blood T cells were stimulated with anti-CD3 mAb followed by cross-linking with secondary antibody and cell lysates were immunoblotted for WASP to investigate whether WASP is degraded after TCR ligation. Immunoblotting with mAb 5A5, which recognizes an epitope in the region corresponding to amino acids 146 to 265, revealed a 62- to 64-kDa band in unstimulated T cells (Fig 1, *A*), as previously observed.[28] Stimulation with anti-CD3 resulted in the appearance of a 55-kDa

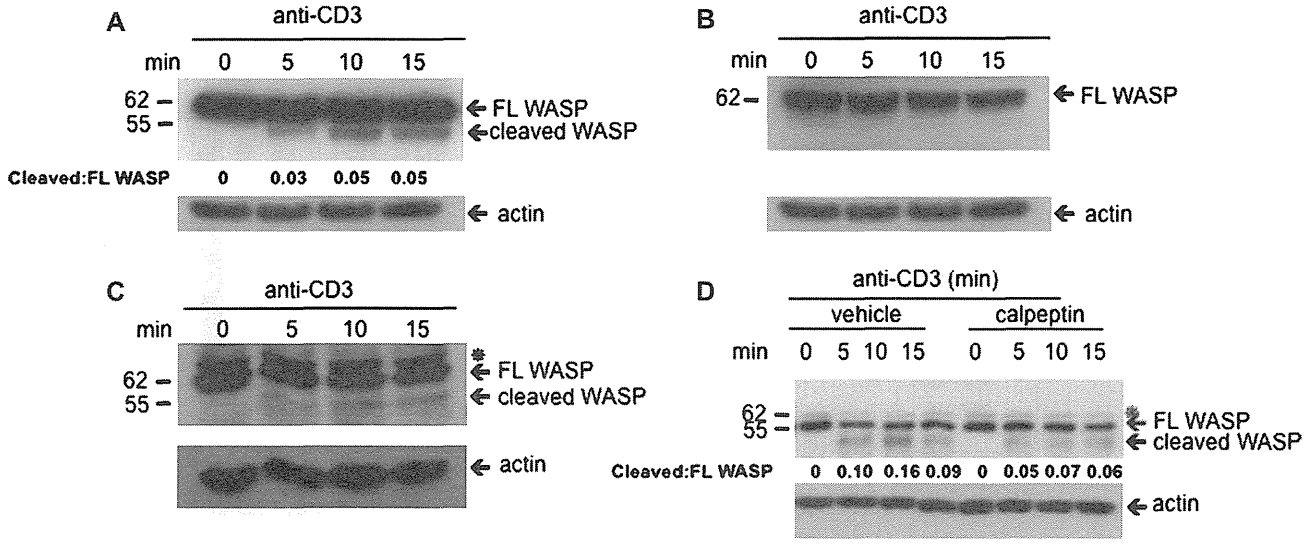

FIG 1. WASP is cleaved by calpain after TCR ligation. **A** and **B,** WASP immunoblot of peripheral blood T cells stimulated for 0 to 15 minutes with anti-CD3 mAb using mAb 5A5 (Fig 1, *A*) or polyclonal antibody K374 (Fig 1, *B*). **C,** WASP immunoblot of Jurkat T cells stimulated with anti-CD3 mAb using mAb 5A5. **D,** Effect of pretreatment for 6 hours with calpeptin on anti-CD3–driven WASP degradation in peripheral blood T cells. Lysates were immunoblotted with mAb 5A5. *Nonspecific band. The positions of molecular weight markers are indicated on the *left* in Fig 1, *A* to *D*. The ratio of cleaved WASP to full-length *(FL)* WASP in Fig 1, *A* and *D*, represents the mean of 5 experiments. Similar results were obtained in Fig 1, *A* to *D*, in 5 independent experiments.

WASP fragment at 5 minutes, which increased at 10 and 15 minutes after stimulation. Scanning densitometric analysis revealed that the intensity of the cleaved WASP band was approximately 5% that of the full-length WASP band at 10 and 15 minutes after stimulation. Similar results were obtained in Jurkat T cells (Fig 1, *C*).

Immunoblotting lysates of T cells with the rabbit polyclonal antibody K374 raised against the C-terminal 20 amino acids of WASP revealed the same 62- to 64-kDa band detected by using mAb 5A5 but did not detect the 55-kDa WASP fragment in anti-CD3–stimulated T cells that was detected by using mAb 5A5 (Fig 1, *B*). Similar results were obtained in Jurkat T cells (data not shown). This result indicates that the 55-kDa WASP fragment lacks the C-terminal VCA domains of WASP (amino acids 421-502) responsible for its actin-polymerizing activity.

Calpain cleaves WASP *in vitro*[29] and contributes to WASP degradation in WIP-deficient T cells.[16,30] To examine whether calpain was responsible for the cleavage of WASP after TCR ligation, T cells were pretreated with the calpain inhibitor calpeptin for 6 hours, washed, and stimulated with anti-CD3 mAb for 5 minutes. Preincubation with calpeptin attenuated by approximately 50% the generation of the 55-kDa WASP fragment in response to anti-CD3 stimulation (Fig 1, *D*), strongly suggesting that calpain mediates the C-terminal truncation of WASP after TCR/CD3 ligation, at least in part.

WASP is ubiquitinated and degraded by the proteasome in T cells after TCR ligation

In the absence of WIP, WASP is degraded by the ubiquitin-proteasome pathway.[16,30] To investigate whether WASP is a substrate for ubiquitination, we incubated *in vitro* transcribed and translated WASP with purified ubiquitin and ubiquitin-conjugating enzymes (mixture of E1, E2, and E3 enzymes), and the reaction mixture was immunoblotted with anti-ubiquitin mAb. WASP was polyubiquitinated in the presence of ubiquitin and ubiquitin-conjugating enzymes, as indicated by an intense high-molecular-weight smear (Fig 2, *A*). Addition of the 26S proteasome fraction to the ubiquitination mixture resulted in marked attenuation of the ubiquitinated WASP smear. These results indicate that after TCR/CD3 ligation, WASP is subject to ubiquitination, which targets it for destruction by the proteasome.

We next examined whether WASP is ubiquitinated in T cells after TCR ligation. Fig 2, *B*, shows the appearance of polyubiquitinated WASP after anti-CD3 mAb stimulation of Jurkat T cells. To examine whether WASP ubiquitinated after TCR ligation is targeted for destruction by the proteasome, Jurkat T cells were pretreated with the proteasome inhibitor MG132 for 6 hours and then stimulated with anti-CD3 mAb for 10 minutes, and WASP immunoprecipitates were prepared from their lysates and probed for ubiquitin. Fig 2, *C*, shows that ubiquitinated WASP was weakly detectable in unstimulated Jurkat cells, but its levels increased after TCR/CD3 stimulation. Pretreatment with MG132 modestly increased the amounts of ubiquitinated WASP in unstimulated Jurkat cells and strongly increased the amounts of ubiquitinated WASP detected after TCR/CD3 ligation. These results indicate that WASP is ubiquitinated and degraded by the proteasome after TCR ligation.

The Cbl family proteins c-Cbl and Cbl-b associate with WASP after TCR ligation and act as E3 ubiquitin ligases for WASP

Members of the Cbl family of E3 ubiquitin ligases are negative regulators in TCR signaling.[31,32] We tested the hypothesis that Cbl proteins might be involved in WASP ubiquitination. We first

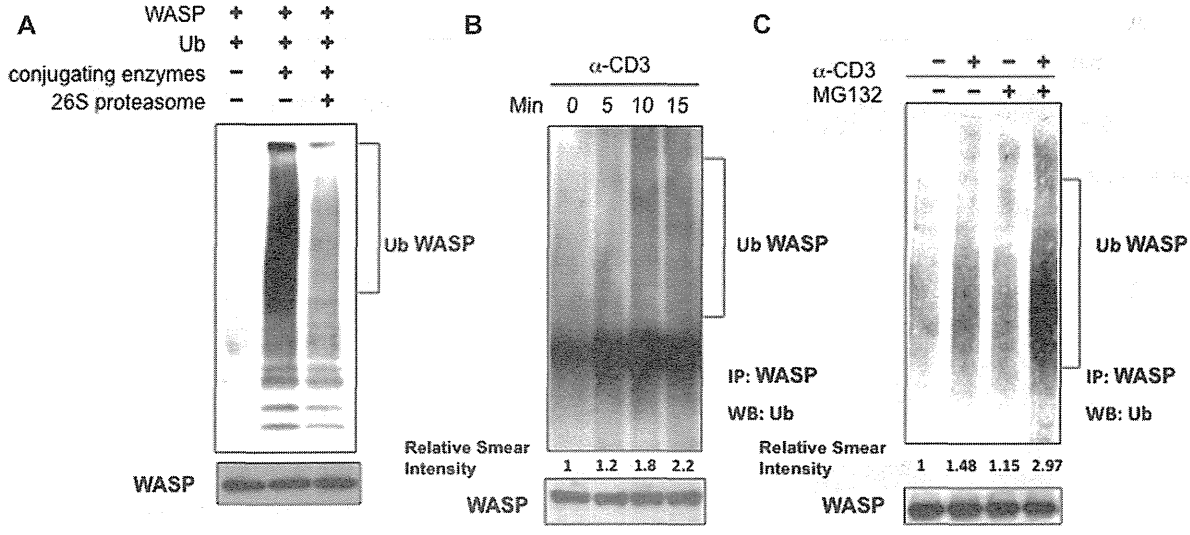

FIG 2. WASP is ubiquitinated and degraded by the 26S proteasome *in vitro* and in anti-CD3–stimulated Jurkat cells. **A,** Ubiquitination of *in vitro* translated purified WASP by ubiquitin-conjugating enzymes and its degradation by the 26S proteasome. Reaction mixtures were probed with anti-ubiquitin. **B,** Generation of ubiquitinated WASP in Jurkat T cells after stimulation with anti-CD3 mAb. WASP immunoprecipitates were probed with anti-ubiquitin mAb. Polyubiquitinated WASP appears as a smear. **C,** Protection of ubiquitinated WASP from degradation by the proteasome inhibitor MG132 in anti-CD3–stimulated Jurkat T cells. Similar results were obtained in Fig 2, *A* to *C*, in 4 independent experiments. The relative smear intensity in Fig 2, *B* and *C*, represents the mean of 4 experiments. *IP*, Immunoprecipitate; *Ub*, ubiquitin; *WB*, Western blot.

investigated whether Cbl proteins and WASP form a complex. WASP immunoprecipitates from Jurkat cell lysates were probed for c-Cbl and Cbl-b. c-Cbl, but not Cbl-b, coprecipitated weakly with WASP in unstimulated Jurkat T cells. TCR ligation increased the association of c-Cbl with WASP. It also induced the association of Cbl-b with WASP at 10 and 15 minutes after stimulation (Fig 3, *A*).

To investigate whether Cbl proteins act as E3 ubiquitin ligases for WASP, we transiently transfected 293T cells with plasmids coding for WT WASP, HA-tagged ubiquitin, FLAG-tagged c-Cbl, or FLAG-tagged Cbl-b. WASP coprecipitated with both c-Cbl and Cbl-b and was polyubiquitinated significantly more when co-transfected with ubiquitin, c-Cbl, and Cbl-b than with ubiquitin and empty vector (Fig 3, *B*).

To examine the role of Cbl-b in WASP ubiquitination after TCR ligation, we used purified T cells from spleens of *Cbl-b*$^{-/-}$ mice. Ubiquitination of WASP after TCR ligation was reduced, although not completely abrogated, in T cells from *Cbl-b*$^{-/-}$ mice (Fig 3, *C*), suggesting that WASP is a substrate for Cbl-b in antigen-stimulated T cells. We could not examine the role of c-Cbl on WASP ubiquitination after TCR ligation because we had no access to T cells from *c-Cbl*$^{-/-}$ mice.

WASP degradation after TCR/CD3 ligation limits TCR/CD3-driven F-actin assembly in T cells

WASP is important for F-actin assembly in T cells.[10] We examined whether WASP degradation after TCR/CD3 ligation regulates TCR/CD3-driven F-actin assembly. Purified T cells from WT and WASP-deficient mice were incubated for 6 hours with calpeptin or left untreated, washed and stimulated with anti-CD3 mAb, and cross-linked with a secondary antibody. The cells were then fixed, permeabilized, stained for F-actin with fluorescein isothiocyanate–conjugated phalloidin, and analyzed by means of flow cytometry. As previously reported, WASP-deficient T cells had a lower F-actin content than WT T cells.[16] TCR/CD3 ligation caused a parallel increase in F-actin levels in both WT and WASP-deficient T cells, which peaked at 5 minutes after stimulation and returned almost to baseline 10 minutes after stimulation. Pretreatment with calpeptin had no effect on F-actin content of the T cells at baseline or at 2 and 5 minutes after stimulation; however, it significantly increased the F-actin content of WT T cells at 10 minutes after anti-CD3 stimulation, maintaining it at almost the peak level achieved at 5 minutes after stimulation. In contrast, pretreatment with calpeptin had no effect on the F-actin content of WASP-deficient T cells 10 minutes after anti-CD3 stimulation. These results suggest that calpain-mediated WASP degradation limits the duration of F-actin assembly after TCR/CD3 ligation.

We next examined whether ubiquitination, which targets WASP for proteosomal degradation, regulates F-actin assembly after TCR/CD3 ligation. Because Cbl-b participates in WASP ubiquitination, we examined F-actin assembly in T cells deficient in Cbl-b. Baseline F-actin content and TCR-driven F-actin assembly were both significantly increased in T cells from c-Cbl-deficient mice compared with T cells from WT control animals (Fig 4, *B*). These results suggest that WASP degradation by ubiquitination regulates baseline and TCR-driven F-actin assembly.

DISCUSSION

Our results demonstrate that TCR ligation triggers the degradation of WASP by calpain-mediated cleavage and Cbl-mediated ubiquitination and subsequent proteasomal degradation. We present evidence that WASP degradation provides a mechanism for limiting the duration of TCR-driven assembly of F-actin.

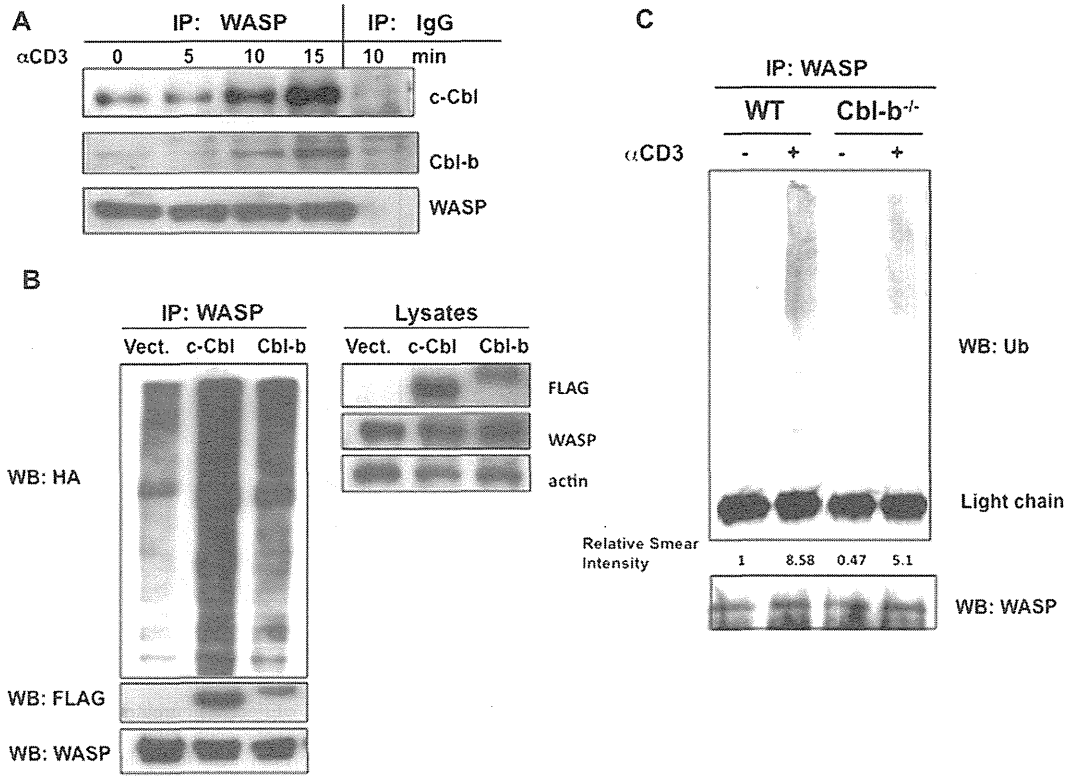

FIG 3. Cbl family E3 ubiquitin ligases associate with and ubiquitinate WASP after TCR ligation. **A,** Western blot analysis of WASP immunoprecipitates from anti-CD3–stimulated Jurkat T cells for c-Cbl and Cbl-b. IgG control antibody precipitates were prepared 10 minutes after anti-CD3 stimulation and used as controls. **B,** Ubiquitination of WASP in 293T cells transfected with WT WASP and HA-tagged ubiquitin plus either FLAG vector alone *(Vect.),* FLAG-tagged c-Cbl, or FLAG-tagged Cbl-b. In the *left panel* WASP immunoprecipitates were probed for HA, FLAG, and WASP. In the *right panel* total lysates were probed for FLAG–c-Cbl or FLAG–Cbl-b, WASP, and actin. **C,** WASP ubiquitination after TCR ligation in T cells from *Cbl-b*$^{-/-}$ mice and WT control animals. WASP immunoprecipitates were probed for ubiquitin. Similar results were obtained in Fig 3, *A* to *C,* in 4 independent experiments. The relative smear intensity in Fig 3, *C,* represents the mean of 4 experiments. *IP,* Immunoprecipitate; *Ub,* ubiquitin; *WB,* Western blot.

TCR/CD3 ligation resulted in the degradation of a small fraction of WASP through calpain-mediated cleavage and the ubiquitin proteasome pathway. We estimated that approximately 5% of WASP is degraded after TCR/CD3 ligation. This is possibly an underestimate because the truncated 55-kDa WASP might be less stable than intact WASP. We could not detect a decrease in the levels of intact WASP in anti-CD3–activated T cells, probably because Western blotting is not sensitive enough to detect a small decrease in protein levels. We were unable to detect the cleaved, C-terminal, approximately 10-kDa fragment using an antibody to the C-terminus of WASP. This is most likely because such a small cleaved fragment would be rapidly degraded in the cell. Normally, WASP is protected from degradation by its partner, WIP.[16] The conformational changes in WASP induced by TCR signaling, which involve a change from an inactive to an active form capable of activating the Arp2/3 complex and F-actin polymerization, possibly increases the susceptibility of WASP to calpain cleavage and to ubiquitination and proteasomal degradation. The observation that WASP is degraded by calpain after TCR ligation is consistent with previous observations that WASP can be degraded in platelet lysates by calpain[29] and that *in vitro* translated WASP is a substrate for calpain I and II.[16] The increase in intracellular Ca^{++} concentration that follows TCR ligation could be the trigger for the Ca^{++}-dependent activation of calpain in anti-CD3–stimulated T cells.

Both c-Cbl and Cbl-b associated with WASP when overexpressed in 293T cells and acted as E3 ubiquitin ligases for WASP ubiquitination *in vitro*. More importantly, WASP ubiquitination after TCR ligation was impaired in Cbl-b-deficient T cells, implicating at least Cbl-b in WASP ubiquitination in T cells. Cbl family proteins act as negative regulators of TCR signaling by virtue of their ability to ubiquitinate LCK and ZAP-70,[33] which are upstream of WASP. Thus Cbl family members might regulate WASP activity indirectly by dampening TCR signaling upstream of WASP, as well as directly by ubiquitinating WASP and targeting it for degradation. Evidence has been presented that the activated WASP phosphorylated at Y291 is a target for ubiquitination.[34] We have also found that inhibition of the proteasome by MG132 increases the amount of tyrosine-phosphorylated WASP in anti-CD3–stimulated cells (see Fig E1 in this article's Online Repository at www.jacionline.org). This observation lends further support to the notion that activated WASP molecules are targets for degradation after TCR ligation.

It is not clear whether the interaction between WASP and c-Cbl and Cbl-b is direct or mediated by other proteins. It has been reported that c-Cbl associates with multiple proteins, which

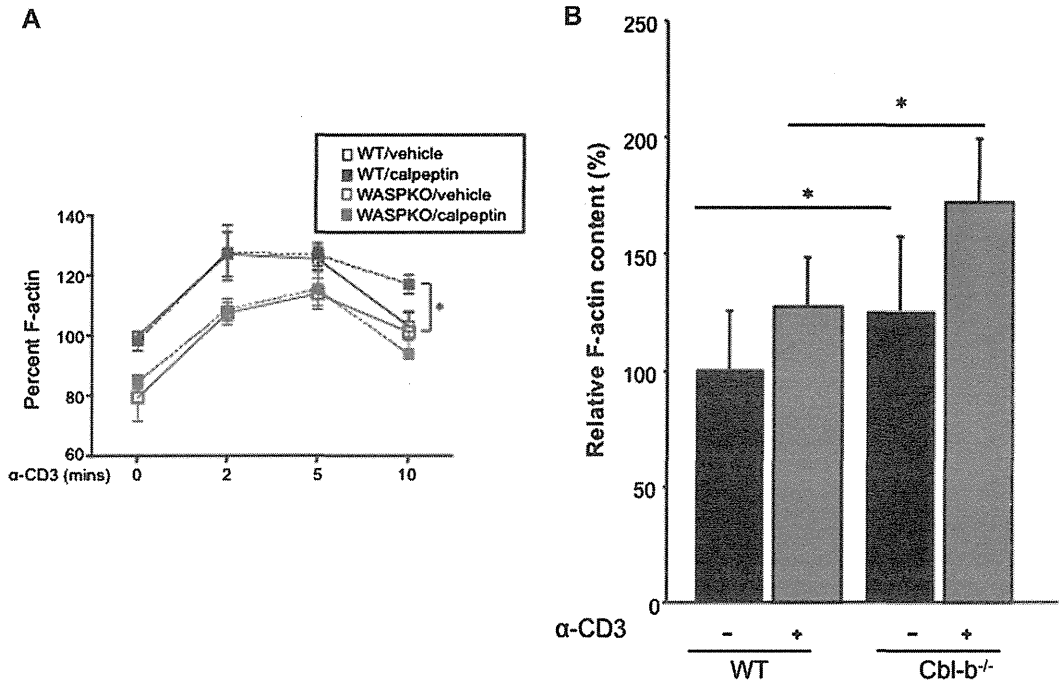

FIG 4. Effect of calpain inhibition and loss of Cbl on F-actin assembly in T cells. **A,** Effect of pretreatment of T cells from WT and WASP knockout mice with calpeptin on TCR-driven assembly of F-actin. Pretreatment with the vehicle dimethyl sulfoxide was used as a control. **B,** TCR-driven assembly of F-actin in T cells from *Cbl-b*$^{-/-}$ mice and WT control animals. T cells were stimulated with anti-CD3 for 10 minutes. Results are expressed as a percentage of the baseline F-actin content in unstimulated WT T cells and represent the means ± SDs of 3 independent experiments. *$P < .01$.

include tyrosine phosphorylated ZAP-70,[35] the adaptor proteins Nck[36] and Grb2[37] through their SH3 domains, CrkL through its SH2 domain,[38] Src tyrosine kinases through their SH3 domains,[39,40] and Vav and the p85 regulatory subunit of phosphatidylinositol-3-OH kinase through their SH2 domains.[41,42] Because these proteins are also reported to associate with WASP, its partner WIP, or both,[17,43] an indirect association of Cbl with WASP cannot be ruled out. Alternatively, c-Cbl and Cbl-b could directly interact with an activated form of WASP, such as tyrosine-phosphorylated WASP. Indeed, while this manuscript was in preparation, it was shown that WASP phosphorylation at tyrosine 291 after TCR activation results in recruitment of Cbl-b.[34]

Our data suggest that the degraded fraction of WASP includes activated WASP. This is supported by the observation that calpain inhibition and lack of the WASP-ubiquitinating E3 ligase Cbl-b resulted in more sustained F-actin assembly in WT T cells after TCR/CD3 ligation. The small fraction of WASP that is cleaved after TCR ligation could be important for F-actin polymerization because of its location close to the TCR. Indeed, we have shown previously that a fraction of WASP translocates together with a fraction of the TCR/CD3 complex to lipid rafts.[17] It is also well known that a fraction of WASP colocalizes with TCR molecules in the immune synapse (IS).[17,44,45] Cbl family molecules, which are also recruited to the IS, where they are activated by LCK and ZAP-70,[46,47] could ubiquitinate WASP molecules recruited to the IS, targeting them for degradation. The IS is a dynamic structure that constantly undergoes protein kinase Cθ–dependent dissolution and WASP/F-actin–dependent reformation of its peripheral supramolecular activation complex.[45] Protein kinase Cθ–dependent dissolution breaks the symmetry of the IS and allows T-cell motility. WASP/F-actin–dependent reformation of the IS is important for the sustained signaling that is necessary for IL-2 production. We speculate that cycles of TCR-triggered recruitment and activation of WASP in the IS followed by local degradation of the activated WASP might be important for IS dynamics and T-cell function.

The observation that baseline F-actin content was increased in *Cbl-b*$^{-/-}$ T cells, but not in calpeptin-treated T cells, suggests that under steady-state conditions, Cbl ubiquitination and proteasome degradation, but not calpain, degrade WASP molecules in activated in T cells. The observation that calpain inhibition had no effect on F-actin assembly in WASP-deficient T cells indicates that calpain regulates F-actin assembly by targeting WASP for degradation. These results strongly suggest that degradation of activated WASP by calpain and by the ubiquitin/proteasome pathway provide an important homeostatic mechanism for terminating signaling to the cytoskeleton after TCR ligation. Furthermore, WASP mutants that are resistant to ubiquitination are associated with enhanced T-cell activation, supporting the notion that WASP degradation limits TCR activation.[34]

Protein cleavage is used by prokaryotes and eukaryotes to activate or terminate signaling. Well-documented examples include the coagulation cascade, the complement activation cascade, degradation of the nuclear factor κB inhibitor IκBα, TNF receptor–associated factor 3, Argonaute, and voltage-gated calcium-channel proteins.[48-53] Degradation of activated WASP might regulate receptor signaling to the cytoskeleton not only in T cells but also in other hematopoietic cells. Such a control mechanism would avoid the potential pathology observed in patients with mutations that cause sustained WASP activation and manifest as X-linked neutropenia.

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Key message

- **TCR signaling causes WASP to be degraded by calpain and by Cbl-family members through ubiquitination and destruction by the proteasome, limiting TCR-driven assembly of F-actin.**

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