have advocated the use of new histological criteria based on $CD8^+$ and MHC-I immunohistochemistry. Examination of the muscle biopsy specimens obtained from anti-SRP-positive patients have usually shown active necrosis and little or no inflammation [9, 10], and the same histological findings were observed in our patient. Based on the histological findings alone, anti-SRP-positive myopathy may be included in a different category from 'histological' PM. In fact, some investigators have considered that anti-SRP antibody is associated with a 'necrotizing myopathy' that differs from 'histological' PM [8–10]. The clinical diagnosis of PM, however, is not based on histological findings alone. The diagnostic criteria proposed by Bohan and Peter [11] are known to be clinically practical, sensitive, and specific, and they have served the community well for nearly three decades [17]. We therefore conclude that 'anti-SRP-positive' should be classified as a particular form of PM.

We concluded that our patient had anti-SRP-positive 'probable PM'. Anti-SRP-positive PM usually affects middle-aged people, however, and our patient is the youngest reported in the literature [5, 6, 8–10]. More importantly, anti-SRP antibody is associated with treatment-resistant and refractory PM, although the clinical features of anti-SRP-positive PM patients show heterogeneity and some of them have a favourable prognosis [9]. It should be realized that anti-SRP-positive PM has sometimes been misdiagnosed as other types of myopathies [8].

Rheumatology key messages

- Anti-SRP antibody is useful for differential diagnosis of myopathies.
- Anti-SRP-positive PM has sometimes been misdiagnosed as other types of myopathies.

Acknowledgements

We thank Dr Ichizo Nishino (National Institute of Neuroscience, National Center of Neurology and Psychiatry) for valuable comments on muscle biopsy and Ms Matsuko Ishida for assisting with the RNA immunoprecipitation assays.

Funding: This work was supported by a grant from the Japanese Ministry of Education, Science, Sports, and Culture, a research grant on intractable diseases from the Japanese Ministry of Health, Labour, and Welfare, and a Neuroimmunological Disease Research Committee grant from the Japanese Ministry of Health, Labour, and Welfare.

Disclosure statement: The authors have declared no conflicts of interest.

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Molecular Pathogenesis of Seipin/BSCL2-Related Motor Neuron Diseases

Daisuke Ito, MD, PhD, and Norihiro Suzuki, MD, PhD

Objective: Heterozygous mutations in the Seipin/BSCL2 gene have recently been identified in two autosomal dominant motor neuron diseases, distal hereditary motor neuropathy type V and Silver's syndrome. Seipin protein is reportedly a transmembrane protein localized in the endoplasmic reticulum (ER). N88S and S90L mutations of this protein disrupt its glycosylation, resulting in its aggregation, but the mechanism of neurodegeneration remains unclear. To clarify the molecular pathogenesis of seipin-related motor neuron diseases, we expressed wild-type and mutant seipin proteins in neuronal and nonneuronal cells.

Methods and Results: Coexpression of human seipin and ubiquitin showed that seipin is polyubiquitinated and its ubiquitination is enhanced by mutation. Treatment of cells with a proteasome inhibitor increased the amounts of mutant seipin in the cells, suggesting that they are degraded through the ER-associated degradation pathway. Immunoprecipitation studies showed that mutant seipin stably binds to the ER chaperone calnexin, indicating accumulation of unfolded mutant seipin in the ER. Furthermore, expression of mutant seipin increased the level of ER stress–mediated molecules and induced apoptosis in cultured cells.

Interpretation: These findings demonstrate that seipin/BSCL2-related motor neuron diseases are novel conformational diseases, and we suspect that they are tightly associated with ER stress–mediated cell death.

Ann Neurol 2007;61:237–250

Silver's syndrome (OMIM #270685), first described by J. R. Silver in 1966,[1] is an autosomal-dominant neurodegenerative disorder characterized by amyotrophy, weakness of the small hand muscles, and spasticity in the lower limbs. This disorder, which has been mapped to chromosome 11q12-q14 by linkage analysis, is also called spastic paraplegia 17 (OMIM #270685)[2,3] and is associated with a phenotypically different motor neuron disease, distal hereditary motor neuropathy type V (dHMNV; OMIM #182960).[4,5] In 2004, Windpassinger and colleagues[5] identified heterozygous mutations (N88S and S90L) in the Berardinelli–Seip congenital lipodystrophy gene (Seipin/BSCL2) that cause both Silver's syndrome and dHMNV.[5] Recent studies of genotype–phenotype correlations have demonstrated enormous phenotypic heterogeneity associated with these mutations, including Silver's syndrome, variants of Charcot–Marie–Tooth disease type 2, dHMNV, and spastic paraplegia, even in identical pedigrees.[6,7] Therefore, there is clinically a wide spectrum of seipin-related motor neuron diseases or neuropathies. In addition, of 90 individuals with the N88S mutation, 4 (4.4%) were asymptomatic and not penetrant[6]; therefore, other genetic background and/or environmental factors must be necessary for the appearance of the modified phenotypes.

Seipin/BSCL2 was originally identified as a candidate gene of congenital generalized lipodystrophy type 2 (OMIM #269700).[8] Individuals with homozygous null mutations in seipin show lipoatrophy affecting the trunk and limbs, increased serum concentrations of triglycerides, insulin resistance, and mental retardation, but not abnormality of motor neurons; however, there is no clinical evidence that heterozygous seipin-related motor neuron diseases are associated with lipodystrophy or abnormal body fat, indicating that mutations associated with seipin-related motor neuron diseases are gain-of-function dominant.

Based on protein sequence analysis, seipin is predicted to be a transmembrane protein located in the endoplasmic reticulum (ER). Furthermore, it has no significant homology to other known functional proteins.[9] The *seipin* gene is highly expressed in most regions of the central nervous system.[5] Although the phenotype of congenital generalized lipodystrophy type 2 suggests that seipin is associated with differentiation of adipocytes, its physiological function remains unknown. Windpassinger and colleagues[5] report that

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Received Oct 22, 2006, and in revised form Oct 22. Accepted for publication Nov 24, 2006.

This article includes supplementary materials available via the Internet at http://www.interscience.wiley.com/jpages/0364-5134/suppmat

Published online Mar 26, 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.21070

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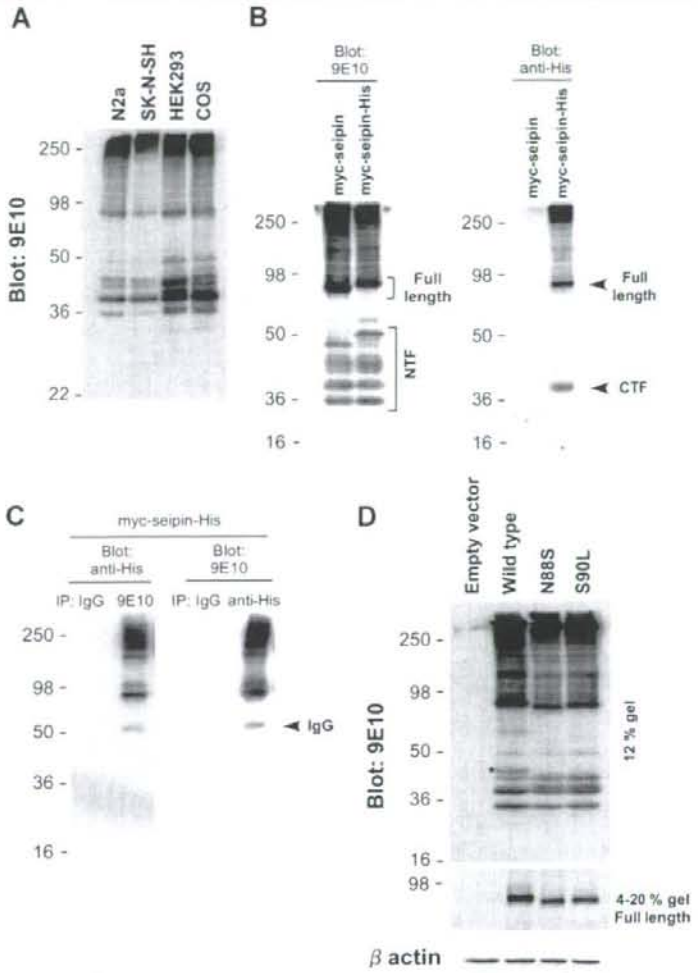

Fig 1. Seipin expression in cultured mammalian cells. (A) Immunoblot analysis of human seipin expression in transiently transfected neuro 2A (N2a), SK-N-SH, human embryonic kidney 293 (HEK293), and COS cells. Cells were transfected with human myc-seipin complementary DNA (cDNA). Detergent lysates prepared from these cells were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with antibody 9E10. (B) Lysates from N2a cells transfected with myc-seipin or myc-seipin-His cDNA were subjected to immunoblot analysis with 9E10 (left) or anti-His antibody (right). (C) Lysates from cells transiently transfected with myc-seipin-His cDNA were subjected to immunoprecipitation with 9E10 (left) or anti-His antibody (right), followed by immunoblot analysis with anti-His antibody (left) or 9E10 (right). (D) N2a cells were transfected with empty vector or myc-wild-type, N88S, or S90L seipin. The lysates were subjected to immunoblot analysis with 9E10. In the bottom panel, the membrane was reprobed with anti–β-actin as an internal loading control (top and bottom: 12% gel; middle: 4–20% gradient gel). Note that the full-length protein of both mutants migrated faster than the full-length wild-type protein in the middle panel, and that the approximately 41kDa N-terminal fragment was absent from the mutant lanes (asterisk) in the top panel. CTF = C-terminal fragment; NTF = N-terminal fragment.

both motor neuron disease–related mutations (N88S and S90L) are located in the N-glycosylation motif (N-X-S/T), suggesting that the improper glycosylation of seipin is closely associated with the pathogenesis of dHMNV and Silver's syndrome. Further investigations are needed to clarify the molecular pathogenesis and devise treatments for these diseases.

Here, we investigated the cellular and biochemical characteristics of seipin protein, as well as the molecular mechanism of neurodegeneration in seipin-related

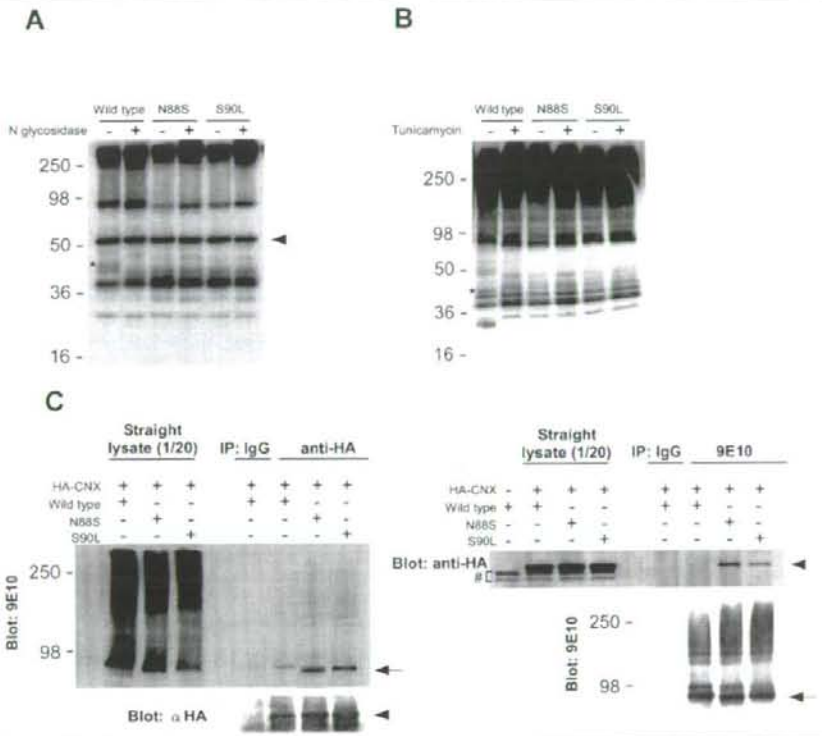

Fig 2. Disturbed N-glycosylation of mutant seipin leading to accumulation of unfolded protein in the endoplasmic reticulum (ER). (A) Cell lysates (100μl) from seipin-expressing neuro 2A (N2a) cells were treated with 10 units of N-glycosidase F at 37°C for 24 hours. Samples were immunoprecipitated with antibody 9E10 and then analyzed by immunoblotting with the same antibody. Arrowhead indicates mouse IgG bands. (B) N2a cells were transfected for 4 hours with myc-seipin plasmid and then treated without or with 2μg/ml tunicamycin. After 18 hours, cell lysates were analyzed by immunoblotting with antibody 9E10. Asterisks in the wild-type lane indicate the approximately 41kDa N-terminal fragment that disappeared after treatment with tunicamycin. (C) Interaction of seipin with calnexin (CNX). N2a cells were cotransfected with myc-wild type, -N88S, or -S90L seipin and hemagglutinin (HA)-CNX. After 48 hours, cell lysates were subject to immunoprecipitation with anti-HA antibody (left) or 9E10 (right), followed by immunoblot analysis with 9E10 (left) or anti-HA antibody (right). In the lower panels, membranes were stripped and reprobed with the opposite antibodies. Number sign indicates two unknown nonspecific bands that reacted with the anti-HA antibody. Note that the mutant interacted more strongly with CNX than the wild-type protein. Arrow and arrowhead indicate full-length seipin and CNX, respectively.

diseases. Overexpressed mutant seipin proteins were highly polyubiquitinated and degraded by the proteasome, and we show that improper glycosylation of mutant seipin exacerbates its misfolding in the ER. Furthermore, we show direct evidence that overexpression of the mutants activate the unfolded protein response (UPR), including upregulation of the proapoptotic transcription factor, CHOP, and cell death through ER stress. These findings suggest a pathogenic mechanism in motor neuron diseases and provide a novel target of therapy for seipin-related motor neuron diseases.

Materials and Methods

Cell Culture and Agents

Neuro 2A (N2a) neuroblastoma cells, HeLa human carcinoma cells, human embryonic kidney 293 cells, and COS African monkey kidney cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum. Human neuroblastoma SK-N-SH cells were cultured in α modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum. Transfections were performed using Lipofectamine and Plus reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Cells were cultured for 48 hours after transfection. N-glycosylation was inhibited with tunicamycin, and the proteasome was inhibited with MG132 (Sigma, St. Louis, MO).

Complementary DNA

In a previous study, seipin was predicted to be a 398-amino acid protein (GenBank accession number NM_032667), but based on the alignment of the human protein with those from other species, it appears that there is a second

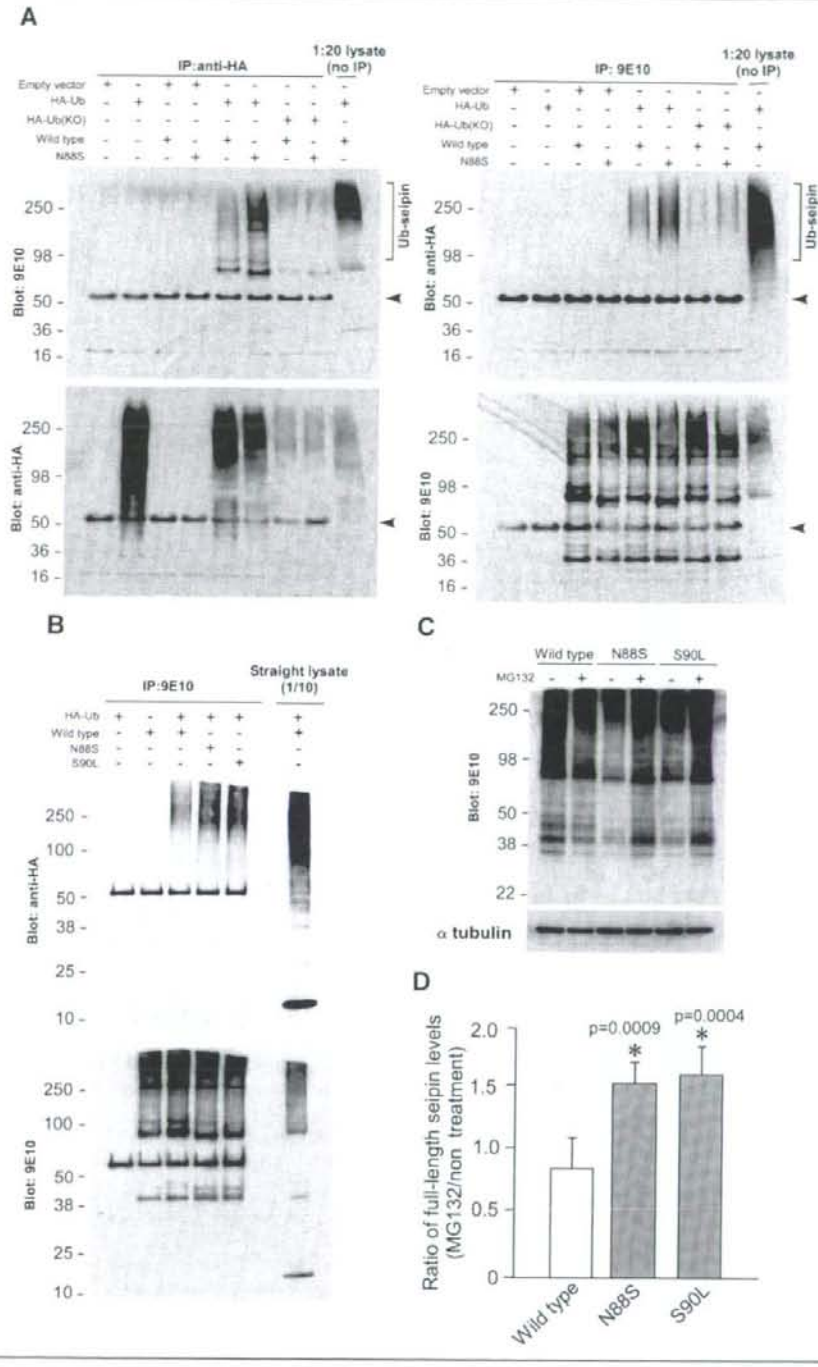
A
IP:anti-HA
1:20 lysate (no IP)
Empty vector
HA-Ub
HA-Ub(KO)
Wild type
N88S
Blot: 9E10
Blot: anti-HA
Ub-seipin
250
98
50
36
16
IP: 9E10
B
IP:9E10
Straight lysate (1/10)
S90L
100
38
25
10
C
Wild type
N88S
S90L
MG132
22
α tubulin
D
Ratio of full-length seipin levels (MG132/non treatment)
p=0.0009
p=0.0004
2.0
1.5
1.0
0.5
0

Figure 3

initiation codon located upstream of the original initiation codon.[9] This N-terminal variation could add another 64 residues to the N terminus. Therefore, we amplified isolated human *seipin* complementary DNA (cDNA) between amino acid residues −64 and 398 by polymerase chain reaction from an SK-N-SH cDNA pool using primers 5′-GTCGAATTCGATGTCTACAGAAAAGGTAGAC-3′ and 5′-GACGGATCCTCAGGAACTAGAGCAGGTGG-3′. The amplified cDNA was then cloned into pBluescript. The mutant *seipin* gene was generated by polymerase chain reaction using the following primers: N88S: 5′-CTGTTGCCAGTGTCTCGCTG-3′ (sense) and 5′-CAGCGAGACACTGGCAACAG-3′ (antisense); and S90L: 5′-CCAATGTCTTGCTGACTAAG-3′ (sense) and 5′-CTTAGTCAGCAAGACATTGG-3′ (antisense). After sequence verification, the cDNA was subcloned into pCS2+MT and pcDNA3.1/V5-His A. hemagglutinin (HA)-tagged human ubiquitin and dog calnexin (CNX) plasmids were gifts from Shigetsugu Hatakeyama (Kyushu University, Fukuoka, Japan) and Ikuo Wada (Fukushima Medical University, Fukushima, Japan), respectively.

Antibodies

We generated a rabbit polyclonal antiserum to a C-terminal peptide of human seipin (AGGALRQRPTCSSS). Other antibodies were purchased from commercial sources as follows: anti-KDEL from Stressgen (San Diego, CA); 9E10 and anti-CHOP/Gadd153 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-HERP antibody from Biomol (Plymouth Meeting, PA); anti–β-actin, anti–α-tubulin, and anti-His clone HIS-1 from Sigma; anti-HA clone 12CA5 from Roche (Basel, Switzerland); polyclonal anti-giantin from Invitrogen; and polyclonal anti-myc antibody from Upstate Biotechnology (Lake Placid, NY).

Fig 3. Overexpressed seipin is ubiquitinated and degraded by the proteasome. (A) Lysates from neuro 2A (N2a) cells cotransfected with myc-seipin and empty vector, hemagglutinin-ubiquitin (HA-Ub), or HA-Ub (knock-out [KO]) were subjected to immunoprecipitation with anti-HA antibody (left) or 9E10 (right), followed by immunoblot analysis with the opposite antibodies. In the lower panels, the membranes were stripped and reprobed with the antibodies used for immunoprecipitation. Arrowheads indicate mouse IgG. (B) Proteins immunoprecipitated with antibody 9E10 precipitates from cells cotransfected with HA-Ub and myc-wild type, N88S, or S90L seipin were analyzed by immunoblotting with anti-HA antibody. In the bottom panel, the membranes were stripped and reprobed with antibody 9E10. Note that the ubiquitination of mutants was significantly higher than the wild type. (C) Effect of a proteasome inhibitor on the amount of seipin protein. N2a cells transfected with myc-wild type, N88S, or S90L seipin were treated with or without 10μg/ml MG132 for 16 hours. Myc-seipin proteins were analyzed by immunoblotting with antibody 9E10. (D) Levels of full-length seipin were assessed by densitometry. Histogram shows the ratio of protein levels (mean ± standard deviation; n = 4), normalized to untreated controls. Asterisks indicate a significant difference versus wild type ($p < 0.001$). Ub-Seipin = ubiquitinated seipin.

Immunoblotting

Cells were briefly sonicated in cold lysis buffer (50mM tris[hydroxymethyl]aminomethane [Tris]-HCl, pH 7.4, 150mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.25% sodium dodecyl sulfate [SDS], 5mM EDTA, and protease inhibitor cocktail [Sigma]). Cell lysates were separated by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4 to 20% Tris-glycine gradient gel (Invitrogen) or 12% Tris-glycine gel, after which proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was incubated with primary antibodies (1:2,500 anti-KDEL, 1:5,000 anti-CHOP, 1:1,000 anti–β-actin, 1:1,000 anti–α-tubulin, 1:1,000 anti-His, 1:1,000 anti-stanniocalcin 2 [anti-STC2], or 1:1,000 anti-HERP), followed by horseradish peroxidase–conjugated secondary antibodies and then visualized using enhanced chemiluminescence reagents (Perkin-Elmer Life Sciences, Boston, MA). Protein levels in Figure 3D were determined by densitometry using an Epson ES-2000 scanner (Tokyo, Japan) and Image J (National Institutes of Health, Bethesda, MD).

Immunoprecipitation

To immunoprecipitate seipin, we incubated cell lysates overnight with the primary antibody and then precipitated with protein A/G-agarose beads (Pierce, Rockford, IL), as described previously.[10] In the experiment shown in Figure 2C, cells were incubated with CHAPS buffer (50mM HEPES [N-2-hydroxyethyl-piperazine-N′-2-ethane-sulfonate], 200mM NaCl, and 2% CHAPS [3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate]) for 30 minutes, centrifuged at 10,000 *g* for 15 minutes at 4°C, and the supernatants were analyzed by immunoprecipitation with 9E10 or anti-HA antibodies and then analyzed by immunoblotting.

Deglycosylation Assay

For the deglycosylation assay, lysates of transiently transfected N2a cells were briefly sonicated in 600μl deglycosidase buffer (0.5% SDS and 0.5% NP-40 in phosphate-buffered saline) and then boiled for 5 minutes. Lysates were treated with N-glycosidase F (Roche) for 24 hours at 37°C according to the manufacturer's instructions, and samples were immunoprecipitated with antibody 9E10 and then analyzed by immunoblotting.

Immunofluorescence

HeLa cells grown on coated coverslips were transfected with the seipin-pCS2+MT plasmid. After 48 hours, cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes and then permeabilized in 0.2% Triton X-100 (Sigma) for 5 minutes. After blocking of nonspecific binding, coverslips were incubated with 1:500 polyclonal anti-myc antibody, 1:1,000 9E10 antibody, or 1:500 SCT14 serum together with organelle marker antibodies (1:1,000 anti-KDEL, 1:1,000 anti-giantin, or 1:1,000 anti-HA) diluted in phosphate-buffered saline containing 0.2% Tween 20 and 3% bovine serum albumin. After three washes, coverslips

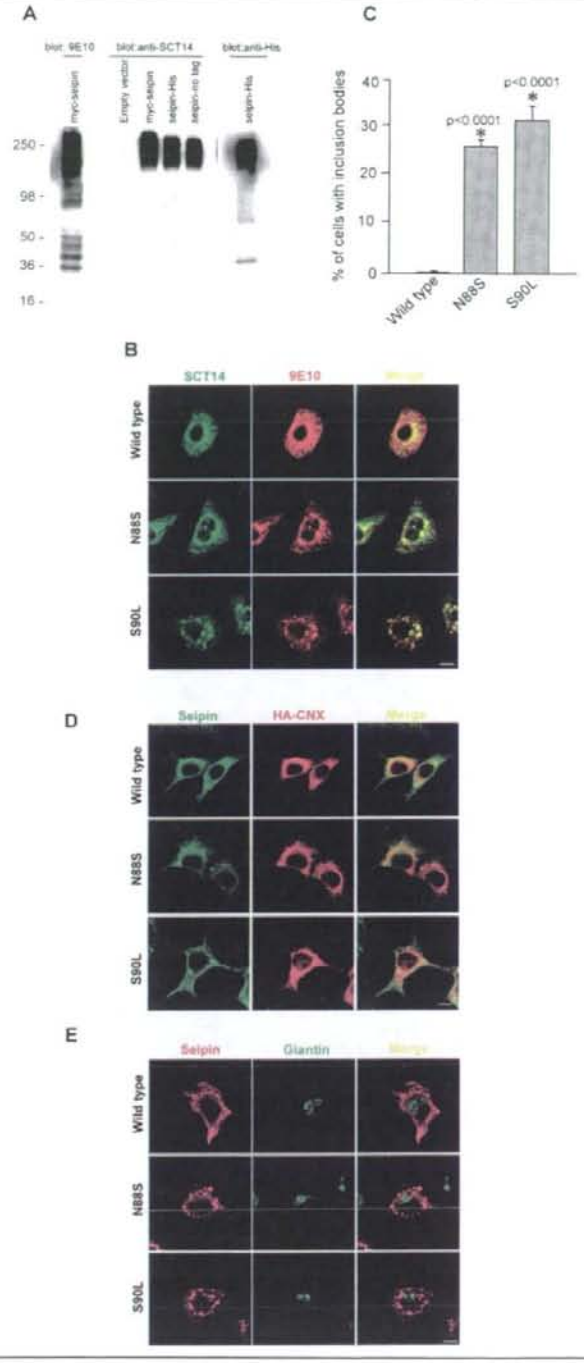
A
blot: 9E10
blot:anti-SCT14
blot:anti-His
myc-seipin
Empty vector
myc-seipin
seipin-His
seipin-no tag
seipin-His
250 -
98 -
50 -
36 -
16 -
C
% of cells with inclusion bodies
40
30
20
10
0
p<0.0001
p<0.0001
*
*
Wild type
N88S
S90L
B
SCT14
9E10
Merge
Wild type
N88S
S90L
D
Seipin
HA-CNX
Merge
Wild type
N88S
S90L
E
Seipin
Giantin
Merge
Wild type
N88S
S90L

Figure 4

were incubated with fluorescein isothiocyanate–conjugated anti–rabbit and Texas Red–conjugated anti–mouse secondary antibodies and mounted. Immunofluorescent staining was examined using an LSM 5-Pascal confocal microscope (Carl Zeiss, Oberkochen, Germany)

Immunohistochemistry of Seipin in Mouse Spinal Cord

The protocol for immunohistochemistry of seipin in mouse spinal cord received prior approval by meeting the Animal Experimentation Guidelines of Keio University School of Medicine. Adult male C57/BL6 mice (8 weeks old; n = 3) were fixed by perfusion with phosphate-buffered saline containing 4% paraformaldehyde. The brains were subsequently removed, and immunohistochemistry was performed on 16μm sections essentially as described previously[11–13] using SCT14 antiserum or the preimmune serum (1:1,000) together with mouse anti–neuronal nuclei (NeuN) monoclonal antibody (1:1,000) (Chemicon, Temecula, CA) or anti-KDEL antibody (1:1,000).

Cell Death Assay

HeLa cells plated on coverslips were transfected as described earlier. After 48 hours, cells were stained with 1.5μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for 15 minutes at room temperature or in terminal deoxynucleotidyl-transferase–mediated dUTP nick end labeling (TUNEL) solution (Roche) for 30 minutes at 37°C, followed by immunofluorescent staining with antibody 9E10 as described earlier.[10] Fluorescent images of three to four random fields were acquired with an Eclipse TE300 fluorescence microscope (Nikon, Kanagawa, Japan) with a 20X or 40X objective, and seipin-positive cells (approximately 300 cells/experiment) and apoptotic cells (Hoechst- or TUNEL-positive condensed nuclei) were counted. Data from independent experiments were expressed as mean ± standard deviation and statistically examined by one-way analysis of variance.

Statistical Analysis

Statistical analysis of the data was performed by one-way analysis of variance with Fisher's protected least-squares difference test using Statview 5.0 system (Statview, Berkley, CA).

Results

Characterization of Seipin Expression in Cultured Cells

To study the biochemical properties of seipin protein in cells, we overexpressed a cDNA-encoding human seipin with an N-terminal 6X Myc tag (myc-Seipin) in various cell lines and analyzed their expression by immunoblotting with a specific antibody (9E10). As shown in Figure 1A, all cells transiently transfected expressed a high-molecular-weight complex, an approximately 74kDa band, and at least 6 bands between approximately 35 and 48kDa. We therefore suspected that seipin is highly modified after translation. To examine this possibility further, we expressed myc-seipin with a C-terminal V5-His tag (myc-seipin-His). Immunoblotting with antibodies against the N and C termini (9E10 and anti-His, respectively) showed a high-molecular-weight complex and an approximately 78kDa band. In addition, an approximately 38kDa band was detected with anti-His antibody, whereas 35- to 48kDa bands were detected with the 9E10 antibody (see Fig 1B). Moreover, immunoprecipitation with either antibody followed by immunoblotting with the other showed a high-molecular-weight complex and an approximately 78kDa band (see Fig 1C). We suspected that the high-molecular-weight complex and the approximately 74kDa (approximately 78kDa for myc-seipin-His) are composed of full-length seipin, whereas the smaller bands are N- and C-terminal fragments of seipin.

Next, we transfected mouse N2a cells with cDNA encoding two mutant forms of seipin (N88S and S90L) to compare their modification and processing with that of wild-type seipin (see Fig 1D). Full-length mutant seipin had a higher mobility on SDS-PAGE than the wild-type protein, as described previously[5] (see Fig 1D, middle). Furthermore, there was an approximately 41kDa N-terminal seipin fragment observed in cells transfected with the wild type that was not observed in cells transfected with either of the mutants (see Fig 1D, top, asterisk in lane 2). Windpassinger and colleages[5] report such a shift in the migration of the mutant seipin and attribute it to improper N-glycosylation. Furthermore, they show that the wild-type and N88S mutant seipin have identical molecular masses after deglycosylation.

To confirm that seipin is N-glycosylated, we examined the effects of two different agents against N-glycosylation, N-glycosidase F, and tunicamycin. Af-

Fig 4. Immunofluorescence analysis of seipin expressed in HeLa cells. (A) Specificity of seipin antiserum SCT14. Neuro 2A (N2a) cells were transfected with plasmids encoding myc-seipin, seipin-His, or seipin without a tag. To determine the titer of SCT14, we analyzed aliquots of detergent lysates by immunoblotting with 9E10, SCT14, or anti-His antibody. (B) HeLa cells transfected with myc-wild-type or N88S seipin were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 (Sigma), and double-labeled with stanniocalcin 14 (STC14) (green) and 9E10 (red). (C) Quantitative analysis of cells with inclusion bodies in HeLa cells expressing wild-type or mutant seipin. Approximately 300 transfected cells from 3 independent experiments were counted. Asterisks indicate statistically significant differences versus wild type ($p < 0.0001$). (D) HeLa cells transiently transfected with hemagglutinin-calnexin (HA-CNX) (endoplasmic reticulum marker) and myc-wild-type or -mutant seipin were immunostained with polyclonal anti-myc antibody (green) and monoclonal anti-HA antibody (red). (E) Cells expressing myc-wild-type or mutant seipin were double stained with 9E10 (red) and polyclonal anti-giantin antibody (green; marker of Golgi apparatus). Scale bar = 10μm.

ter treatment with these reagents, the apparent molecular weight of full-length wild-type seipin was reduced, and the approximately 41kDa N-terminal fragment was converted to 35- to 38kDa polypeptides for both the wild-type and mutant proteins (Figs 2A, B). Therefore, wild-type seipin is N-glycosylated and cleaved into N-terminal fragments, whereas the mutants are not N-glycosylated but appear to undergo normal proteolytic processing.

Glycosylation is known to be important for the proper folding and maturation of glycoproteins. For instance, it is well known that inhibition of N-glycosylation by tunicamycin causes accumulation of unfolded protein in the ER. In the ER, the chaperones Bip, calreticulin, and CNX transiently bind to newly synthesized glycoprotein intermediates, guiding their proper folding.[14] Previous studies have demonstrated that these chaperones bind for an extended period to mutant proteins that fail to fold.[14–16] To clarify the folding state of mutants, we therefore examined the interaction between seipin and CNX, which is membrane-bound molecular chaperone in the ER.[17,18] We cotransfected N2a cells with HA-CNX and myc-seipin and examined their coimmunoprecipitation with anti-HA or 9E10 antibodies. We found that the interaction of CNX with N88S and S90L seipin was more stable than the interaction with wild-type seipin (see Fig 2C). Thus, our results suggest that the N88S and S90L mutations interfere with glycosylation and lead to production of unfolded protein in the ER.

Ubiquitination and Proteasomal Degradation of Seipin

As shown in Figure 1, when overexpressed, a portion of myc-seipin appeared as a high-molecular-weight weight complex on SDS-PAGE. Seipin also appeared as a high-molecular-weight complex when stably expressed without a fusion tag (see Supplementary Information, Fig S1). Given that other proteins associated with neurodegeneration, for example, Parkin and polyglutamine, are ubiquitinated in transfected cells and affected brain,[19–21] we suspected that this high-molecular-weight complex consists of polyubiquitinated seipin. Therefore, we examined the role of ubiquitination in N2a cells cotransfected with plasmids expressing wild-type or mutant myc-seipin and HA-ubiquitin (HA-Ub) or a lysine-less ubiquitin mutant (HA-Ub [knock-out]) that inhibits ubiquitin chain elongation.[22] Immunoprecipitation with anti-HA or 9E10 antibody, followed by immunoblotting with the opposite antibody, showed a high-molecular-weight band when wild-type myc-seipin was coexpressed with HA-Ub, but not HA-Ub (knock-out) (Fig 3A). Furthermore, immunoprecipitation with anti-His antibody against the C terminus of seipin using lysates from cells coexpressing HA-Ub and seipin-His also showed a ubiquitinated high-molecular complex (see Supplementary Information, Fig S2), indicating full-length seipin is ubiquitinated. Interestingly, the level of ubiquitination of both mutants was noticeably greater than that of the wild type (see Fig 3B).

Misfolded or unassembled proteins in the ER are eliminated by the ER-associated degradation (ERAD) system, which is tightly associated with the ubiquitin-proteasomal system (UPS).[23] We therefore examined the effect of MG132, an inhibitor of proteasome-mediated degradation, on the levels of wild-type and mutant seipin expressed in N2a cells. The levels of both mutant seipin proteins were markedly increased by treatment with MG132 (see Fig 3C). Densitometric analysis showed that the ratio of the levels in MG132-treated versus untreated cells was 0.882 ± 0.234 (mean ± standard deviation) for wild type, 1.530 ± 0.123 for N88S, and 1.615 ± 0.1.88 for S90L (see Fig 3D). Collectively, these results indicate that mutant seipin is constitutively degraded through ubiquitin-proteasome pathway because of its misfolding in the ER.

Subcellular Localization of Seipin

Windpassinger and colleagues[5] report that a seipin-green fluorescent protein fusion mainly localizes in the ER, and that mutant seipin appears to be rapidly concentrated in areas resembling aggresomes. To explore this in more detail, we performed double-immunofluorescence studies using several subcellular markers, including expressed HA-CNX, giantin, Mitotracker (Molecular Probes, Eugen, OR), and early endosome antigen I (EEA1), which are markers of the ER, Golgi apparatus, mitochondria, and early endosomes, respectively. For these studies, we generated a polyclonal antiserum (SCT14) against the C terminus of human seipin. Immunoblot analysis of transfected cell lysates confirmed that both SCT14 antisera uniquely react with the high-molecular-weight form of overexpressed seipin (Fig 4A). Unexpectedly, however, the C-terminal fragment of untagged seipin was not detected with this antibody, even when we used it for immunoprecipitation followed by immunoblotting (data not shown). Although it is possible that the titer of the SCT14 antibody is low, it is also possible that the untagged C-terminal fragment is rapidly degraded after processing.

Double-immunofluorescent staining with 9E10 and SCT14 in HeLa cells expressing wild-type myc-seipin showed typically reticular patterns in the cytoplasm that mostly overlapped (see Fig 4B). The majority of wild-type myc-seipin colocalized with HA-CNX (see Fig 4D), and some of it was found in the cytoplasm outside the ER. We did not find significant overlap between staining for seipin and the Golgi apparatus (see Fig 4E), mitochondria, or EEA1 (see Supplementary Figs S3 and S4). In contrast, both forms of mu-

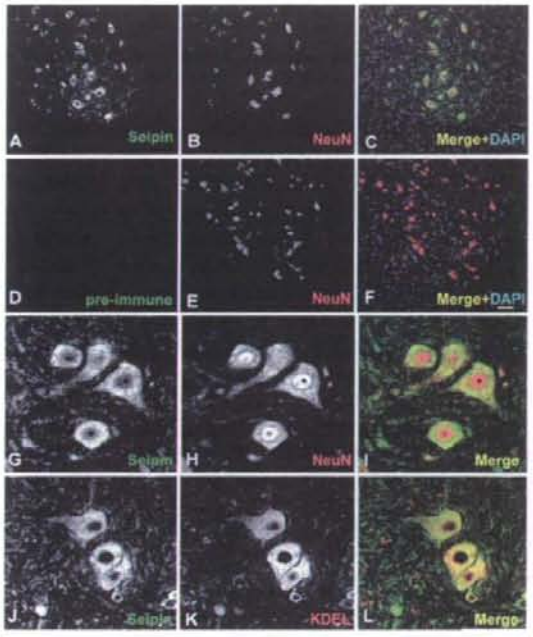

Fig 5. Immunohistochemical analysis of seipin expression in mouse spinal cord. Sections were stained with anti–neuronal nuclei (anti-NeuN) antibody (neuronal marker) and SCT14 antiserum (A–C, G–I) or preimmune serum (D–F). (A–F) Cells were counterstained with 4',6-diamino-2-phenylindole (DAPI), showing anterior horns of spinal cords. (G–I) Higher magnification under the confocal laser scanning microscope. Note the strong seipin immunoreactivity in the spinal cord motor neurons. (J–L) Double-labeling with anti-KDEL antibody (red) and SCT14 antiserum (green). This panel (J–L) shows the colocalization of endoplasmic reticulum marker and seipin protein in motor neurons. Scale bar = 50μm.

tant seipin form large perinuclear inclusions similar to aggresomes[5] (see Figs 4B, C). These inclusions are localized outside the ER (see Fig 4D), Golgi apparatus (see Fig 4E), mitochondria, and EEA1 (see Supplementary Figs S3 and S4). Of approximately 300 transfected cells in 3 independent experiments, 25 to 30% of those expressing mutants contained inclusions, whereas inclusions were nearly undetectable in cells expressing the wild-type protein (mean ± standard deviation; wild-type: 0.32 ± 0.28%, n = 1,254; N88S: 24.95 ± 1.10%, n = 1,224; S90L: 30.56 ± 3.65%, n = 1151; see Fig 4C). Notably, double immunofluorescence using the 9E10 and SCT14 antibodies demonstrated that these inclusions contained full-length or both C- and N-terminal fragments of seipin (see Fig 4B). Although it is often difficult to discriminate between inclusion bodies and Golgi apparatus by single staining,[24] Figure 4E clearly shows that the inclusion bodies were distinct from the Golgi apparatus.

Next, we performed immunohistochemical analysis to determine where seipin is expressed in motor neurons because its localization is altered in seipin-related motor neuron diseases. Seipin immunoreactivity was present in NeuN-positive cells of the anterior horns of the spinal cord and clearly colocalized with staining for the ER marker KDEL (Fig 5). These in vivo findings indicated that seipin protein is expressed in spinal cord motor neurons and probably in the ER as found in cultured cells.

Mutant Seipin Expression Activates the Unfolded Protein Response and Induces Cell Death

Our findings suggested that mutant, misfolded seipin accumulates in the ER, and we speculated that this can be toxic to cells. We performed immunofluorescence studies using an anti-KDEL peptide antibody, which recognized ER chaperones Grp94 and BiP in N2a cells. We found that HeLa cells transfected with mutant seipin are stained more strongly than cells transfected with wild-type seipin (Fig 6A), suggesting that mutant seipin activates the UPR. To confirm this, we transiently transfected N2a cells with wild-type, N88S, or S90L seipin and compared the levels of known UPR markers, including the BiP, Grp94, PERK-ATF4 pathway-mediated glycoprotein, STC2,[10] the proapoptotic transcription factor CHOP, and the ERAD component HERP.[25] All UPR markers were clearly upregulated in cells expressing mutant forms of seipin, but low levels of these proteins were found in cells expressing empty vector or wild-type seipin (see Fig 6B). Because CHOP is one of the most important mediators of ER stress–mediated apoptosis,[26–28] we performed quantitative analysis of its expression in transfected cells by immunoblotting. Densitometric analysis showed that, compared with wild-type cells, CHOP expression was 2.09 ± 0.67-fold greater in cells transfected with N88S and 1.85 ± 0.48-fold greater in cells transfected with S90L (see Fig 6C). Collectively, these results suggest that expression of mutant seipin induces ER stress.

To determine whether expression of mutant seipin induces cell death, we transfected HeLa cells with seipin for 48 hours and then assessed the extent of cell death by both Hoechst 33342 and TUNEL staining. As shown in Figure 7, there was no significant difference in the extent of apoptosis in cells transfected with vector alone (pCS2+MT; 5.1 ± 1.4% by Hoechst staining and 5.6 ± 2.5% by TUNEL staining) or wild-type seipin (8.9 ± 2.4% by Hoechst staining and 6.8 ± 0.7% by TUNEL staining), whereas approximately 20% of N2a cells expressing N88S and S90L seipin died at 48 hours after transfection (N88S: 22.6 ± 2.9% by Hoechst staining and 17.6 ± 4.1% by TUNEL staining; S90L: 21.8 ± 5.6% by Hoechst staining and 18.2 ± 3.2% by TUNEL staining), indi-

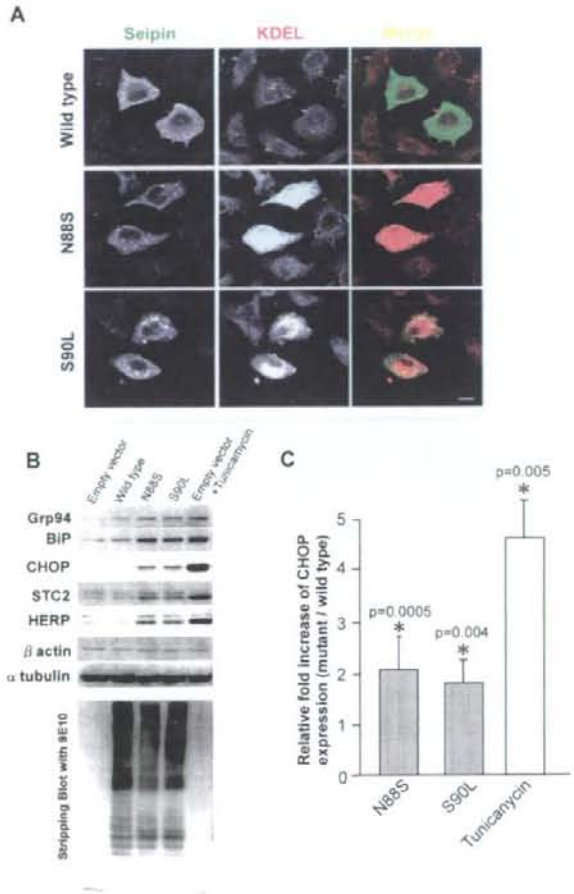

Fig 6. Effect of N88S and S90L mutation on induction of unfolded protein response (UPR) molecules. (A) HeLa cells were transfected with wild-type or mutant myc-seipin and double-immunolabeled with antibodies against Myc (green) and KDEL (red). All images were acquired by confocal microscopy under the same conditions. Note that cells expressing mutant seipin showed stronger staining with the anti-KDEL antibody. (B) Immunoblot analysis of UPR molecules Grp 94, BiP, CHOP, stanniocalcin 2 (STC2), and HERP. Neuro 2A (N2a) cells expressing empty vector (lane 1; negative control) or empty vector exposed to 2μg/ml tunicamycin for 16 hours (lane 5; positive control) were lysed 48 hours after transfection. Lanes 2, 3, and 4 are lysates from N2a cells transfected with wild-type, N88S, and S90L seipin, respectively. β-Actin and α-tubulin served as internal loading controls. In the bottom panel, the membranes were reprobed with antibody 9E10 to confirm similar expression of seipin. The results are representative of three independent experiments. (C) Quantitative analysis of CHOP protein induction. Immunoblots were scanned and analyzed by densitometry. CHOP expression was normalized by an internal control (α-tubulin or β-actin). Graph shows the fold increase in CHOP expression compared with that in the wild type. Values represent means ± standard deviation of seven independent experiments for the mutant and three experiments for tunicamycin treatment. Asterisks indicate a significant difference versus wild type ($p < 0.005$). Scale bar = 10μm (A).

cating that expression of mutant seipin induces apoptosis. Taken together, our findings demonstrated that misfolded mutant seipin accumulates in the ER and leads to cell death as a result of ER stress. We suspect that this mechanism causes the neurodegeneration in dHMNV and Silver's syndrome.

Discussion

In addition to playing a role in calcium storage and signaling, the ER provides a specialized environment for the folding and maturation of transmembrane and luminal proteins. Physiological, pathological, and experimental conditions that perturb ER function cause the accu-

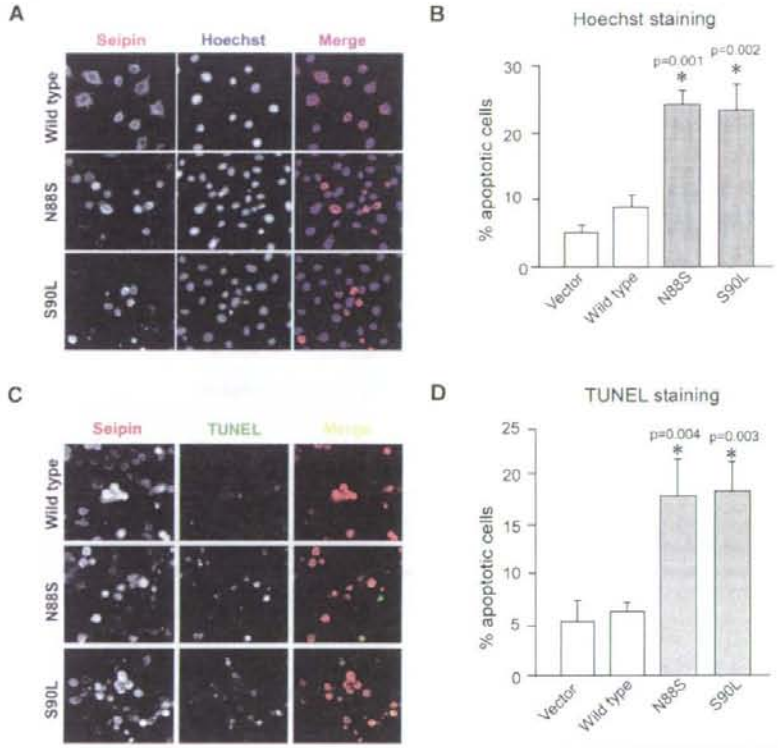

Fig 7. Mutations in seipin induce apoptosis in transfected HeLa cells. HeLa cells grown on glass coverslips were transfected with empty vector (pCS2+MT), myc-wild-type plasmid, or myc-mutant seipin plasmids. After 48 hours, coverslips were washed in phosphate-buffered saline (PBS) and stained with Hoechst 33342 (blue) or terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL; green) to detect dead cells, and then stained with antibody 9E10 (red). Images were acquired with a fluorescence microscope, and seipin-positive and dead cells were counted. (A, C) Top panels show representative images of wild-type– and mutant-transfected cells stained with Hoechst 33342 (A) or TUNEL (C). (B, D) Percentage of cells undergoing apoptosis was calculated from the number of cells with condensed nuclei as shown by Hoechst 33342 staining (B) or by TUNEL staining (D) divided by the number of seipin-positive cells. Three fields were counted in each experiment. Graph shows the mean ± standard deviation form three independent experiments. Data was analyzed by one-way analysis of variance with Fisher's protected least-squares difference test. Asterisks indicate a significant difference from wild type ($p < 0.005$).

mulation of misfolded proteins within the ER, inducing ER stress, which activates an adaptation program, termed the UPR.[29,30] If ER stress is prolonged and the defect in protein folding is not corrected, the ER activates a unique pathway that leads to cell death through apoptosis. Thus, on ER stress, cell fate depends on the balance of two opposite pathways: adaptation and cell death. Furthermore, misfolded protein is selected and translocated across the ER membrane into the cytosol by the ERAD system, leading to degradation through the UPS.[23] This connection between the ER and proteasomal system is essential for the maintenance of protein quality within the lumen of the ER.

Growing evidence indicates that various neurodegenerative diseases, such as Alzheimer's disease, polyglutamine diseases, prion disease, Parkinson's disease, and stroke, may have common pathological mechanisms, specifically, protein misfolding, aggregation, and accumulation in affected brain regions, resulting in neuronal dysfunction.[10,13,20,31–33] Therefore, dysfunction of the protein quality-control system and ER stress are thought to be common features of pathological processes in neurological diseases.[29,30,34]

This report documents several lines of evidence that UPS and ER stress play an important role in the pathogenesis of seipin-related motor neuron diseases (Fig 8). First, transiently expressed human seipin appears to be proteolytically cleaved into an N-terminal fragment, and full-length seipin is polyubiquitinated in cultured neuronal and nonneuronal cells. N88S and S90L mutations of seipin, which disrupt the sites of N-glycosylation, enhance ubiquitination and degrada-

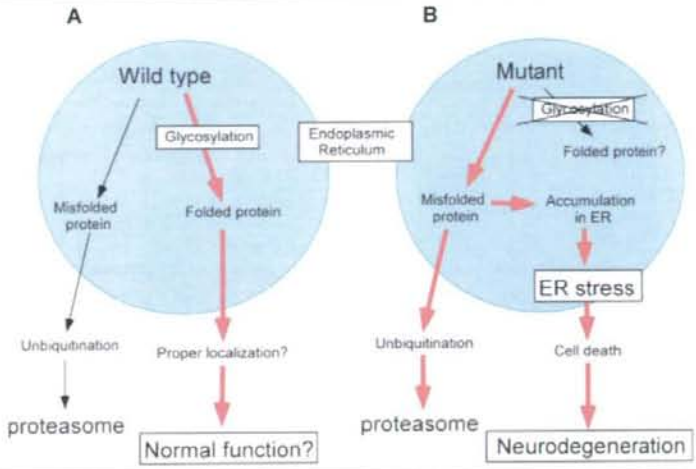

Fig 8. Possible mechanisms of seipin-related motor neuron diseases. (A) Wild-type seipin is glycosylated and correctly folded by chaperones in the endoplasmic reticulum (ER). A small amount is misfolded, ubiquinated, and degraded by proteasome. Folded seipin is transferred to the proper subcellular location, where it exhibits its physiological function. (B) Mutant seipin, whose N-glycosylation is disrupted, does not fold correctly, causing it to accumulate in the ER. Although the ubiquitin-proteasomal system eliminate some of the mutant misfolded protein from the ER, excessive accumulation leads to ER stress, resulting in neuronal cell death.

tion by UPS. Based on these results, we speculate that ERAD is involved in the degradation of misfolded seipin protein (see Figs 1 through 3). Second, immunohistochemical analysis using a specific antiserum against seipin shows that seipin protein is expressed in spinal cord motor neurons, which are the main cells affected in seipin-related motor neuron diseases (see Fig 5). Third, these mutants appear to be improperly folded, resulting in their accumulation in the ER, which activates the UPR. Furthermore, Hoechst and TUNEL staining demonstrate that overexpression of mutant seipin induces apoptosis in cultured cells (see Figs 6 and 7). Although these findings should be verified using autopsy samples or in a transgenic mouse model, our results suggest that seipin-related motor neuron diseases, including dHMNV and Silver's syndrome, are novel conformational diseases,[35] and we speculate that the pathological process of these diseases is tightly associated with ER stress–mediated cell death.

Familial amyotrophic lateral sclerosis is linked to a mutation in the Cu,Zn-superoxide dismutase 1 (SOD1), and recent evidence suggests that this is due to sustained ER stress.[36] Activation of UPR and caspase-12 in the ER has been reported during presymptomatic disease in transgenic rodent models of amyotrophic lateral sclerosis,[36–38] suggesting that mutant SOD1 has a direct adverse effect on ER function. Furthermore, mutant SOD1 is polyubiquitinated and degraded by the proteasome, and the sustained expression of mutant SOD1 leads to the death of motor neurons.[39–42] Therefore, mutant seipin and SOD1 may have similar biochemical roles in neurodegeneration, and we propose that the ER stress–mediated pathway for cell death is a novel target for the treatment of motor neuron disease.

Recent studies have reported that the autophagy-lysosomal pathway for degrading cellular proteins also plays a critical role in the clearance of aggregate-prone proteins, such as polyglutamine and α-synuclein.[43–45] Ravikumar and coworkers[46] report that inhibition of mammalian target of rapamycin, which enhances autophagy, attenuates the accumulation of huntingtin and protects against polyglutamine toxicity in a mouse model of Huntington's disease. Therefore, in future studies, we will examine whether autophagy and the lysosome also participate in the metabolism of aggregated seipin.

Whether the formation of inclusion bodies is pathogenic or protective in neurodegenerative diseases remains to be determined. Our study demonstrated that approximately 30% of cells expressing mutant seipin contain cytoplasmic inclusions outside ER, which is similar to Windpassinger and colleagues' findings.[5] So far, it is unclear whether seipin inclusions are biochemically similar to so-called aggresomes,[24] which are reportedly observed in cells expressing polyglutamine, α-synuclein, and Parkin.[47–49] Furthermore, there is no evidence of inclusion bodies in motor neurons from tissue samples of patients with dHMNV or Silver's syndrome. A recent study shows that inclusion bodies reduced the intracellular levels of diffuse huntingtin and prolonged survival, suggesting that inclusion bod-

ies might protect neurons[50]; however, whether this is a common mechanism of protection in other neurodegenerative diseases remains unclear. Therefore, clarification of the relation between inclusion body formation and cell fate in seipin-related motor neuron diseases should help understand the pathogenesis of neurodegenerative diseases characterized by protein aggregates.

Finally, chemical chaperones, which promote proper protein folding by altering the conditions of the ER, are being used to treat other conformational diseases including ΔF508-homozygous cystic fibrosis and α_1-antitrypsin deficiency.[51–53] In addition, chemical agents that selectively inhibit the dephosphorylation of eIF2α and protect cells from ER stress have been recently identified and are expected to be useful in the treatment of diseases involving ER stress.[54] It will be important to determine whether application of these agents modulates misfolded protein- and ER stress–mediated apoptosis associated with protein aggregation in neurodegenerative diseases. Although further studies are needed to elucidate the molecular pathogenesis of motor neuron diseases including seipin-related diseases, we could hope that promotion of mutant protein folding and degradation in ER will delay or prevent neurodegeneration in motor neuron diseases.

We are grateful to Dr I. Wada for providing the HA-CNX plasmid and thank Dr S. Hatakeyama for providing the HA-Ub and HA-Ub (knock-out) plasmids. We also thank Dr G. Thinakaran for providing cell lines and thoughtful discussion.

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特集　目と脳のアンチエイジング

7

パーキンソン病は予防可能か？

The Protective Factors against the Risk of Parkinson's Disease

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Key Words
- Parkinson's disease
- environmental risk factors
- smoking
- coffee
- alcohol

Summary

The cause of Parkinson's disease is considered to involve both genetic and environmental risk factors. Smoking and coffee drinking have consistently identified to have protective effects in recent epidemiologic studies.

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はじめに

Q：パーキンソン病は予防可能か？

A：現在，パーキンソン病（Parkinson's disease：PD）やアルツハイマー病などの神経変性疾患で予防が可能なものはない。

神経変性疾患では，神経系のある特定の細胞群が選択的かつ進行性に障害される。各疾患では，どの神経系に障害が起こり，どのようなメカニズムが病態に関与しているかの詳細が徐々に解明されつつある。しかし，神経障害の選択性と進行性という最も本質的な問題は依然として解明されていない。現在の対症療法を越える治療法の最終的な目標は病気の進行抑制，疾患の完全な治癒であり，これは将来の大きな課題となっている。

疾患の確実な予防法は，その原因の解明が前提となる。原因究明の研究手段の一つに，発症に関連する環境因子の調査がある。PDに関しても，近年の疫学的な研究によって危険因子と予防因子が多数指摘されている。現時点で，どのようなライフスタイルとPDの発症が関連しているか？　という質問に対する答えはある程度用意されている。したがって，パーキンソン病は予防可能か？　という質問に対しては，PDの発症危険因子を可能な限り避け，予防因子を意識することが，現時点での解答と考えられる。本稿では，このような視点からPDの発症にかかわる各因子を記載する。

パーキンソン病の疾患概念と臨床症状

PDは，安静時振戦，筋強剛，無動／寡動，姿勢反射障害の4大徴候，さらに特徴的な歩行障害を呈する錐体外路系の神経変性疾患である。この運動症状は，中脳の黒質にあるドパミン神

経細胞が進行性に変性し，その結果として線条体のドパミンが減少することにより出現する。しかし，ドパミン神経細胞の変性について正確なメカニズムは解明されていない。また，従来PDは運動障害を中心とする疾患と考えられてきたが，最近では疾患概念が大きく変貌しつつある。臨床的に非運動症状の重要性が強く認識され，精神症状（うつ状態などの気分障害，認知症，幻覚・妄想），睡眠障害，自律神経障害（起立性低血圧，排尿障害），感覚障害（痛みや嗅覚障害）などのさまざまな病態が注目されている。病理学的にドパミン系以外の神経系の障害は以前から指摘されてきたが，やはりPDの中核症状となる運動障害にはドパミン系が関与しており，発症機序も黒質の変性を中心に研究が進められている。

PDの運動症状は，ふるえや動作緩慢，姿勢や歩行の異常など，正常の加齢変化による運動機能の低下に重なる部分が多い。黒質線条体系のドパミン神経細胞は，加齢に伴い徐々に減少していく。病理学的には，ドパミン神経細胞が正常の40%以下に減少すると，また生化学的にはドパミンやその合成酵素が正常の20%以下に減少すると，PD症状が出現するとされる。正常の加齢変化では80歳を超えるとドパミン神経細胞が正常の60%以下になるが，PDでは何らかの原因で神経細胞の変性が早く進行し発症につながると考えられている（図1）。

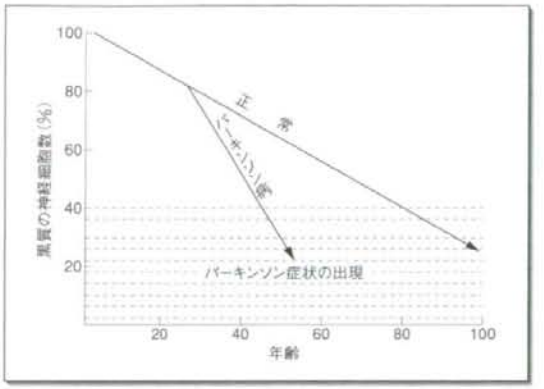

図1．加齢とパーキンソン病における黒質の神経細胞減少
（柳澤信夫 編：パーキンソン病―診断と治療―．東京，金原出版，7，2000より引用）

パーキンソン病の疫学

PDの多くは中年期以降に発症し，人口の高齢化に伴い患者数が増加しており，今後もさらに増加していくことが予想される。

1．有病率

我が国でのPDの有病率は，人口10万人当たり約100～130人と推定され，欧米と比較してやや少ないが大きな差はないと考えられている。PD患者数は全人口の約0.3%，年齢65歳以上の人口では3%に達し，年齢（aging）はPD発症に関する明らかな危険因子であり，PDの有病率は社会の高齢化に伴って今後増加していく可能性がある。

2．男女差

我が国では女性が男性の約2倍多い傾向がある。海外の報告の多くでは男性のほうが多いとされ，男女差は一致せず，その理由も明らかではない。

3．人種差と地域差

PDは以前，黒色人種には少なく，白色人種に多く，黄色人種はその中間と考えられていた。黒質のドパミン神経細胞はメラニンを多く含んでおり，皮膚のメラニン含量との関連も推論されていたが，現在では人種差はほとんどないと考えられている。米国在住の黒色人種のPD有病率（人口10万人対）は341人で，米国の白色人種と差がなかったが，ナイジェリアの黒色人種では有病率が67人と少なく，人種差よりもむしろ環境因子の関与が示唆されている。

パーキンソン病の発症にかかわる因子

現在，PDの発症機序には，遺伝的

素因と環境因子の相互作用が関連すると考えられている。環境因子として多くのものが提唱されており，特に農薬や喫煙，カフェイン飲料などが注目されるが，PD 発症の直接の原因として確立されたものはいまだない。

1．遺伝的な因子

遺伝的な素因に関しては多数の検討がある。なかでも，α-シヌクレインと呼ばれる蛋白の遺伝子多型が PD 発症の危険因子として注目されている。PD の黒質神経細胞では，Lewy 小体と呼ばれるα-シヌクレイン陽性の細胞内凝集体がみられ，α-シヌクレインの蓄積とドパミン神経の変性との関連性が指摘されている。遺伝子多型がα-シヌクレインの発現量増加に関与して危険因子となる可能性が考えられている[1]。

2．危険因子としての環境因子

（1）職業

農業従事者では若年での PD 発症が多く，かつ発症のリスク自体も高いとされるが，その原因として殺虫剤・除草剤への暴露（次項 2）との関連性が疑われている。また溶接工でも若年発症が多いとする報告があり，金属への暴露（項目 3）との関連で注目されるが，その後，溶接業務との関連性を否定する検討もある。医療従事者や教職，法律家，科学者，宗教に関連した職業，また高学歴が PD 発症リスクを関連するとされるが，その原因は明らかではない。

（2）殺虫剤・除草剤への暴露

農薬の中でもパラコートが危険因子として注目され，台湾の稲作農家で20年以上の暴露歴がある場合，PD 発症のリスクが 6 倍以上との報告がある。PD の動物モデルでもパラコートは黒質線条体のドパミン神経細胞を選択的に障害し，α-シヌクレイン陽性の細胞内凝集体が出現することが確認されている。

（3）金属への暴露

鉛，銅，鉄，マンガンなどは危険因子として知られている。特に鉛と鉄，鉄と銅の組み合わせでの暴露が長期に及ぶと PD 発症のリスクが上昇するとの報告がある。最近，これらの金属がα-シヌクレインの構造変化や凝集反応を引き起こす可能性が示唆されている。

（4）肥満

PD 患者は発症後に体重減少 "痩せ" を認めることが多いが，PD 発症のリスクとしてはむしろ肥満が指摘されている。特に中心性肥満が，あるいは BMI（body mass index）が独立した危険因子であるとの報告もある。

（5）頭部外傷

10以上の臨床研究があるが，現時点で PD 発症と頭部外傷の関連性には議論がある。最近でも，健忘や意識消失を伴う軽度から中等度の非開放性頭部外傷の既往と発症の関連が，双生児において指摘されている。

（6）その他

農村地域での居住や，井戸水の使用（特に20歳以前の井戸水摂取）との関連性も指摘されている。

3．防御因子としての環境因子

（1）喫煙

過去50年間に行われた40以上の臨床研究の結果，現在，喫煙と PD 発症リスクの低下の関連は疫学的手法によってほぼ確立されている[2][3]。しかも，タバコの消費量が多いほどリスクの低下が明らかで，用量依存性も確認されている。過去の喫煙歴もリスクを低下させる。もちろん，喫煙者がそれに関連した疾患によって短命であるため，高齢で発症する PD 患者が少ないといった単純な図式ではない。通常，PD 発症率は加齢によって上昇するが，喫煙者では増加がみられず，喫煙そのものが PD 発症を遅らせていると指摘する報告もある。双生児における検討でも，喫煙の防御因子としての作用が確認されている。タバコに含まれる化学物質は4,000以上とされるが，なかでもニコチンが防御因子の候補として注目されている。ニコチンは線条体の神経細胞を刺激し，かつ抗酸化作用をもつため，実験モデルでの神経障害に対する保護作用が確認されている。また，PD の動物モデルでドパミン神経細胞の障害を引き起こす反応にかかわるモノアミン酸化酵素B（monoamine oxidase B；MAO-B）の活性が，喫煙者で40%低下していることも知られている。

（2）コーヒー

コーヒーなどのカフェイン飲料が PD 発症を低下させることも，10以上の臨床研究で確認されている[2][4]。PD の動物モデルで，カフェインがドパミン神経細胞の保護効果を示すとの

データがあり，作用機序としてアデノシンA_{2A}受容体の阻害を介するメカニズムなどが考えられている。

（3）飲酒

アルコール依存の既往があるとPD発症が少ないとの報告があるが，通常量の飲酒習慣と発症リスクの関連性は証明されていない[5]。ワインやリキュールと比較し，ビールを飲むことでPD発症率が低下する傾向を指摘するものもある。機序として，ビール摂取に伴う尿酸値の上昇と，フリーラジカルに対する細胞保護作用の関連が考えられている。

（4）非ステロイド性抗炎症薬（NSAIDs）

抗炎症・抗酸化作用をもつNSAIDsとPD発症に関する検討も多くなされ，発症リスクの低下を指摘するものもある。しかしデータの詳細をみると，関連性がないとする報告[4]から，アスピリンのみ，あるいはイブプロフェンのみが発症リスクの低下と関連する，また男性のみで関連性があるなど，結果は一致していない。

（5）運動習慣

小規模の臨床研究がいくつかあるが，一致した結論は得られていない。そのうち前向き研究は2つのみで，一方は，運動習慣が発症リスクを男性においてのみ60%低下させることを示したが，女性では確認されなかった。もう一方の研究は男性のみを対象としたが，運動習慣によるリスクの低下は確認されなかった。

（6）食事

全脂質摂取量を検討した小規模研究がいくつかあるが，一致した結論は得られていない。オランダのRotterdam studyは，食事から摂取される全脂質と不飽和脂肪酸が多いほどPD発症のリスクが低下していることを示した。不飽和脂肪酸は細胞膜の重要な構成成分で，抗炎症作用をもち，またドパミン神経にかかわる内因性のカンナビノイドの前駆体で，PD発症に関連している可能性がある。また女性では，総コレステロール値が高いと，PD発症のリスクが低下することも指摘されている。

（7）ビタミンB_6

前述のRotterdam studyでは，食事から摂取されるビタミンB_6が喫煙者において発症リスクを有意に低下させるとしている。ビタミンB_6の効果にも抗酸化作用が予想されている。

（8）その他

PDの治療や進行を抑制させる薬として，ビタミンEやコエンザイムQ_{10}も検討されている。なかでもコエンザイムQ_{10}は強力な抗酸化作用をもち，1,200mg/日の内服による治療効果も報告されているが，証明されるには至っておらず，現時点では予防効果も不明である。

4．パーキンソン病の病前性格と嗜好品

前述のように，喫煙やコーヒーの嗜好がPD発症リスクを低下させることが，多くの疫学的研究結果として報告されている。このデータを根拠として，ニコチンやカフェインなど特定の化学物質がドパミン神経変性に保護的に作用している可能性が，動物モデルなどでも精力的に検討されている。しかし，PD患者では特徴的な病前性格が指摘されており，これが嗜好品の選択などの行動に影響していると解釈する視点もある。すなわち，PD患者は“仕事をベッドの中に持ち込むように勤勉”で，“タバコや酒に溺れることは滅多にない”尊敬すべき特質をもっているとされ，病前性格の検討でも，“几帳面”“抑うつ的”で“社会性はもつものの，他者との関わりについては消極的”であり，“用心深い”“冒険を好まず柔軟性が低い”ことなどが指摘されている。したがって，喫煙，飲酒，コーヒーなどの嗜好に関しても“厳格”“消極的”である可能性がある[6]。運動症状が出現してPDと診断される以前に，このような性格・心理的な変化が生じ，行動，思考や習慣の様式に影響している可能性についても慎重に検討する必要があろう[7]。

おわりに

PD発症の防御因子として，喫煙とコーヒーは特に注目されている。もちろん筆者は，PD予防目的として喫煙を勧めようとは考えない。喫煙が虚血性心疾患，脳血管障害，悪性腫瘍などのさまざまな病態にかかわる危険因子であることは疑いの余地がないからである。本文中にも記載したように，ニコチンなどのタバコに含まれる防御因子が将来分離される可能性を期待したい。その他，多くの防御因子が指摘されているが，本文中でも触れたように病因や予防効果が確立されたものはな