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Original article

The axonal damage marker tau protein in the cerebrospinal fluid is increased in patients with acute encephalopathy with biphasic seizures and late reduced diffusion

Naoyuki Tanuma [a,h,*], Rie Miyata [b,h], Satoko Kumada [c], Masaya Kubota [d], Jun-ichi Takanashi [e], Akihisa Okumura [f], Shin-ichiro Hamano [g], Masaharu Hayashi [h]

[a] *Department of Pediatrics, Tokyo Metropolitan Fuchu Medical Center for the Disabled, Japan*
[b] *Department of Pediatrics, Tokyo Kita Shakai Hoken Hospital, Japan*
[c] *Department of Neuropediatrics, Tokyo Metropolitan Neurological Hospital, Japan*
[d] *Department of Neurology, National Center for Child Health and Development, Japan*
[e] *Department of Pediatrics, Kameda Medical Center, Japan*
[f] *Department of Pediatrics, Juntendo University, School of Medicine, Japan*
[g] *Division of Neurology, Saitama Children's Medical Center, Japan*
[h] *Department of Clinical Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Japan*

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Abstract

Acute encephalopathy with biphasic seizures and late reduced diffusion (AESD) is a recently clinicoradiologically-established encephalopathy syndrome. In the present study, we examined the levels of cerebrospinal fluid (CSF) tau protein, a marker of axonal damage, in 11 patients with AESD. CSF tau levels were normal on day 1 and increased from day 3 of the disease between the initial and the secondary seizures. Magnetic resonance imaging (MRI) reveals reduced diffusion in the subcortical white matter during days 3–7. Two patients showed elevated tau protein prior to the diffusion abnormality of subcortical white matter on MRI. Levels of CSF neuron specific enolase (NSE), a neuronal marker, were elevated in only two out of seven patients with AESD, and CSF tau levels were also increased in these patients. Our results indicated that tau protein is a more sensitive marker than NSE and axonal damage causes the conspicuous MRI findings in AESD patients. A therapeutic strategy for axonal protection should be developed to prevent severe neurological impairment of AESD patients.
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Keywords: Acute encephalopathy; Tau protein; Axonal damage

1. Introduction

Tau protein is a microtubule-associated protein found predominantly in neuronal axons. It plays an important role in maintaining the cytoskeleton and axonal transport. It is released into the interstitial fluid compartment during neuroaxonal disintegration and diffuses into the cerebrospinal fluid (CSF). Increased levels of CSF tau are found in various pathological conditions, such as traumatic brain injury (TBI) [1], amyotrophic lateral sclerosis (ALS) [2] and multiple sclerosis (MS) [3–5]. Thus CSF tau protein is considered to be a useful biomarker of axonal damage.

Acute encephalopathy with biphasic seizures and late reduced diffusion (AESD) is recently described by Takan-

* Corresponding author. Address: Department of Pediatrics, Tokyo Metropolitan Fuchu Medical Center for the Disabled, 2-9-2, Musashidai, Fuchu, Tokyo 183-0042, Japan. Tel.: +81 42 323 5115x5232; fax: +81 42 322 6207.
E-mail address: Naoyuki_Tanuma@member.metro.tokyo.jp (N. Tanuma).

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ashi et al. [6,7]. This new encephalopathy syndrome is characterized by a prolonged (>30 min) febrile seizure (FS) on day 1, followed by secondary seizures (most often in a cluster of complex partial seizures) on days 4–6. Magnetic resonance imaging (MRI) shows no abnormality during the first 2 days, but reveals reduced diffusion in the frontoparietal subcortical white matter during days 3–9. This diffusion abnormality disappears between days 9 and 25, resulting in cerebral atrophy, and affected children have subsequently variable levels of neurological sequelae. The cause of the MRI findings in AESD remains unknown, and it is important to diagnose AESD as early as possible because the initial prolonged seizure is not easily distinguishable from convulsive status epilepticus.

In the present study, we examined the levels of CSF tau protein in patients with AESD and found that CSF tau levels were increased from day 3 between the initial and the secondary seizures. Our results suggest that axonal damage is the cause of the MRI findings in AESD.

2. Methods

2.1. Subjects

We studied 11 patients with AESD aged 10 months to 3 years of age (mean age of 1.4 ± 0.7 years) and 31 control children aged 1 month to 16 years (mean age of 3.5 ± 5.4 years). The control group consisted of children who presented with fever and required analysis of CSF to exclude the presence of central nervous system (CNS) infection. They were later found to be free of such infection based on the results of CSF examination and negative clinical findings including febrile convulsions.

The clinical diagnosis of AESD was made according to the biphasic clinical course and characteristic MRI findings [6]. Clinical information on these patients is shown in Table 1. The study protocol was approved by the Human Ethics Review Committee of our institution.

2.2. Sample collection and measurement of CSF tau protein levels

CSF samples were obtained from each subject and immediately stored at −80 °C until analysis. The parents of patients with AESD and normal controls understood the purpose of this study and approved the offer of CSF samples.

Levels of CSF human total tau protein were determined using an enzyme-linked immunosorbent assay (ELISA) kit from BioSource International, Inc., Camarillo, California, USA. Neuron-specific enolase (NSE) levels were measured by a standard radioimmunoassay (RIA). All the analyses were performed in triplicate and in a blinded fashion.

3. Results

3.1. Clinical features of patients with AESD

Infectious agents were identified in 9 out of 11 patients and human herpes virus (HHV)-6 was the most common pathogen (63.6%). The outcome of the 11 patients ranged from almost normal to severe mental retardation.

The representative MRI (Patient 7) was shown in Fig. 1. MRI on day 3 (2 days after the initial prolonged seizure) showed no acute parenchymal lesion in diffusion-weighted image (DWI) (Fig. 1A) or in T2-weighted image (Fig. 1D). Subcortical white matter lesions were observed on day 5 (2 days after the second seizure) and were most conspicuous on DWI (Fig. 1C). The subcortical high signal intensity on DWI disappeared (Fig. 1E) and cerebral atrophy was observed on day 20.

3.2. CSF tau protein levels in normal controls and AESD patients

The kinetics of CSF tau protein levels in normal controls are shown in Fig. 2. In the early infantile period, the level of CSF tau was above 1000 pg/ml (Fig. 2A). This level reduced to below 500 pg/ml at around 1 year of age (Fig. 2B). After 1 year of age, the mean level of CSF tau was 439.6 ± 146.4 pg/ml (mean ± SD) (range; 12–630 pg/ml, $n = 11$).

We next examined the levels of CSF tau protein in patients with AESD (Fig. 3 and Table 1). Although the sampling day of each patient varied from day 1 to day 7, the level of CSF tau was normal on day 1, increased from day 3 and remained above 1000 pg/ml thereafter (Fig. 3). We confirmed elevated tau protein prior to the diffusion abnormality of subcortical white matter on MRI in two patients (Patients 7 and 9).

We also examined the levels of CSF NSE in seven patients with AESD and controls. The average level of CSF NSE in controls is 10.4 ± 3.94 ng/ml (mean ± SD). CSF NSE was elevated in two patients (Patients 8 and 10). Although CSF tau protein level was increased in three (Patients 2, 7 and 9) out of seven patients, CSF NSE was within normal range in these patients.

Regarding the relationship between the level of CSF tau and clinical prognosis, there was no correlation between them (Table 1). Although two patients had high CSF NSE levels, one was without neurological sequelae (Patient 10) and the other had mental retardation (Patient 8).

4. Discussion

In the present study, we demonstrated that the level of CSF tau was elevated in early infantile normal con-

Table 1
Clinical profiles of the 11 patients with AESD.

Patients	Age/sex	Pathogen	Outcome	CSF sampling day	Diffusion abnormality on MRI	Abnormal signal lesion	Tau (pg/ml)	NSE (ng/ml)
1	1y0 m/F	HHV-6	Mild MR (DQ 69.2)	Day 1 Day 3	Day 3	Bil. F. WM	714.5 2376	n.d. n.d.
2	1y4 m/F	Unknown	Psychomotor retardation, epilepsy	Day 1 Day 5	Day 5	Bil. WM (sparing peri-Roland)	412.1 2025.1	4.4 14
3	10mo/F	HHV-6	Without neurological sequelae	Day 1	Day 5	Bil. F. WM	436.7	4.9
4	1y2 m/M	HHV-6	MR (speech delay)	Day 1	Day 5	Bil. F. WM	467	5
5	1y4 m/F	HHV-6	Mild MR (speech delay)	Day 1	Day 6	B. F., T., P., O. WM, GM	379.9	n.d.
6	3y1 m/F	Influenza A	Severe MR (DQ 26), epilepsy	Day 1	Day 7	Bil. F., P., O. WM, GM	416.6	n.d.
7	1y10 m/F	Unknown	Severe MR, hypotonia	Day 3	Day 5	Bil. WM (sparing peri-Roland)	2014.7	9.8
8	1y2 m/M	HHV-6	MR	Day 3	Day 3	Bil. F.	7986.2	25
9	11mo/F	HHV-6	Without neurological sequelae	Day 5	Day 6	RT. F., T. WM	2036	15
10	1y0 m/F	HHV-6	Without neurological sequelae	Day 6	Day 6	Bil. F. WM	1885.6	32
11	1y11 m/M	Influenza B	Without neurological sequelae	Day 7	Day 6	Bil. F. WM	2411.7	n.d.

HHV-6, human herpes virus-6; MR, mental retardation; DQ, Development quotient; Bil., bilateral; rt, right; F., frontal; T., temporal; P., parietal; O., occipital; WM, white matter; GM, gray matter; n.d., not done.
The average level of CSF tau in controls after 1 year of age was 439.6 ± 146.4 pg/ml (mean ± SD) (range; 12–630 pg/ml, $n = 11$).
The average level of CSF NSE in controls is 10.4 ± 3.94 ng/ml (mean ± SD).

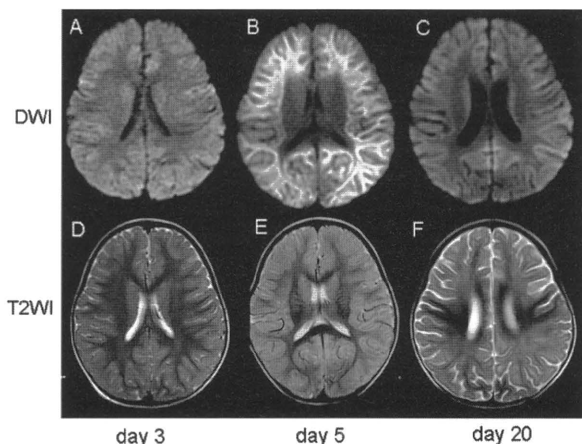

Fig. 1. Representative MRI of a patient with AESD (Patient 7) on days 3 (A and D), 5 (B and E) and 20 (C and E). MRI on day 3 showed no abnormality on diffusion-weighted image (A) or T2-weighted image (D). MRI on day 5 revealed reduced diffusion in the subcortical white matter with sparing of the peri-Rolandic region (B) and T2 prolongation (E). Diffusion-weighted image on day 20 showed mild cerebral atrophy with no signal abnormalities (C). T2-weighted image on day 20 showed T2 prolongation in the subcortical white matter (F).

trols and rapidly reduced after 6–8 months of age. The precise reason for this finding is unknown. However, one possible explanation is that the high level of CSF tau in the early infantile period may be related to myelination, which is most active during this period. Tau protein is also present in oligodendrocytes that make myelin [8,9], and is integral to neuronal process development [10]. Our results suggest that tau protein may be released into the CSF during myelination. Further analysis will be required to confirm this hypothesis.

The most important finding of this study is that the level of CSF tau was normal on day 1 and increased from day 3 between the initial and the secondary (late) seizures in AESD patients. In addition, CSF tau did not increase in patients with febrile seizures without encephalopathy (data not shown). Thus result suggests that the axonal damage in AESD patients begins at least between day 1 and day 3, resulting in a diffusion MRI abnormality thereafter. We also examined NSE, a glycolytic enzyme predominantly present in neurons and endocrine cells. NSE is another index of organic neuronal damage and its CSF levels are increased in several neurological diseases [11,12]. However, the level of

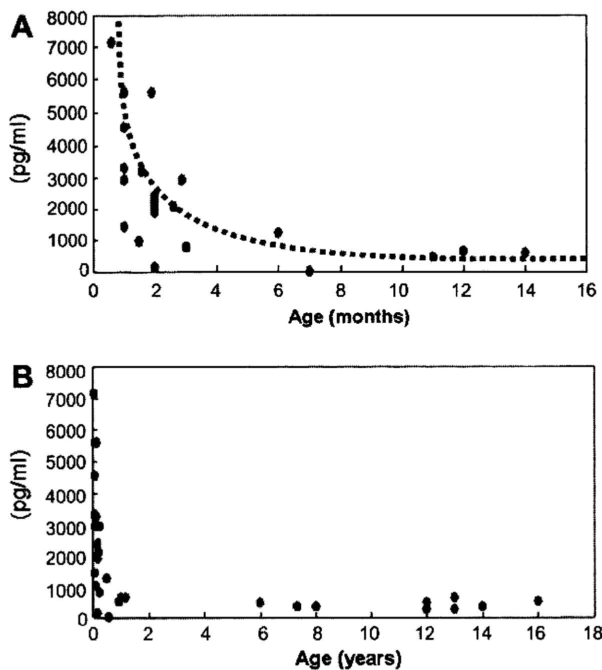

Fig. 2. The kinetics of CSF tau protein levels in normal controls. The level of CSF tau was above 1000 pg/ml in the early infantile period and rapidly reduced to below 500 pg/ml at around 1 year of age (A). Above 1 year of age, the mean level of CSF tau was 363.4 ± 174.7 pg/ml (mean ± SD) (range; 12–618 pg/ml, $n = 11$) (B).

CSF NSE was elevated in only two patients with AESD, although CSF tau levels were increased in patients with normal CSF NSE levels. These results indicate that tau protein is a more sensitive marker than NSE. MRI analysis revealed that reduced diffusion disappears between days 9 and 25, finally resulting in cerebral atrophy. It is likely that axonal damage is the cause of conspicuous diffusion abnormality and late cerebral atrophy in AESD patients.

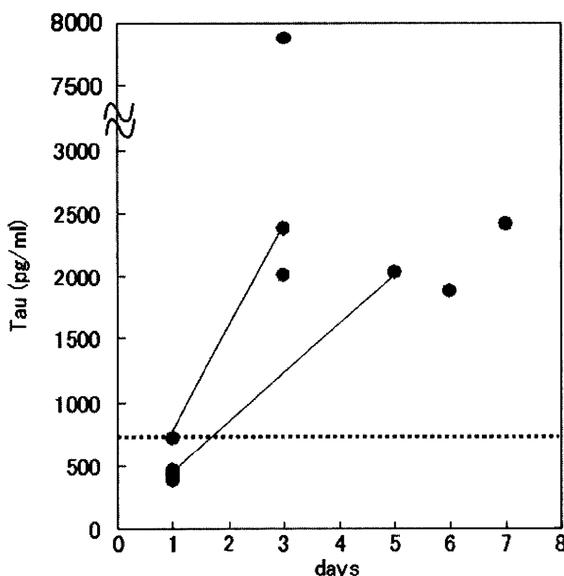

Fig. 3. The levels of CSF tau protein in patients with AESD. The level of CSF tau was normal on day 1, increased from day 3 and remained over 1000 pg/ml thereafter. The dotted line indicates the cut-off value (mean + 2 SD of normal controls). The solid line indicates CSF samples from the same patents (Patients 1 and 2) on a different day.

Although the cause of the MRI findings of AESD remains unknown, excitotoxity is one of the candidates [6,13,14]. MR spectoroscopy revealed that glutamine/glutamate complex (Glx) was elevated during the first week in AESD, although normal Glx concentrations were found in patients with prolonged febrile seizures [6,14]. These findings suggest that excitotoxic neuronal damage is likely to play an important role in the pathogenesis of AESD. The pathomechanisms of excitotoxicity are complex involving glutamate overload, ionic channel dysfunction, calcium overload, mitochondriopathy, proteolytic enzyme production and activation of apoptotic pathways [15]. In addition, glutamate excitotoxicity is considered to be one of the major causes of axonal degeneration in multiple sclerosis [16]. These findings raise the possibility that excitotoxicity may result in not only neuronal damage but also axonal damage in AESD.

Regarding neurological outcome of AESD patients, we first expected that higher tau levels would be associated with more severe brain damage and a worse outcome, because initial ventricular CSF tau levels are correlated with 1-year neurological outcome in patients with traumatic brain injury [1]. Unfortunately, we could not predict the neurological prognosis on day 1 from CSF tau levels. However, three patients with neurological sequelae (Patients 1, 7 and 8) had high CSF tau levels, at least on day 3. By contrast, patients without neurological sequelae (Patients 9, 10 and 11) also had high CSF tau levels, but their levels were elevated after day 5. Thus, it is likely that high CSF levels at an early time point would be associated with a worse outcome. We also examined the levels of CSF tau protein in other type of encephalopathy. Increased CSF tau was also observed in patients with other type of encephalopathy, transverse myelitis and encephalitis (unpublished data), especially in severe cases. In contrast, all the cases in AESD have increased CSF tau after day 3 even if they have no neurological sequelae. It will be useful to further investigate the time course of CSF tau levels in the same patient; alternatively, a larger study would be needed.

Recently, the identification of strategies to protect axons from degeneration in multiple sclerosis patients has emerged as a major research priority [17]. Black et al. reported that the sodium channel blocker phenytoin provides protection for spinal cord axons in long-term monophasic and in chronic-relapsing murine experimental autoimmune encephalomyelitis (EAE) [18] and withdrawal of phenytoin is accompanied by acute exacerbation of EAE [19]. Their results demonstrate the efficacy of phenytoin as a neuroprotectant in

EAE. Intravenous phenytoin is also used in treatment of convulsive status epilepticus (CSE) [20]. In Japan, intravenous phenytoin is less frequently used than midazolam, because of its side effects venous irritation and phlebitis [21]; however, phenytoin (or fosphenytoin) is a second-line antiepileptic drug for CSE in the USA and European countries [20]. These observations together with our results raise the possibility that intravenous phenytoin may prevent axonal damage, if we use it more frequently for the treatment of CSE, especially the initial prolonged febrile seizure in AESD patients. A therapeutic strategy for axonal protection should be developed to prevent severe neurological impairment of AESD patients.

In summary, we demonstrated that CSF tau levels were increased prior to the diffusion abnormality of subcortical white matter on MRI in AESD patients. Our result suggests that axonal damage causes the conspicuous MRI findings.

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Case report

Low-dose levodopa is effective for laryngeal dystonia in xeroderma pigmentosum group A

Rie Miyata [a,*], Toru Sasaki [b], Masaharu Hayashi [c], Satoshi Araki [d], Masayuki Shimohira [d], Jun Kohyama [a]

[a] *Department of Pediatrics, Tokyo Kita Shakai Hoken Hospital, 4-17-56 Akabanedai, Kita-ku, Tokyo 115-0053, Japan*
[b] *Department of Otorhinolaryngology, Tokyo Kita Shakai Hoken Hospital, 4-17-56 Akabanedai, Kita-ku, Tokyo 115-0053, Japan*
[c] *Department of Clinical Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan*
[d] *Department of Pediatrics, Tokyo Medical and Dental University, Tokyo, Japan*

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Abstract

Xeroderma pigmentosum (XP), a genetic disorder in DNA nucleotide excision repair, is characterized by skin hypersensitivity to sunlight and progressive neurological impairment. Laryngeal dystonia and vocal cord paralysis are complications that can arise in older XP group A (XPA) patients. We report three patients with XPA being administered low-dose levodopa (0.3–1.5 mg/kg/day) for laryngeal dystonia. Patients were aged from 13 to 18 years, exhibited paroxysmal choking and inspiratory stridor, and were diagnosed with laryngeal dystonia. Two XPA patients responded to low-dose levodopa, and paroxysmal choking and involuntary movements resolved, although one of the two patients showed incomplete resolution due to suspected vocal cord paralysis. The other patient was unable to tolerate the medication because of a transient decrease of muscle tone in the extremities. We previously reported a decreased immunostaining of dopaminergic (DA) terminals in the basal ganglia of XPA patients, which may be involved in laryngeal dystonia. Low-dose levodopa has been reported to alleviate DA receptor supersensitivity in tic patients, while laryngeal dystonia occurs in patients with tardive dyskinesia caused by DA receptor supersensitivity. Thus, low-dose levodopa may improve laryngeal dystonia by alleviating DA receptor supersensitivity in XPA patients. We recommend that low-dose levodopa be used for treatment of paroxysmal respiratory disturbances and/or involuntary movements in XPA patients.
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Keywords: Xeroderma pigmentosum; Laryngeal dystonia; Levodopa; Dopamine; Basal ganglia

1. Introduction

Xeroderma pigmentosum (XP), a genetic disorder in DNA nucleotide excision repair, is characterized by skin hypersensitivity to sunlight and progressive neurological impairment [1]. The common complementation subgroup of XP in Japan is group A (XPA). XPA leads to severe neurological disorders including mental deterioration, cerebellar ataxia, extrapyramidal abnormalities, and neuronal deafness; however, there are no effective treatments for such symptoms [2]. Laryngeal dystonia is characterized by paroxysmal stridor due to vocal cord dystonia, and is observed in extrapyramidal disorders [3]. Laryngeal dystonia and vocal cord paralysis are complications that can arise in older XPA patients and can be life threatening [4,5]. We previously demonstrated selective damage to the dopamine (DA) neurons in the substantia nigra of XPA cases at autopsy [6]. Herein, we report three patients with clinically or genetically confirmed XPA who received low-dose levodopa for laryngeal dystonia. In two of the three patients, laryngeal dystonia and involuntary movements in the

* Corresponding author. Tel.: +81 3 5963 3311; fax: +81 3 5963 6678.
E-mail address: rie88miyata@ybb.ne.jp (R. Miyata).

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upper extremities were relieved. Parental consent in addition to the approval of the chief of the hospital ethical committee was obtained for all cases.

2. Case report

Patient 1 was an XPA female aged 18 years and 8 months with a homozygous splicing mutation in intron 3. She began using a hearing aid for neuronal deafness at 8 years, she developed rigidity in the extremities in her teens, and had received anticonvulsants since she was 13 years. Brain MRI demonstrated diffuse atrophy in the cerebrum, cerebellum, and brainstem. At 16 years, she began to show dysphagia and myoclonic movements during wakefulness in the upper extremities, and started medication for a neurogenic bladder. A surface electromyogram was not performed. She developed nighttime inspiratory stridor, and laryngoscopy (LS) was performed at 17 years. On LS, vocal cord movement was restricted, and the glottis tended to be fixed in the midline during inspiratory and expiratory phases (Fig. 1A), although no morphological abnormalities were found in the arytenoid processes. She began to experience paroxysmal choking during meals and sleep at 17 years and 2 months, with subsequent LS revealing restricted movements in the vocal cord. We suspected that her symptoms were due to laryngeal dystonia, and started low-dose therapy of levodopa (0.3 mg/kg/day) at 17 years and 10 months. Her nighttime inspiratory stridor, paroxysmal choking, and myoclonic movements of the upper extremities resolved, and LS showed a partial improvement in vocal cord opening (Fig. 1B). The patient developed transient, mild motor weakness in the extremities a few days after the start of levodopa, which disappeared spontaneously. She suffered from viral respiratory infection at 18 years and 1 month, during which nighttime inspiratory stridor reappeared, and the levodopa dose was increased to 0.6 mg/kg/day. Both inspiratory stridor and dysphagia then resolved, and vocal cord adduction and abduction were completely normalized on LS (Fig. 1C). The respiration and vocal cord movements on LS remain normal at 20 years.

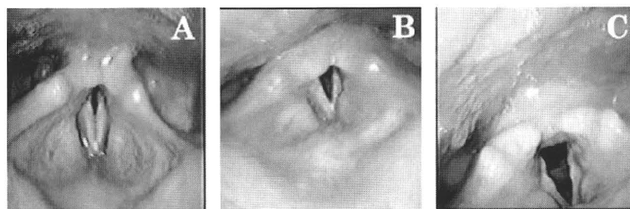

Fig. 1. Laryngoscopy findings during inspiratory phase in patient 1. (A) At 17 years of age, the glottis was fixed in the midline. (B) At 18 years of age, 3 months after the initiation of levodopa, the vocal cord showed restricted opening. (C) At 18 years of age, after the dose of levodopa was increased, the vocal cord showed normal opening.

Patient 2 was a male XPA patient aged 18 years and 10 months without genetic analysis. He developed rigidity in the extremities and hand tremors during wakefulness in his teens. A surface electromyogram was not performed. He first presented with inspiratory stridor after suffering respiratory infections at 12 years. Nighttime inspiratory stridor began when he was 13. Brain MRI was not performed due to severe dyspnea caused by the sedation. When he was 17, the patient developed paroxysmal choking during his meals and sleep, and awoke during his sleep due to dyspnea. On LS, vocal cord movements were restricted, the glottis tended to be fixed in the midline during inspiratory and expiratory phases, and there was mucosal swelling in the arytenoid processes. We diagnosed him with laryngeal dystonia, and he was treated with low-dose levodopa (0.3 mg/kg/day) beginning at 18 years and 2 months. Since neither inspiratory stridor during night nor the LS findings showed any changes, the dose of levodopa was increased to 0.6 mg/kg/day after 2 months. Inspiratory stridor at night, paroxysmal choking during his sleep, and hand tremors resolved, although choking during meals persisted. At 20, he is being treated with levodopa (1.5 mg/kg/day), and the dyspnea and abnormal movements of vocal cord on LS are gradually aggravating.

Patient 3 was an XPA male aged 13 years and 3 months with a homozygous splicing mutation in intron 3. He exhibited nighttime choking at 9 years. He developed mild rigidity in the lower extremities from the age of 10 years. At 12 years and 3 months, inspiratory stridor appeared occasionally during his meals, occurring suddenly in the absence of antecedent events. We diagnosed the patient with laryngeal dystonia, and he was treated with low-dose levodopa (0.5 mg/kg). Although he was able to walk independently before the initiation of levodopa, he began to frequently stagger and stumble. Levodopa was discontinued after 4 days at parental request. Limb weakness continued to aggravate, however, and he began to frequently drop objects and could not walk without support. He required a wheel chair for transfer and parental assistance during meals. It took 3 months for complete recovery of his motor abilities. Since he did not visit our hospital, the detailed neurological examination was not performed until the complete improvement of motor weakness. Neither brain MRI nor LS was performed.

3. Discussion

Laryngeal dystonia is caused by disturbed synergy of the laryngeal muscles during phonation or respiration [7]. Laryngeal electromyography is useful for the diagnosis of laryngeal dystonia, but was unable to be performed in our patients due to the risks and lack of consent. Botulinum toxin has been used for treatment of laryngeal dystonia in adults, while anticholinergics, benzodiazepines, dopamine-depleting agents, and baclo-

fen have also been evaluated, although their efficacy is uncertain.

Expression of tyrosine hydroxylase in the nigrostriatal input, intrastriatal connections, and striatal outputs was previously shown to be impaired in XPA autopsy cases [6], and is likely to be involved in laryngeal dystonia [3]. In Tourette's syndrome, functional neuroimaging studies have suggested DA receptor supersensitivity in the basal ganglia, while low-dose levodopa can alleviate DA receptor supersensitivity and decrease tics in some patients [8]. The DA receptor supersensitivity in the basal ganglia caused by neuroleptics and antipsychotics can lead to tardive dyskinesia [9], and patients with tardive dyskinesia have been reported to suffer from laryngeal dystonia [10]. Speculatively, low-dose levodopa may ameliorate laryngeal dystonia by means of alleviating DA receptor supersensitivity in the basal ganglia in XPA patients. However, the mechanism of how low-dose levodopa alleviated myoclonus and tremor remains unclear.

In our patients, the paroxysmal event-specific occurrence of inspiratory stridor and choking strongly indicated laryngeal dystonia. However, patient 2 may have also suffered from vocal cord paralysis [5], as indicated by insufficient response to treatment. Patients 1 and 2 responded to low-dose levodopa with resolution of paroxysmal choking and involuntary movements, without serious adverse effects. Patient 3 showed decreased limb muscle tone and was unable to tolerate medication. Speculatively, levodopa may have moderated his muscle tone increase due to DA supersensitivity, and unmasked muscle hypotonia caused by a peripheral nerve lesion and cerebellar atrophy. Since motor weakness just after the start of levodopa was transient in patient 1, muscle hypotonia may have improved after a longer-term of levodopa.

We suggest that low-dose levodopa can be effective for intractable neurological disorders in XP patients, and recommend the introduction of low-dose levodopa, under close observation, for paroxysmal respiratory disturbances and/or involuntary movements.

Conflicts of interest

There are no conflicts of interest to declare.

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DNA 修復異常症における DNA 損傷応答の違いによる脳神経病変への関与 —ATLD, NBS, XPA の神経病変

中根裕信[1]、Erin R.P. Shull[2]、Youngsoo Lee[2]、栗政明弘[3]、稲賀すみれ[1]、名黒知徳[1]、田中亀代次[4]、Peter J. McKinnon[2]

[1]鳥取大学　医学部ゲノム形態学分野、[2] Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, Tennessee　38105, USA、[3]鳥取大学　大学院医学系研究科 機能再生医科学専攻 遺伝子医療学、[4]大阪大学　大学院生命機能研究科　個体機能学講座　細胞機能学研究室

DNA 二重鎖切断に対する細胞の DNA 損傷応答に重要な ATM(ataxia telangiectasia, mutated)と MRN 複合体(Mre11/RAD50/NBS1)は、ATM の異常で毛細血管拡張性運動失調症(AT)、Mre11 の異常で AT バリアント (ATLD)あるいは NBS1 の異常でナイミーヘン症候群(NBS)の原因となる。AT, ATLD は神経細胞の変性を、NBS は小頭症を神経病変の特徴として示す。この病変の違いを理解するため、hypomorphic mutation をもつ生後 5 日令 $Mre11^{ATLD1/ATLD1}$ マウスと $Nbs1^{\Delta B/\Delta B}$ マウスに X 線を照射したところ、$Mre11^{ATLD1/ATLD1}$ マウス小脳は DNA 損傷によるアポトーシスが殆ど生じず抵抗性を示した。その抵抗性は ATM 活性化の欠如と Chk2・p53 がリン酸化されないためであり、$Mre11^{ATLD1/ATLD1}$｜DNA ligase IV(Lig4)(-/-) 2 重欠損胎児マウスからも明らかになった。一方、$Nbs1^{\Delta B/\Delta B}$ マウス小脳では、ATM の活性化の欠如にかかわらずアポトーシスは対照と同様に生じた。成獣脳の Lig4 をコンディショナルに破壊した $Lig4^{Nes\text{-}Cre}$ マウスは、生きて重篤な小頭を示し、加齢した脳に DNA 損傷が蓄積した。$Lig4^{Nes\text{-}Cre}$ の小頭は、Atm(-/-)及び $Mre11^{ATLD1/ATLD1}$ の遺伝的背景で改善されたが $Nbs1^{\Delta B/\Delta B}$ では変わらなかった。ATLD と NBS の脳神経の DNA 損傷シグナルへの応答の違いが細胞死に影響し、各神経病変の違いとなる可能性を示した(G&D,23:171,2009)。

他の DNA 修復異常症として、ヌクレオチド除去修復(NER)に異常をもつ A 群色素性乾皮症(XP)は皮膚癌に加え小頭症、精神遅滞、小脳失調等の神経症状を示す。この遺伝子を欠く Xpa マウスは、紫外線等で皮膚癌を生じるが神経症状はない。近年、X 線で生じる酸化的 DNA 損傷サイクロプリンが NER 特異的に修復されることが報告された。生後 5 日令 Xpa マウスに X 線照射すると、対照に比べ小脳の神経細胞死が増加していた。現在、神経細胞の DNA 損傷応答について解析中である。DNA 修復異常症における DNA 損傷応答の違いによる脳神経病変への関与を解析することで、新たな神経変性の分子病態が明らかになることが期待される。

key word: A-T-like disease (ATLD)、Nijmegen breakage syndrome (NBS)、DNA damage signaling、Apoptosis、Microcephaly

Differential DNA damage signaling accounts for distinct neural apoptotic responses in DNA repair deficient mice of ATLD, NBS, XPA, XPG

- Insight from neuropathology of DNA repair-deficiency disorders -

Hironobu Nakane

Division of Genome Morphology, Department of Functional, Morphological and Regulatory Science, Faculty of Medicine, Tottori University, Nishi-cho 86, Yonago, Tottori 683-8503, Japan

The MRN complex (Mre11/RAD50/NBS1) and ATM (ataxia telangiectasia, mutated) are important for the cellular response to DNA double strand breaks(DSB). ATM defect causes ataxia telangiectasia (A-T), while MRN dysfunction can lead to A-T-like disease (ATLD) or Nijmegen breakage syndrome (NBS). Neuropathology is a manifestation of these diseases, whereby neurodegeneration occurs in A-T and ATLD while microcephaly characterizes NBS. To understand the contrasting neuropathology resulting from Mre11 or Nbs1 hypomorphic mutations, we analyzed neural tissue from $Mre11^{ATLD1/ATLD1}$ and $Nbs1^{\Delta B/\Delta B}$ mice after genotoxic stress. We found a pronounced resistance to DNA damage-induced apoptosis after ionizing radiation or DNA ligase IV (*Lig4*) loss in the $Mre11^{ATLD1/ATLD1}$ nervous system that was associated with defective Atm activation and phosphorylation of its substrates Chk2 and p53. Conversely, DNA damage-induced Atm phosphorylation was defective in $Nbs1^{\Delta B/\Delta B}$ neural tissue, although apoptosis occurred normally. We also conditionally disrupted Lig4 throughout the nervous system using Nestin-cre ($Lig4^{Nes\text{-}Cre}$), and while viable, these mice showed remarkable microcephaly and a prominent age-dependent accumulation of DNA damage throughout the brain. Either $Atm^{-/-}$ or $Mre11^{ATLD1/ATLD1}$ genetic backgrounds, but not $Nbs1^{\Delta B/\Delta B}$, rescued $Lig4^{Nes\text{-}Cre}$ microcephaly. Thus, DNA damage signaling in the nervous system is different between ATLD and NBS and probably explains their respective neuropathology [1].

Xeroderma pigmentosum (XP) is an autosomal recessive disorder characterized by a high frequency of skin cancer on sun-exposed areas and consists of seven genetic complementation groups (groups A-G). XP patients of some groups exhibit neurological complications. XP has a defect in the early step of nucleotide excision repair (NER) which functions to excise bulky lesions, UV-induced photoproducts, from DNA. In the NER, XPA is

involved in the sensing of DNA damage through interaction with other NER proteins. XP-A patients exhibit progressive neurological symptoms such as microcephaly, mental retardation, cerebellar ataxia, sensorineuronal deafness, and peripheral neuropathy. However, neither the normal function of XPA in the nervous system nor the biological basis of the neurodegeneration in XP is known. In *Xpa*-deficient mice [*Xpa* (-/-) mice], susceptibility to UV- and chemical carcinogen-induced skin cancer was greatly increased with no neurological defects [2,3]. It was reported that 8,5'-(S)-cyclo-2'-deoxyadenosine, unique oxidative DNA damage that was formed spontaneously and can be formed by ionizing radiation (IR), is repaired by NER [4]. Therefore, such oxidative DNA damage may be responsible for the neurological abnormalities in XP-A patients.

We investigated the effect of IR on the developing nervous system in *Xpa* (-/-) mice. More apoptotic neurons in the developing cerebellum of *Xpa* (-/-) mice were observed than that of control mice at 4hr after IR. These results suggest that developing cerebellar neurons of *Xpa* (-/-) mice had a hypersensitivity to oxidative DNA damage. XPA may function to protect proliferating neurons from oxidative DNA damage during cerebellar development.

Cockayne syndrome (CS) is a disease characteristic of a photosensitivity, severe growth failure with microcepahly, a limited life span, and progressive neurological dysfunction without cancer predisposition. Most CS patients belong to either group CS-A or CS-B with mutations in the *CSA* or *CSB* gene, which is required for the preferential removal of UV-induced lesions in the transcribed strands of genes by the transcription-coupled repair(TCR) process in NER. Rare CS patients in three complementation groups of XP(XP-B, XP-D, XP-G) also show characteristic features of CS, the so-called XP-CS. XPB and XPD are subunits of TFIIH, which is a multifunctional complex associated with basal transcription, NER, and the cell cycle. XPG is a structure-specific DNA endonuclease that functions in NER and forms a stable protein complex with TFIIH in the regulation of gene expression. The *Xpg* null mice exhibited postnatal growth failure, neurological abnormalities with microcephaly, life-shortening. These are similar to clinical symptoms of the CS, XP-G/CS patients [5,6]. In addition, deletion of *Csb* and deletion of exon 15 of *Xpg*(*Xpg del.ex15*) had a mild or no effect on mice like *Xpa* (-/-) mice. However, combining these mutations with deletion of *Xpa* (*Xpa* | *Csb* , *Xpa* | *Xpg del.ex15*) led to severe phenotypes like *Xpg* null mice [6]. Recently, it was reported that XPD protein complex containing MMS19 was involved in chromosome segregation. This function was independent of TFIIH, although XPD was a subunit of TFIIH. The frequency of abnormal cell division and abnormal cell nuclei was

increased in XP-D/CS and XP-D cells [7]. These findings may help us to understand the pathology of microcephaly not only in *Xpa* (-/-) mice and *Xpg* null mice but in XP-A and CS patients. We need to do further analysis of microcephaly in the *Xpa* (-/-) mice and *Xpg* null mice.

In conclusion, our data show that the disease-causing mutations in MRN lead to defective ATM-dependent DNA damage-induced signal transduction in the nervous system, whereby the relative activation of apoptosis accounts for the differential neuropathology of these syndromes. Thus DNA repair deficient mice such as DNA DSB repair, NER provide a valuable animal model to study DNA damage signaling pathways involved in microcephaly during brain development.

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Retrovirus-mediated transduction of a short hairpin RNA gene for *GRP78* fails to downregulate GRP78 expression but leads to cisplatin sensitization in HeLa cells

TOSHIKAZU SUZUKI[1,2], JUN LU[1], GAOFENG HU[1], KAZUKO KITA[1] and NOBUO SUZUKI[1]

[1]Department of Environmental Biochemistry, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670; [2]Department of Health and Nutrition, Wayo Women's University, 2-3-1 Konodai, Ichikawa, Chiba 272-8533, Japan

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Abstract. Glucose-regulated protein 78 (GRP78) is expressed abundantly in various types of cancer cells and is believed to contribute to chemotherapeutic resistance. In this study, we investigated the effect of a continuous approach for the expression of a short hairpin RNA (shRNA) targeted to *GRP78* with retrovirus transduction on the sensitivity to the anticancer drugs VP-16 and cisplatin. The reduction of GRP78 expression failed, and the expression of *GRP94* and *P5* chaperon mRNA increased; this increase was associated with a mild activation of the unfolded protein response in HeLa cells, which were stably transduced with *GRP78* shRNA gene. The transduced cells exhibited similar sensitivity to VP-16-induced cell death when compared to control *GFP* shRNA gene-transduced cells. However, sensitivity to cisplatin-induced cell death was higher in *GRP78* shRNA gene-transduced cells compared to control cells. These results demonstrate that the continuous or prolonged approach targeting GRP78 confers sensitization of HeLa cells to cisplatin independently of the down-regulation of GRP78 expression. The role of the unfolded protein response in sensitization to cisplatin is discussed.

Introduction

Cancer cells in poorly vascularized solid tumors are often subjected to stressful microenvironments, including hypoxia, low pH, and the deprivation of glucose and other nutrients (1). These microenvironments disrupt protein folding in the endoplasmic reticulum (ER) (2,3). The accumulation of unfolded proteins in the ER activates the unfolded protein response (UPR) pathways and enhances the expression of ER chaperone proteins, including glucose-regulated protein (GRP) 78 and GRP94 (4-6). There are a number of studies stating that overexpression of GRP78 and GRP94 frequently occurs in various types of cancer, including prostate, breast, lung, esophageal, gastric, colon, brain and liver cancers (7-14).

GRP78 associates with nascent proteins, facilitates protein folding, prevents intermediates from aggregating, and targets misfolded proteins for proteasome degradation (15). GRP94 is thought to facilitate the assembly or oligomerization of folding intermediates in the ER (16). Both GRP78 and GRP94 also bind Ca^{2+}, help to immobilize Ca^{2+}, and maintain ER calcium homeostasis (4). With these properties, induction of GRP78 and GRP94 is involved in the protection of tumor cells from death under stressful microenvironmental conditions. Furthermore, overexpression of GRP78 and 94 may confer chemotherapeutic resistance to solid tumors (5,17). Knockdown of GRP78 or GRP94 expression has been shown to sensitize cancer cells to chemotherapeutic agents and ionizing radiation (13,18-23). Additionally, tumor cells derived from *GRP78* heterozygous mice grew more slowly and showed enhanced apoptosis compared with tumor cells from *GRP78* wild-type mice (24). Therefore, targeting GRP78/GRP94 expression or activity may be a promising anticancer strategy.

There is a possibility that the reduction of GRP78 expression increases the amount of misfolded or aggregated proteins, activates the UPR signaling pathway and enhances the expression of UPR-induced genes, including ER chaperone proteins, because GRP78 itself serves as a negative modulator of the UPR signaling pathways by binding to three ER stress sensor proteins: activating transcription factor 6, inositol-requiring 1 protein (Ire1p) and protein kinase RNA-activated-like ER kinase (PERK) (25).

We previously observed enhanced expression of *GRP94*, ER-localized DnaJ homologue 4 *(ERdj4)* and *P5* chaperone mRNA, as well as activation of the *GRP78* promoter, when the expression of GRP78 was suppressed by a small interfering RNA (siRNA) in HeLa cells (26). This finding suggests that the approach of prolonging suppression of GRP78 expression

Correspondence to: Dr Toshikazu Suzuki, Department of Health and Nutrition, Wayo Women's University, 2-3-1 Konodai, Ichikawa, Chiba 272-8533, Japan
E-mail: t-suzuki@wayo.ac.jp

Dr Nobuo Suzuki, Department of Environmental Biochemistry, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
E-mail: nobuo@faculty.chiba-u.jp

Key words: glucose-regulated protein 78, short hairpin RNA, the unfolded protein response, cisplatin sensitivity

may fail to sensitize cells to anticancer drugs. On the contrary, it might confer chemotherapeutic resistance. To search for the possibility that such unintended consequences might occur under *GRP78*-targeted anticancer therapy, we used a retrovirus vector containing an shRNA-expressing gene targeted to *GRP78*. Transduction of an shRNA gene enabled us to suppress the expression of the target gene specifically and stably (27,28). Retrovirus-mediated transduction is widely used to introduce genetic material efficiently and stably into the genome of any dividing cell type (29). HeLa cells were infected with the *GRP78* shRNA vector and the stably transduced cells were selected. We then analyzed the expression of the UPR-activated genes, including *GRP78* and *GRP94*, and sensitivity to two representative anticancer drugs, VP-16 and cisplatin, in the stable *GRP78* shRNA gene-transduced cells.

Materials and methods

Plasmid construction and siRNA. The retrovirus vector pSUPER.retro (27), containing the H1-RNA promoter, was obtained from OligoEngine (Seattle, WA, USA). The pSUPER.retro vector was digested with *Bgl*II and *Hin*dIII and the following pairs of annealed oligos were ligated into the vector to create pSUPER.retro-shGRP78, pSUPER.retro-shGRP94 and pSUPER.retro-shGFP, respectively: 5'-gat ccc ccA GTG TTG GAA GAT TCT GAT ttc aag aga ATC AGA ATC TTC CAA CAC Ttt ttt gga aa-3' and 5'-agc ttt tcc aaa aaA GTG TTG GAA GAT TCT GAT TCT GAT Ttc tct tga aAT CAG AAT CTT CCA ACA CTg gg-3'; 5'-gat ccc ccG AAG AAG CAT CTG ATT ACC ttc aag aga GGT AAT CAG ATG CTT CTT Ctt ttt gga aa-3' and 5'-agc ttt tcc aaa aaG AAG AAG CAT CTG ATT ACC tct ctt gaa GGT AAT CAG ATG CTT CTT Cgg g-3'; and 5'-gat ccc ccG CAA GCT GAC CCT GAA GTT Ctt caa gag aGA ACT TCA GGG TCA GCT TGC ttt ttg gaa a-3' and 5'-agc ttt tcc aaa aaG CAA GCT GAC CCT GAA GTT Ctc tct tga aGA ACT TCA GGG TCA GCT TGC ggg-3'. The 19 nt *GRP78* and *GRP94* target sequences and the 20 nt *GFP* target sequences are indicated in capitals in the oligonucleotide sequences. The target sequences for *GRP78* and *GRP94* were reported previously (26). The predicted sequences of the short hairpin transcripts of *GRP78* shRNA, *GRP94* shRNA and *GFP* shRNA are shown in Fig. 1A.

Adenovirus vector constructs were generated by excising shRNA-expressing cassette fragments from the retrovirus vectors with *Eco*RI and *Hin*dIII and blunt-ended with Klenow enzyme. These fragments were ligated into the *Bam*HI- and *Xba*I-digested, and blunt-ended pENTR-U6 entry vector (Invitrogen, Carlsbad, CA, USA), yielding pENTR-shGRP78, pENTR-shGRP94 and pENTR-shGFP. Each shRNA-expressing cassette sequence in the pENTR vectors was transferred to an adenovirus vector, pAd/BLOCK-iT™ -DEST, using Gateway® LR Clonase™ Enzyme mix (Invitrogen), yielding pAd/shGRP78, pAd/shGRP94 and pAd/shGFP.

Cell culture, transfection and viral infection. The cervical tumor cell line, HeLa S3, was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The packaging cells for adenovirus production, 293A, were purchased from Invitrogen. Phoenix-Ampho packaging cells for retrovirus production (ATCC catalogue number SD 3443) were obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa cells were cultured in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical, Tokyo, Japan) containing 10% (v/v) calf serum (Invitrogen). Phoenix and 293A packaging cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical) supplemented with 0.1 mM MEM non-essential amino acids (Invitrogen), 60 μg/ml kanamycin (Sigma, St. Louis, MO, USA) and 10% (v/v) fetal bovine serum (Roche Diagnostics, Mannheim, Germany). All cell lines were grown at 37˚C in a humidified atmosphere containing 5% CO_2 and subcultured when confluence was reached.

Amphotropic retroviral supernatants were produced by transfection of each of the pSUPER.retro shRNA-expressing vectors into Phoenix-Ampho packaging cells using Lipofectamine™ 2000 reagent (Invitrogen). At 24 h post-transfection, the culture medium was refreshed, and the cells were incubated for a further 24 h. The culture medium was then filtered through a 0.45 μm filter, and each viral supernatant (Ret/shGRP78, Ret/shGRP94 and Ret/GFP) was used for infection of cells with polybrene solution (4 mg/ml). HeLa cells were infected for 5 h and allowed to recover for 48 h in fresh medium. The infected cells were cultured in the presence of 0.8 μg/ml puromycin for 8 days to remove uninfected cells. The stably infected cells were then used in experiments.

High titer adenoviral stocks ranging from 2-5x10^8 pfu/ml were produced by transfection of each of the pAd/shRNA-expressing vectors into 293A packaging cells according to the manufacturer's instructions for the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen). HeLa cells were infected with the each of the adenoviral stocks (Ad/shGRP78, Ad/shGRP94 and Ad/shGFP) at an MOI of 50 for 24 h. The infected cells were replaced with fresh medium, cultured for a further 24 h and used for Western blot analysis.

Western blot analysis. Western blot analysis was carried out as described previously (26). Whole cell protein samples were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Detection of GRP78, GRP94, the cleaved form of caspase-3 and ß-actin proteins was performed with mouse anti-GRP78 (Assay Design, Ann Arbor, MI, USA), rat anti-GRP94 (Assay Design), rabbit anti-cleaved caspase-3 (Trevigen, Gaithersburg, MD, USA) and mouse anti-ß-actin (MP Biomedicals Inc., Aurora, OH, USA) monoclonal antibodies, respectively. The antigen-antibody complexes were detected by horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, Buckinghamshire, UK), anti-rabbit IgG (GE Healthcare) or anti-rat IgG (Assay Design) and visualized using the ECL system (GE Healthcare) according to the manufacturer's instructions.

RT-PCR. Isolation of total RNA, first-strand cDNA synthesis and RT-PCR analysis was carried out as described previously (26). The following sets of primers were used: 5'-GTT CTT CAA TGG CAA GGA ACC ATC-3' and 5'-CCA TCC TTT

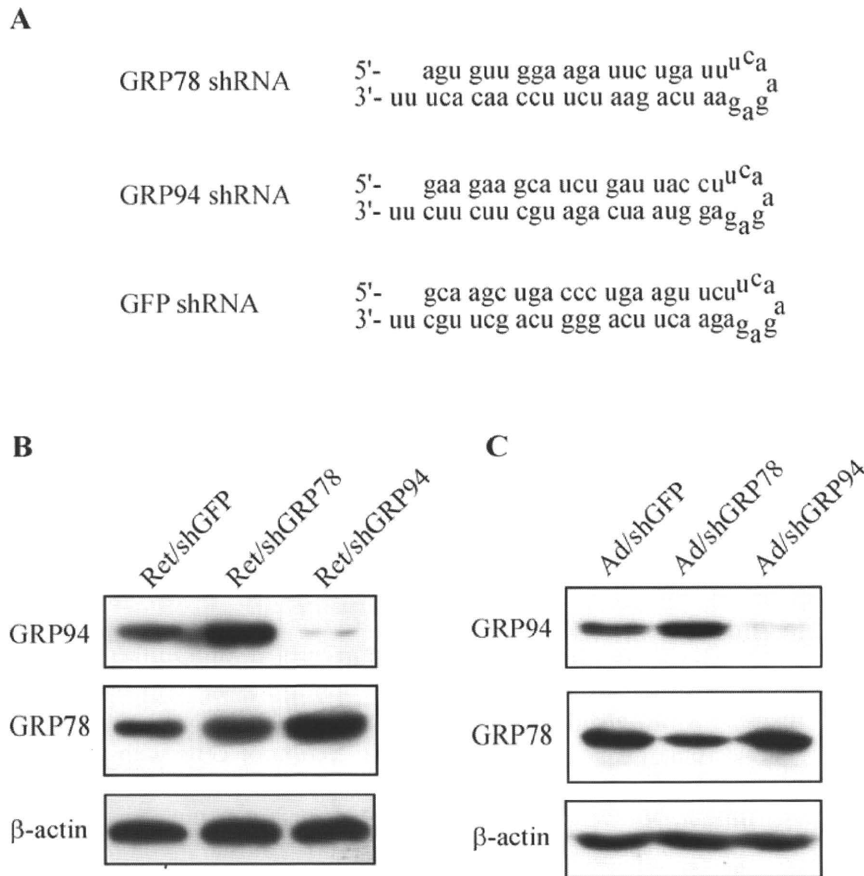

Figure 1. Effect of *GRP78* shRNA gene transduction on GRP78 and GRP94 protein levels in HeLa cells. (A) Sequences of the predicted shRNA specific to *GRP78*, *GRP94* and *GFP* (control) mRNA. (B) Stable transduction of the *GRP78* shRNA gene by a retrovirus vector failed to suppress the expression of GRP78 protein but increased the expression of the GRP94 protein. Cell lysates prepared from the surviving population of the transduced cells were analyzed by Western blot analysis. The amount of actin was used as a loading control. Ret/shGFP, Ret/shGRP78, and Ret/shGRP94 represent the retrovirus vector-transduced cells, respectively. (C) Transient expression of *GRP78* shRNA with an adenovirus vector suppressed the expression of GRP78 protein and increased the expression of GRP94. Cell lysates prepared 2 days after infection with adenovirus were analyzed by Western blot analysis. Ad/shGFP, Ad/shGRP78, and Ad/ shGRP94 represent the adenovirus vector-infected cells, respectively.

CGA TTT CTT CAG GTG-3' for *GRP78*; 5'-AAG AAC CTG CTG CAT GTC ACA GAC-3' and 5'-ATG GGC TCC TCA ACA GTT TCA GTC-3' for *GRP94*; 5'-TTC CAG ACT GAT CCA ACT GCA GAG-3' and 5'-AGC TGT GCC ACT TTC CTT TCA TTC-3' for *CHOP*; 5'-TCG GAG GGT GCA GGA TAT TAG AAA-3' and 5'-GGA TCC AGT GTT TTG GTT TTG ACC-3' for *ERdj4*; 5'-GAA AGG CGA GTC TCC TGT GGA TTA-3' and 5'-AGA GCT CCC TGA GAA ACT CGT TGA-3' for *P5*; 5'-GAC CTC AAC TAC ATG GTC TAC ATG-3' and 5'-TGT CGC TGT TGA AGT CAG AGG AGA C-3' for glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*; and 5'-CCT TGT AGT TGA GAA CCA GG-3' and 5'-GGG GCT TGG TAT ATA TGT GG-3' for X-box binding protein 1 *(XBP1)*. The PCR products were visualized after electrophoresis on 1.5% (w/v) agarose gels by ethidium bromide staining. To detect the spliced form of *XBP1* cDNA, samples were electrophoresed on 4% polyacrylamide gels to separate the spliced form from the unspliced form.

Determination of drug sensitivity. Cell viability after treatment with VP-16 or cisplatin was determined by a colony-formation assay. Cells were seeded onto 60-mm dishes at a density of 200-300 cells per dish. The next day, VP-16 (0-40 μM) or cisplatin (0-20 μM) were added to the cell cultures. One hour after incubation, drug-containing media were replaced with fresh MEM containing 10% calf serum, and the cells were cultured for 2 weeks to form colonies. Colonies comprising >50 cells were counted after staining with 0.1% (w/v) methylene blue tetrahydrate. The survival fraction of each drug concentration was calculated as the ratio of colony numbers in the drug-treated samples to those in mock-treated samples.

Results

Expression of GRP78 is not suppressed in GRP78 shRNA gene-transduced HeLa cells. We isolated HeLa cells transduced with the *GRP78* shRNA gene by retrovirus infection with Ret/shGRP78. We also isolated *GRP94* and *GFP* shRNA gene-transduced HeLa cells by infection with Ret/shGRP94 and Ret/GFP, respectively, for comparison. After removal of the uninfected and infected but inactivated fraction of cells by puromycin selection, the presence of retroviral DNA within the cellular genome was confirmed by PCR (data not shown). Total cell lysates were prepared from polyclonal pools of the transduced cells, and the expression of GRP78 protein was determined by Western blot analysis

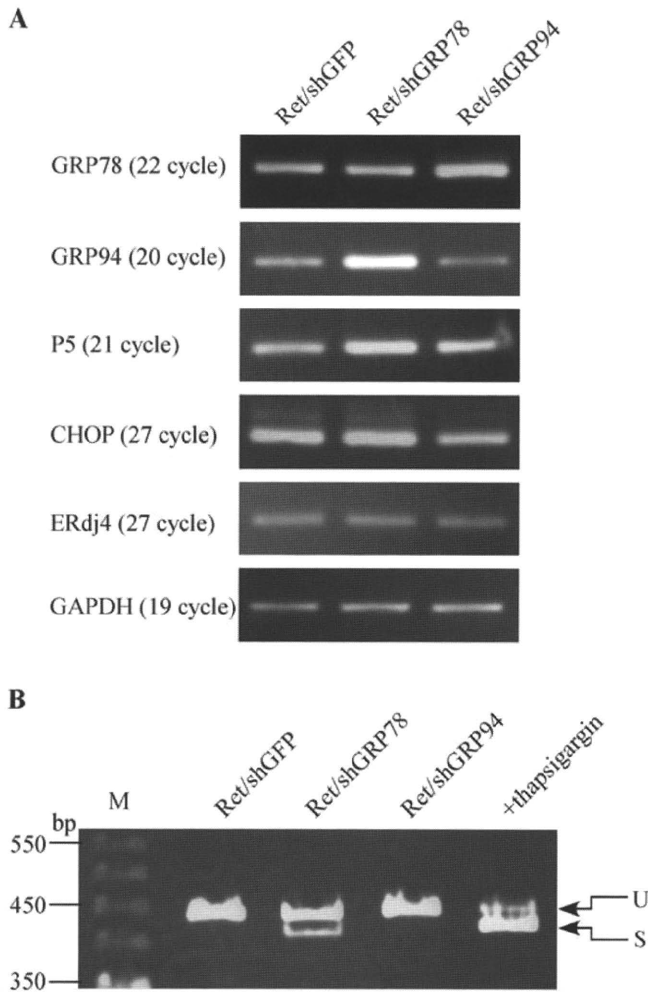

Figure 2. mRNA expression of *GRP78, GRP94* and UPR-induced genes in Ret/shGRP78-, Ret/shGRP94- and control Ret/shGFP-transduced HeLa cells. (A) The mRNA levels of *GRP78, GRP94, P5, CHOP* and *ERdj4* were analyzed by RT-PCR. The PCR cycles indicated in brackets were predetermined to allow semiquantitative comparisons among cDNAs developed from identical reverse transcriptase reactions. The reference gene, GAPDH, was amplified as an internal control. (B) Stable expression of *GRP78* shRNA partially activates the Ire1p-XBP1 pathway. RT-PCR analysis of *XBP1* mRNA was performed as previously described. The PCR products were separated on a 4% polyacrylamide gel and visualized by ethidium bromide staining. The positions of the unspliced (442 bp) and spliced (416 bp) products are shown as U and S, respectively. RNA samples isolated from thapsigargin-treated (1 μM for 8 h) cells were used as a positive control for activation of the Ire1p-XBP1 pathway.

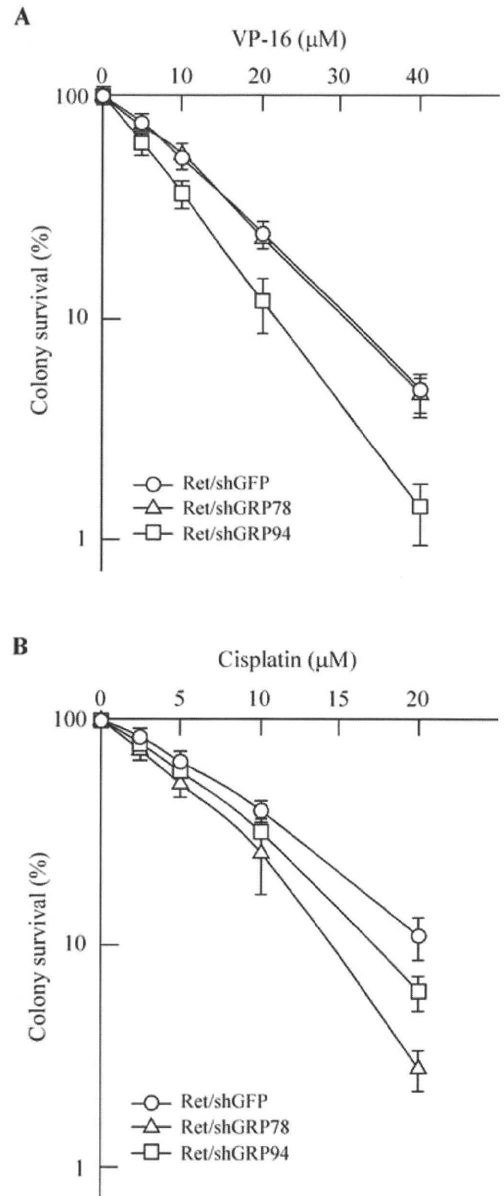

Figure 3. Comparison of sensitivity to VP-16 (A) and cisplatin (B) in Ret/shGRP78-, Ret/shGRP94- and control Ret/shGFP-transduced HeLa cells by a colony survival assay. Cells were exposed to VP-16 or cisplatin for 1 h at the different concentrations indicated. Values represent the percentage of colony numbers relative to those of mock-treated cells. Values are the mean ± SD of three independent experiments.

(Fig. 1B). Contrary to our expectations, the amount of GRP78 protein was not reduced, but rather appeared to have slightly increased in Ret/shGRP78-transduced cells. Additionally, the amount of GRP94 had increased in the Ret/shGRP78-transduced cells. On the other hand, a marked knockdown of GRP94 protein was observed in Ret/shGRP94-transduced cells, although the expression of the GRP78 protein appeared to have increased.

To determine whether the *GRP78* shRNA can inhibit the expression of the target protein, the gene cassette was inserted into an adenovirus vector and used for transient expression of *GRP78* shRNA. Adenovirus vectors containing *GRP94* or the *GFP* shRNA gene were also used as controls. HeLa cells express high levels of coxsackie-adenovirus-receptor and showed highly-efficient adenovirus-vector-mediated transient transgene expression (30,31). Two days after infection with each shRNA-expressing adenovirus vector at a MOI of 50, the expression of the targeted protein had decreased in both Ad/shGRP78- and Ad/shGRP94-infected cells, as expected (Fig. 1C). This result demonstrates that the *GRP78* shRNA designed by us can actually inhibit expression of the target protein when transiently introduced. The expression of the GRP94 protein also increased with Ad/shGRP78 infection, similar to when the cells were transfected with *GRP78* siRNA (26). These results suggest that stable transduction of the *GRP78* shRNA gene with the retrovirus vector is not capable of suppressing GRP78

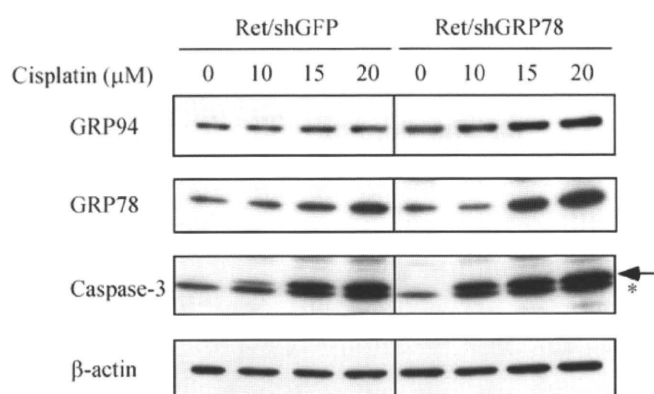

Figure 4. Activation of caspase-3 by cisplatin is enhanced in Ret/shGRP78-transduced HeLa cells. Cells were exposed to cisplatin for 1 h at the different concentrations indicated, cultured for a further 23 h in normal culture media, and then analyzed by immunoblotting using anti-GRP94, anti-GRP78, anti-cleaved form of caspase-3 and anti-actin antibodies. The amount of actin was used as a loading control. Bands representing the cleaved form of caspase-3 are indicated by an arrow. The asterisk indicates non-specific bands.

expression in HeLa cells, even if the gene can suppress the expression of the target protein when it is transiently introduced.

Expression of UPR-induced genes increases in GRP78 shRNA gene-transduced HeLa cells. One possible explanation for the lack of suppression of GRP78 in Ret/shGRP78-transduced cells may be the activation of the UPR, as upregulation of UPR-induced genes and activation of UPR-dependent transcription was observed when HeLa cells were transfected with a *GRP78* siRNA (26). Next, we analyzed the expression of three representative UPR-induced mRNAs, in addition to *GRP78* and *GRP94*, by RT-PCR analysis in Ret/shGRP78-transduced cells (Fig. 2A). The levels of *GRP94* and *P5* mRNA increased more than two-fold, whereas the levels of *CHOP* and *ERdj4* mRNA did not increase significantly. Reduction of *GRP78* mRNA expression was not observed in Ret/shGRP78-transduced cells. The mRNA levels of *P5, CHOP* and *ERdj4* did not increase in Ret/shGRP94-transduced cells.

We previously reported that the spliced form of *XBP1* mRNA, which increased after Ire1p activation and produces the active form of the XBP1 protein necessary for the induction of UPR-induced genes, increased in *GRP78* siRNA-transfected HeLa cells (26). To ascertain that this pathway is also activated in Ret/shGRP78-transduced cells, we analyzed the amount of the spliced form of *XBP1* mRNA by RT-PCR (Fig. 2B). As a positive control for UPR activation, thapsigargin-treated HeLa cells were also used. The amount of spliced mRNA (Fig. 2B) increased in Ret/shGRP78-transduced cells, although the extent of the increase was smaller than in thapsigargin-treated cells.

These results suggest that the UPR is somewhat activated and that UPR-induced genes, including GRP78 itself, are moderately upregulated in Ret/shGRP78-transduced cells. It should be noted that the increased expression of *GRP78* mRNA, as well as GRP78 protein, was observed in Ret/shGRP94-transduced cells (Fig. 1B and 2A). This increase by the suppression of GRP94 may occur independently of UPR activation, as expression of other UPR-induced genes was not upregulated and the spliced form of *XBP1* mRNA did not increase in the Ret/shGRP94-transduced cells.

Sensitization to cisplatin by transduction of the GRP78 shRNA gene without suppression of GRP78 expression. Prolonged *GRP78* shRNA expression could not maintain GRP78 knockdown because the levels of GRP78 protein in Ret/shGRP78-transduced cells was similar to that in control Ret/shGFP-transduced cells. It is possible that continuous treatment for suppression of GRP78 function may activate the UPR and result in an adverse overexpression of GRP78, allowing the treated cancer cells to survive and maintain ER-homeostasis. Such an adaptation to suppression might also contribute to the resistance of cells to chemotherapy and radiotherapy.

To determine the effect of UPR activation on Ret/shGRP78-transduced cells and their sensitivity to anticancer drugs, we treated the cells with various concentrations of VP-16 and cisplatin, which are clinically and widely used as anticancer drugs and whose sensitivities were reported to depend on the expression levels of GRP78 and GRP94 (13,19,22,23). Sensitivity to VP-16 was similar between Ret/shGRP78- and control Ret/shGFP-transduced cells (Fig. 3A). However, surprisingly, sensitivity to cisplatin was higher in Ret/shGRP78-transduced cells than in Ret/shGFP-transduced cells regardless of similar GRP78 protein levels in both cells (Fig. 3B). Colony survival data for Ret/shGRP94-transduced cells confirmed that knockdown of GRP94 increased sensitivity to VP-16 as previously reported (Fig. 3A) (18). Knockdown of GRP94 also sensitized HeLa cells to cisplatin although to a lesser extent than with *GRP78* shRNA gene transduction (Fig. 3B).

To further confirm cisplatin sensitization by *GRP78* shRNA expression without GRP78 knockdown, the amount of the active form of caspase-3 was analyzed by Western blot analysis (Fig. 4). Compared with Ret/shGFP-transduced cells, the levels of active caspase-3 were higher in Ret/shGRP78-transduced cells when treated with various concentrations of cisplatin for 24 h. In addition, induction of both GRP78 and GRP94 by cisplatin was higher in Ret/shGRP78-transduced cells than in control Ret/shGFP cells, implying increased sensitivity to cisplatin in the Ret/shGRP78-transduced cells. These results suggest that *GRP78* shRNA expression sensitized HeLa cells to cisplatin not via suppression of GRP78 but through mild activation of the UPR.

Discussion

A number of studies have reported that the downregulation of GRP78 expression with siRNA or antisense DNA sensitized cancer cells to chemotherapeutic agents, including VP-16 and cisplatin (13,19,22,23). The reports suggested that GRP78 itself contributed to the resistance to those agents and could be a potential target for enhancing chemosensitivity of cancer cells. However, we previously reported that knockdown of GRP78 activated the UPR (26). Since the UPR is involved not only in apoptosis but also cell survival (6), the possibility that GRP78-targeted anticancer therapies might

induce unfavorable events cannot be excluded, especially if the treatment is performed continuously or repeatedly.

In this study, we stably transduced HeLa cells with a *GRP78* shRNA-expressing gene and found that this continuous approach for GRP78 knockdown was not effective, even if the same *GRP78* shRNA gene can actually reduce the expression of GRP78 when transiently transduced with an adenovirus vector system (Fig. 1). Moreover, the expression of *GRP94* and *P5* mRNAs increased and was accompanied by UPR activation in the Ret/shGRP78-transduced cells (Fig. 2). The reason why the expression of GRP78 did not decrease in the transduced cells is unclear. One possible explanation is that only the population of cells that were adapted for cell growth remained after puromycin selection, while the cells with suppressed GRP78 expression died due to strong UPR activation. This outcome is similar to what is observed in cells transfected with *GRP78* siRNA (26). If so, the approach for GRP78 knockdown or inactivation should not be used continuously or repeatedly because it may no longer sensitize the cells to anticancer drugs. Indeed, sensitivity to VP-16-induced cell death in Ret/shGRP78-transduced cells was similar to that in control Ret/shGFP-transduced cells (Fig. 3A).

Nevertheless, sensitivity to cisplatin was still higher in Ret/shGRP78-transduced cells when compared with control Ret/shGFP-transduced cells (Figs. 3B and 4). This could be because suppression of GRP78 expression is not involved in the sensitization to cisplatin by shRNA expression or siRNA treatment targeted to *GRP78*, but activation of the UPR is involved. Disruption of GRP78 metabolism by the expression of *GRP78* shRNA may be involved in the activation of the UPR in Ret/shGRP78-transduced cells, although the apparent protein levels of GRP78 in the transduced cells were similar to those in control Ret/shGFP-transduced cells. This idea is further supported by several studies that reported higher sensitivity to cisplatin-induced cell death following upregulation of GRP78 with 2-deoxyglucose or 6-amino-nicotineamide treatment in human colon cancer cells, human ovarian cancer cells and Chinese hamster V79 cells (32-34). In contrast, induction of ER chaperone proteins, including GRP78, by treatment with 2-deoxyglucose or A23817 conferred resistance of V79 and SK-MES-1 human lung cancer cells to doxorubicin, vincristine and VP-16 (35-37).

In addition, celecoxib, a non-steroidal anti-inflammatory drug that activates the UPR, potentiated the anticancer effect of cisplatin in vulvar cancer cells independently of cyclo-oxygenase (38,39). Considering all of the various published studies, the possibility that the continuous but mild activation of the UPR by the expression of *GRP78* shRNA can sensitize cancer cells to cisplatin is conceivable. Cisplatin has the unique ability to activate apoptosis via both DNA damage-dependent and -independent pathways, and the ER is likely its non-nuclear target (40). Induction of apoptosis by VP-16 requires nuclear events; cisplatin induced the activation of caspase-3 in enucleated cells (cytoplasts), but VP-16 did not (40). Therefore, the ability to activate two pathways for apoptosis may be involved in sensitization to cisplatin when the UPR is activated.

In summary, retroviral-mediated stable transduction of a *GRP78* shRNA gene failed to suppress the expression of GRP78 in HeLa cells. Upregulation of *GRP94* and *P5* mRNAs with a mild activation of UPR was observed in the transduced cells. However, sensitivity to cisplatin-induced cell death was higher in Ret/shGRP78-transduced cells than in control cells, suggesting that the activation of UPR is involved in sensitization. These results may offer a new chemotherapeutic approach using cisplatin.

Acknowledgements

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小児の発達症候学

ヒトの随意運動の発達

久保田雅也

随意運動の諸相

- 随意運動とはその名のごとくわれわれが意図して（随意的に）身体を動かす（あるいは止める）ことである．
- しかしわれわれの身体を駆動した意図はどこから生まれ何によって制御されているのか，そもそもその随意性はどこまで意識できるのか，意識できない部分はどういう神経学的基盤を有するのか，「心」と「身体」の関係はどこまで記述できるのかなど，答えの不明な問いは際限なく出てくる．
- ここでは随意運動の諸相を Tourette 症候群，9 か月乳児，表情筋の収縮に情動性と随意性で解離のみられた症例から神経学的基盤を考察してみる．

Tourette 症候群（TS）にみる随意運動の発達

- マリナーズのイチローが，「2009 年の WBC 最後の打席が最も雑念の多かった打席であった」と述懐していた．卓越した打撃技術にとっても雑念[*1]は大きな影響を与えるということであろう．
- それでもイチローはヒットを打ち，英雄となった．
- イチローが獲得していた打撃の内部モデルは，雑念に影響されないほど身体がおぼえ込んでいたということかもしれない．
- この「身体がおぼえ込む」「身体が勝手に反応する」ということは随意運動が複雑になればなるほど必要になってくる．
- 熟練の過程は意識した運動から無意識の運動への変化，自動化の過程である．
- 「意識して意のままに」動かすことを「随意性」とすると「勝手に反応した身体」は「随意性」をはずれる．
- この「随意性」とは何であろうか．「随意性」と「不随意性」は「意識」と「無意識」と同様，お互いを排除する概念なのであろうか．
- Tourette 症候群（TS）の主たる徴候としてのチックは，瞬き，顔しかめ，肩をすくめる，咳払い，発声などさまざまであるが，これらは一定時間止めようと思えば止めることが可能なので，随意性があるといえる．
- しかし無理に止めるとその後増加し，止め続けることは困難となって随意性の及ばない状況となるが，ミオクローヌスなどのようなまったくの不随意運動とも異なる[*2]．
- 多くの TS の患者ではチックの出現前に「前兆としての衝動やもやもやした不快感[*3]」が現れる．
- TS の本来の不随意性はこの不快な前兆にあり，チック自体の随意性は大きく損なわれてはいないとする説もある．

WBC：World Baseball Classic

*1
内部からの己の技術に対するゆらぎや外部からの期待やゆさぶり．

*2
随意性とも不随意性とも確定しにくい運動を準随意運動（quasi-volitional movement）と呼ぶこともある．

*3
premonitory urge, premonitory sensation.