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ORIGINAL ARTICLE

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Localization of Serine Racemase and Its Role in the Skin

Ran Inoue[1], Yoko Yoshihisa[2], Yosuke Tojo[3,7], Chieko Okamura[3,7], Yuzo Yoshida[3,7], Jiro Kishimoto[3,7], Xinghua Luan[1,8], Masahiko Watanabe[4], Mineyuki Mizuguchi[5], Yuko Nabeshima[5], Kenji Hamase[6], Kenji Matsunaga[2], Tadamichi Shimizu[2] and Hisashi Mori[1]

D-Serine is an endogenous coagonist of the *N*-methyl-D-aspartate (NMDA)–type glutamate receptor in the central nervous system and its synthesis is catalyzed by serine racemase (SR). Recently, the NMDA receptor has been found to be expressed in keratinocytes (KCs) of the skin and involved in the regulation of KC growth and differentiation. However, the localization and role of SR in the skin remain unknown. Here, using SR-knockout (SR-KO) mice as the control, we demonstrated the localization of the SR protein in the granular and cornified layer of the epidermis of wild-type (WT) mice and its appearance in confluent WT KCs. We also demonstrated the existence of a mechanism for conversion of L-serine to D-serine in epidermal KCs. Furthermore, we found increased expression levels of genes involved in the differentiation of epidermal KCs in adult SR-KO mice, and alterations in the barrier function and ultrastructure of the epidermis in postnatal day 5 SR-KO mice. Our findings suggest that SR in the skin epidermis is involved in the differentiation of epidermal KCs and the formation of the skin barrier.

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INTRODUCTION

The *N*-methyl-D-aspartate (NMDA)–type glutamate receptor is one of the three major subtypes of ionotropic glutamate receptors and has a prominent role in the central nervous system (Nakanishi, 1992; Bliss and Collingridge, 1993; Ozawa *et al.*, 1998). Recent studies have demonstrated that in addition to being expressed in central nervous system, the NMDA receptor is also expressed in keratinocytes (KCs) in the human skin and is involved in the regulation of KC growth and differentiation and in the maintenance of cutaneous barrier homeostasis (Fuziwara *et al.*, 2003; Nahm *et al.*, 2004).

The NMDA receptor is a heteromeric complex consisting of a GluN1 subunit and at least one of the four types of the GluN2 subunit (Kutsuwada *et al.*, 1992). D-Serine, a D-amino acid abundant in the mammalian brain, is an endogenous ligand of the glycine site of the NMDA receptor (Hashimoto *et al.*, 1992). The activation of NMDA receptors requires, besides the binding of glutamate to the GluN2 subunit, the binding of glycine or D-serine to the glycine site on the GluN1 subunit (Dingledine *et al.*, 1999). D-Serine synthesis is catalyzed by serine racemase (SR), an enzyme that is highly abundant in the brain and directly converts L-serine to D-serine (Wolosker *et al.*, 1999). D-Serine is efficacious in potentiating the activity of NMDA receptors (Fadda *et al.*, 1988; Matsui *et al.*, 1995), and its reduction was demonstrated to greatly decrease NMDA receptor activity (Mothet *et al.*, 2000). We previously demonstrated that NMDA receptor–mediated excitotoxicity can be attenuated in SR knockout (SR-KO) mice (Inoue *et al.*, 2008). Although the expression and roles of the NMDA receptor in the skin have been demonstrated, it is still unclear whether SR is localized in the skin epidermis and whether a mechanism of conversion of L-serine to D-serine by SR exists in KCs.

In this study, using SR-KO mice as the control, we localized SR expression by immunohistochemistry and confirmed the localization of this protein in the epidermis of wild-type (WT) mice and cultured WT KCs. We further investigated the conversion of L-serine to D-serine in cultured WT and

[1]*Department of Molecular Neuroscience, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan;* [2]*Department of Dermatology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan;* [3]*Shiseido Innovative Science Research and Development Center, Yokohama, Japan;* [4]*Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan;* [5]*Department of Structural Biology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan and* [6]*Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan*

[7]*In accordance with Shiseido's company policy on limitation of animal experiments, these authors performed only in vitro assays, such as measurement of amino acids and quantitative real-time PCR analysis.*

[8]*Current address: Department of Neurology and Institute of Neurology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China*

Correspondence: Hisashi Mori, Department of Molecular Neuroscience, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan. E-mail: hmori@med.u-toyama.ac.jp

Abbreviations: K10, keratin 10; KC, keratinocyte; P5, postnatal day 5; PBS, phosphate-buffered saline; NMDA, N-methyl-D-aspartate; SC, stratum corneum; SG, stratum granulosum; SR, serine racemase; SR-KO, serine racemase–knockout; TEWL, transepidermal water loss; TGase 3, transglutaminase 3; WT, wild type

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SR-KO KCs, the expression of proteins involved in KC differentiation, the ultrastructure of the epidermis, and the function of the epidermal barrier.

RESULTS

SR protein expression in epidermis and cultured KCs

We first examined the SR protein expression in the epidermis of the skin by western blot analysis. The SR protein signal was detected in the epidermis of WT mice, but not in the epidermis of SR-KO mice (Figure 1a). The expression level of SR protein in the epidermis was ~100 times lower than that in the brain (data not shown). We next examined the localization of the SR protein in the skin by immunofluorescence staining. As shown in Figure 1b, SR immunopositivity was detected in the granular and cornified layers of the skin in WT mice but not in those of SR-KO mice. To determine SR localization in the epidermal subcompartments, the differentiation markers involucrin and keratin 10 (K10), the former normally present in the granular and cornified layers (Tharakan *et al.*, 2010) and the latter in the spinous and granular layers (Fuchs *et al.*, 1992), were selected for their double immunofluorescence staining with SR. The double staining showed that some immunopositivity signals of SR overlapped with those of involucrin (Figure 1c) but rarely with those of K10 (Figure 1d), suggesting that SR is present mainly in the cornified layer and partially in the granular layer.

We further examined SR protein expression in the cultured KCs derived from the skin of WT or SR-KO mice. In agreement with the finding of localization of SR in the granular and cornified layers of the skin in WT mice, SR protein expression was detected in confluent cultured WT KCs, but not in growth-phase KCs of this genotype (Figure 2a). In contrast, no SR was detected in either the growth-phase or confluent SR-KO KCs (Figure 2a). Consistent with the findings of immunocytochemical analysis, western blot analysis revealed the absence of SR in the lysates from growth-phase KCs derived from either WT or SR-KO mice. SR immunopositivity signals were detected in the lysates from confluent WT KCs, but not in the lysates from confluent SR-KO KCs (Figure 2b).

Synthesis of D-serine in cultured KCs derived from skin

The ability of SR to convert L-serine to D-serine was analyzed using confluent KCs derived from the skin of WT or SR-KO mice. Under our culture conditions without addition of L-serine, the concentration of intracellular D-serine was significantly higher in WT KCs than in SR-KO KCs (Figure 3a, left). There was no significant difference in intracellular L-serine concentration between WT and SR-KO KCs (Figure 3a, middle). The addition of 10 mM L-serine to the culture medium resulted in larger increases in intracellular D-serine and L-serine concentrations in WT than in SR-KO KCs (Figure 3b, left and middle). The concentration ratio of D-serine to total serine was significantly higher in WT KCs than in SR-KO KCs under both culture conditions—that is, with and without the addition of L-serine (Figure 3a and b, right).

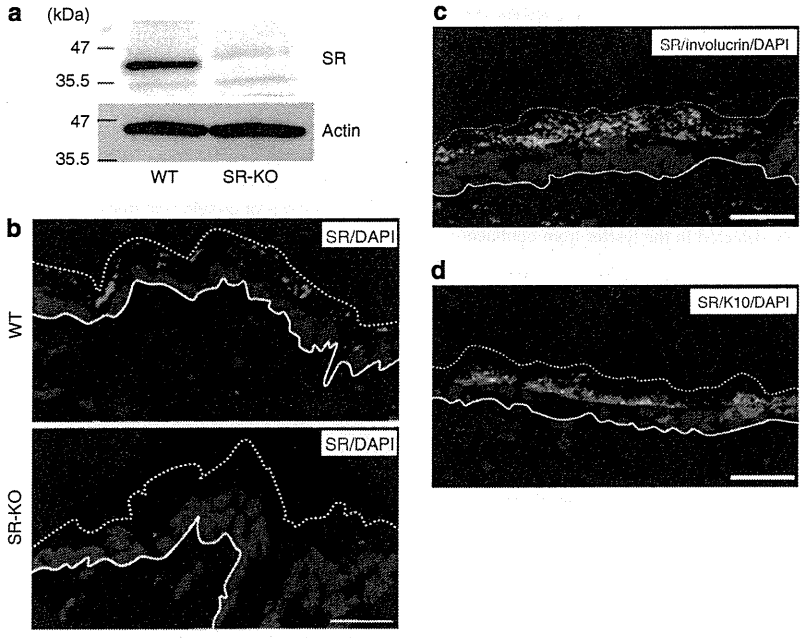

Figure 1. Expression of serine racemase (SR) in mouse skin. (**a**) Western blot analysis of epidermal proteins in wild type (WT) and SR-knockout (SR-KO) mice using anti-SR and anti-actin antibodies. (**b**) Immunohistochemical staining of skin from WT and SR-KO mice with anti-SR antibody (green). SR immunopositivity was detected in the granular and cornified layers of the skin from WT mice but not from SR-KO mice. (**c**, **d**) Double immunofluorescence staining of skin from WT mice using anti-SR (green) and anti-involucrin (**c**, red) or anti-K10 antibodies (**d**, red). K10, keratin 10. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). The dotted lines indicate the skin surface. The lines indicate the border between the epidermis and the dermis. Scale bars = 20 μm.

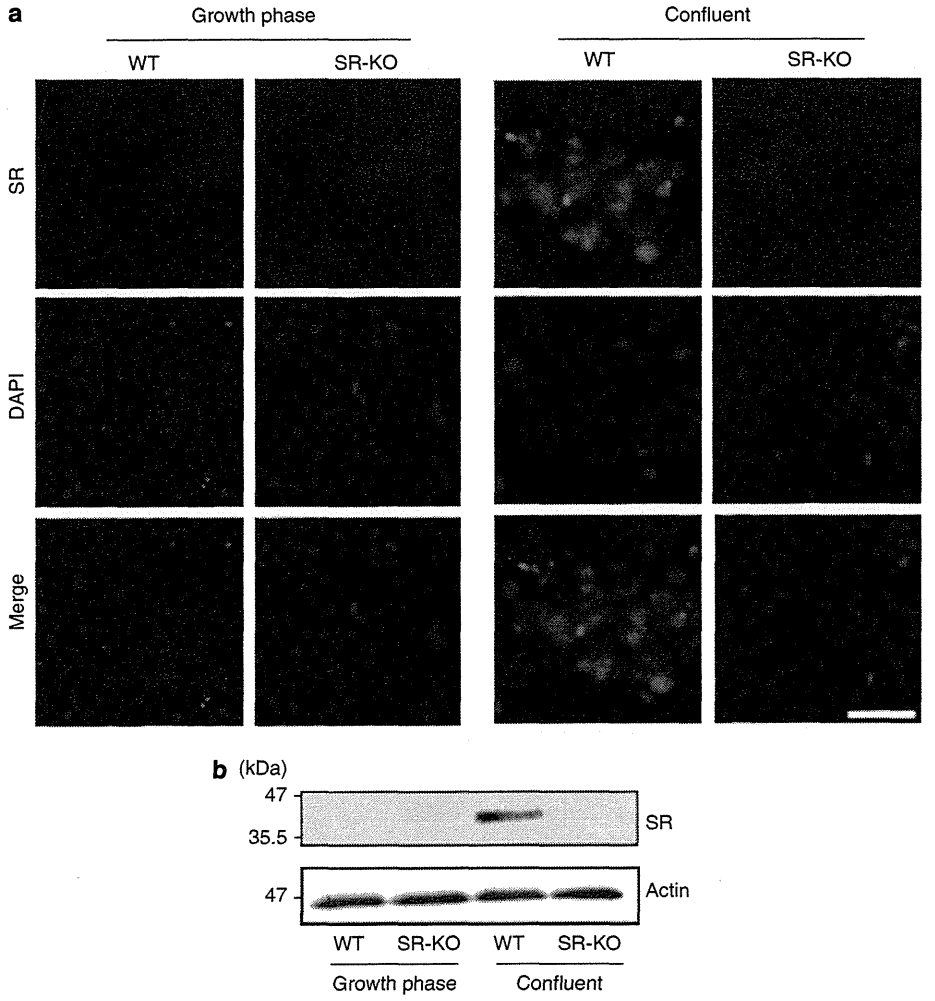

Figure 2. Expression of serine racemase (SR) in skin-derived cultured keratinocytes (KCs). (**a**) Immunofluorescence staining of SR (green) in growth-phase (left columns) and confluent KCs (right columns) derived from skin of wild type (WT) and SR-knockout (SR-KO) mice. SR immunopositivity was only detected in confluent WT KCs. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Scale bar = 100 μm. (**b**) Western blot analysis of proteins in the lysates from growth-phase and confluent KCs using anti-SR and anti-actin antibodies. The positions of protein size markers are indicated on the left side. The SR protein band of ~38 kDa was only detected in the lysates from confluent WT KCs.

Expression levels of mRNAs and proteins involved in differentiation of epidermal KCs

On the basis of the localization of SR in the cornified and granular layer of the epidermis, we examined by quantitative real-time PCR the mRNA expression levels of filaggrin, involucrin, and loricrin in the epidermis of WT and SR-KO mice that are involved in the differentiation of epidermal KCs (Steinert and Marekov, 1995). The mRNA expression level of transglutaminase 3 (TGase 3), an enzyme involved in the formation of the epidermal barrier (Nemes and Steinert, 1999; Hitomi, 2005), was also examined. The mRNA expression levels of involucrin and TGase 3 in the epidermis of SR-KO mice were significantly higher than those in the epidermis of WT mice (Figure 4). The expression levels of these protein markers and K10 were further examined by immunohistochemistry. Among the examined proteins, the involucrin (Figure 5a and b) and K10 (Figure 5a and c) proteins showed significantly higher immunopositivity signal intensities in SR-KO mice than in WT mice. There were no marked differences in the immunopositivity signal intensities of filaggrin and loricrin between WT and SR-KO mice (data not shown).

Barrier function of skin

On the basis of the above changes observed in the SR-KO epidermis, we determined whether deletion of SR results in alteration in the barrier function of the skin. Because we detected SR expression in the skin of WT mice on postnatal day 5 (P5) (data not shown), skin permeability, transepidermal water loss (TEWL), and recovery of disrupted skin barrier were examined in newborn and P5 WT and SR-KO mice. The skin of either WT or SR-KO newborn mice was not permeable to toluidine blue (Supplementary Figure S1 online). However, the

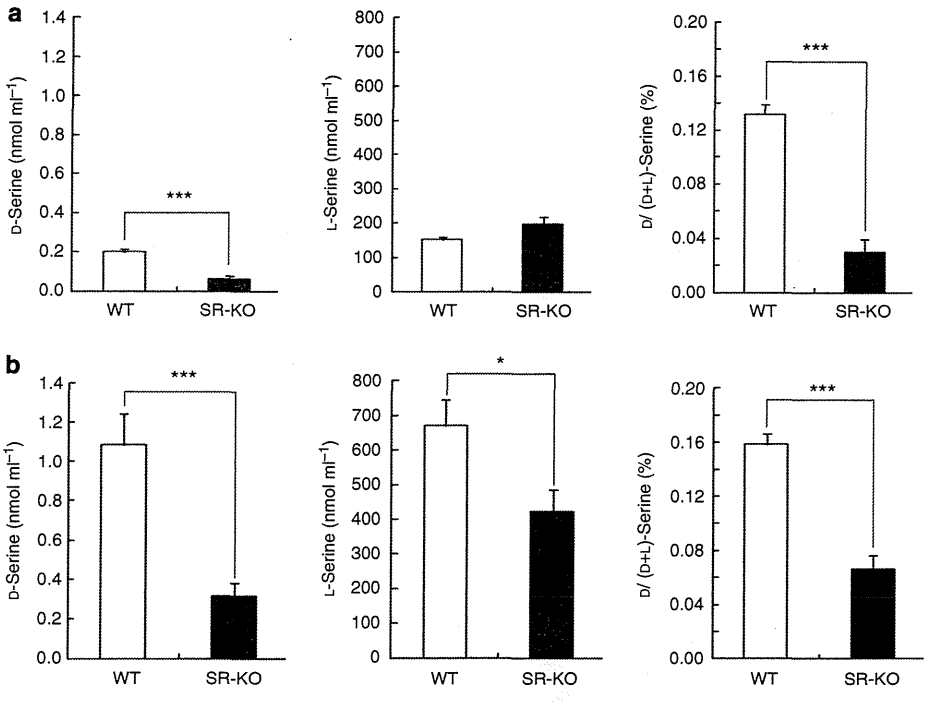

Figure 3. Concentrations of intracellular D-serine and L-serine in wild type (WT) and serine racemase–knockout (SR-KO) keratinocytes (KCs) cultured in medium with and without the addition of L-serine. (**a**) Concentrations of intracellular D-serine and L-serine in extracts of KCs cultured in medium without addition of L-serine. (**b**) Concentrations of intracellular D-serine and L-serine in extracts of KCs cultured in medium with addition of 10 mM L-serine. Data are presented as mean ± SEM ($n=7$). *$P<0.05$; ***$P<0.001$; two-tailed Student's *t*-test.

level of TEWL in SR-KO mice was significantly higher than that in WT mice (Figure 6a). In the assay of barrier recovery, SR-KO mice exhibited significantly lower recovery rates at 4 and 6 hours after tape stripping than did WT mice (Figure 6b).

We also assessed the healing rate of wounds produced in the dorsal skin of adult WT and SR-KO mice, and found no marked difference in the time needed for complete healing of wounds between the two genotypes (Supplementary Figure S2 online).

Ultrastructural analysis of epidermis

The SR-KO mice did not show any gross histological abnormality in the skin examined with hematoxylin and eosin staining (data not shown). The dorsal skin obtained from P5 mice was further examined by electron microscopy. Although no marked change was observed in the formation of lamellar bodies, the exocytosis of lamellar granules, and the formation of lamellar bilayers in the epidermis of SR-KO mice (Supplementary Figure S3 online), the number of stratum corneum (SC) layers in this genotype of mice was significantly decreased when compared with WT mice (Figure 6c–e). Moreover, in the transition zone of the stratum granulosum (SG) of the SR-KO epidermis, the keratohyalin granules were markedly enlarged (Figure 6f and g).

DISCUSSION

The epidermis of the skin functions as a barrier against the environment through the uppermost layer of terminally differentiated, denucleated KCs, namely, the cornified layer (Nemes and Steinert, 1999; Candi *et al.*, 2005), that forms the end point of epidermal differentiation and barrier formation. In this study, we found the localization of SR protein expression in the granular and cornified layers of the skin in WT mice, changes in the expression levels of markers of differentiation (involucrin, TGase 3, and K10) in SR-KO mice, and the alteration of the barrier function in SR-KO mice. These findings imply that SR is involved in the terminal differentiation of KCs and the formation of the epidermal barrier. Confluency is a stage representing the terminal differentiation of cultured WT KCs (Botta *et al.*, 2012). In our *in vitro* assay, SR immunopositivity was identified only in confluent WT KCs but not in growth-phase KCs that further suggests an association between SR expression and the terminal differentiation of KCs.

In view of the SR immunopositivity, SR seems predominantly expressed in the cornified layer, and with terminal differentiation many functional proteins in epidermal KCs are degraded, including the NMDA receptor. The question arising here is whether the racemization catalyzing the conversion of L-serine to D-serine exists in the epidermis. Although we were unable to directly examine SR activity in the cornified layer, we speculate about the function of SR in the epidermis on the basis of the following reasons. First, from the determined molecular weight of the SR protein (~38 KDa) from the skin, the protein is expected to be of full-length size and not degraded. Second, the overlapping of SR and involucrin expressions indicates that SR may at least be partially

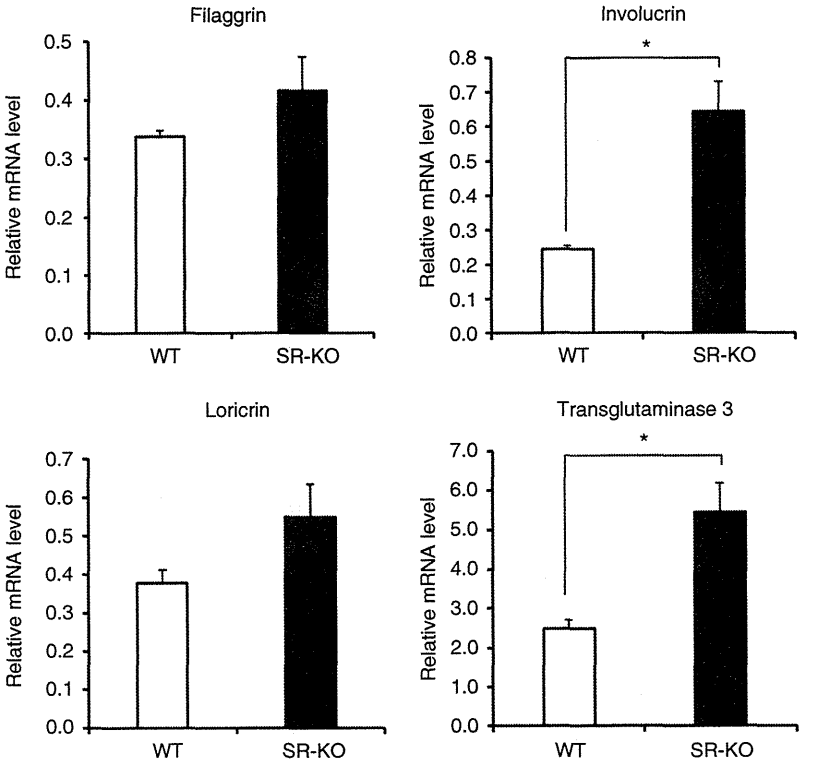

Figure 4. The mRNA expression levels of filaggrin, involucrin, loricrin, and transglutaminase 3 (TGase 3) in the epidermis as determined by quantitative real-time PCR analysis. The relative mRNA expression levels of filaggrin, involucrin, loricrin, and TGase 3 in the epidermis of wild type (WT) and serine racemase–knockout (SR-KO) mice were normalized against the level of glyceraldehyde-3-phosphate dehydrogenase mRNA. The mRNA expression levels of involucrin and TGase 3 in the epidermis of SR-KO mice were significantly higher than those of WT mice. Data are presented as mean ± SEM ($n = 3$). *$P < 0.05$; two-tailed Student's *t*-test.

expressed in the granular layer. Third, the increased involucrin, TGase 3, and K10 expression levels in SR-KO epidermis indicate the association of SR function with the differentiation of KCs in the granular layer. Finally, our *in vitro* assay demonstrated the existence of a mechanism for the conversion of L-serine to D-serine through racemization by SR in the epidermal KCs. These findings suggest that SR and D-serine are required for KC differentiation and the maintenance of the physiological function of the skin. One study demonstrated that an enzyme isolated from frog skin secretions catalyzes the isomerization of L-amino acids in peptides to the D-type (Jilek *et al.*, 2005) that further suggests the importance of D-amino acids and racemization in skin functions.

Besides converting L-serine to D-serine, SR has the activity operating in reverse racemase mode, converting D-serine to L-serine (Foltyn *et al.*, 2005). The condensation reaction between L-serine and palmitoyl-CoA, catalyzed by serine palmitoyltransferase, is the first step in the *de novo* biosynthesis of ceramides (Holleran *et al.*, 1990; Hanada, 2003; Breiden and Sandhoff, 2013). As serine palmitoyltransferase strictly uses L-serine as its amino acid substrate (Hanada *et al.*, 2000), it is possible that SR in SG may have a role in the synthesis of ceramides by catalyzing the mutual conversion of L-serine and D-serine to maintain an appropriate level of L-serine.

The increased level of TEWL and the significantly reduced rates of barrier recovery in P5 SR-KO mice reveal an alteration in the barrier function of the SR-KO skin. Formation of the skin barrier requires not only the formation of the SC lipid-enriched extracellular matrix, but also the corneocyte formation (Hohl, 1990; Nemes and Steinert, 1999). During the final stages of epidermal differentiation, outer SG cells transform into anucleate corneocytes, with highly resilient cornified envelopes. The significant decrease in the number of SC layers observed in SR-KO mice is assumed to result from the impairment in this transformational process that consequently exerts an influence on the barrier function of the epidermis or its recovery after acute disruption by tape stripping. The influx of calcium ions into KCs through the NMDA receptor has been shown to have an important role in KC differentiation. In one pharmacological study, blockade of keratinocytic NMDA receptors with MK-801 suppressed the expression of differentiation markers such as K10 and filaggrin (Fischer *et al.*, 2004a, b). Furthermore, parakeratotic cornification was demonstrated to be associated with the reduced level of NMDAR1(GluN1) expression (Fischer *et al.*, 2004b). Taken

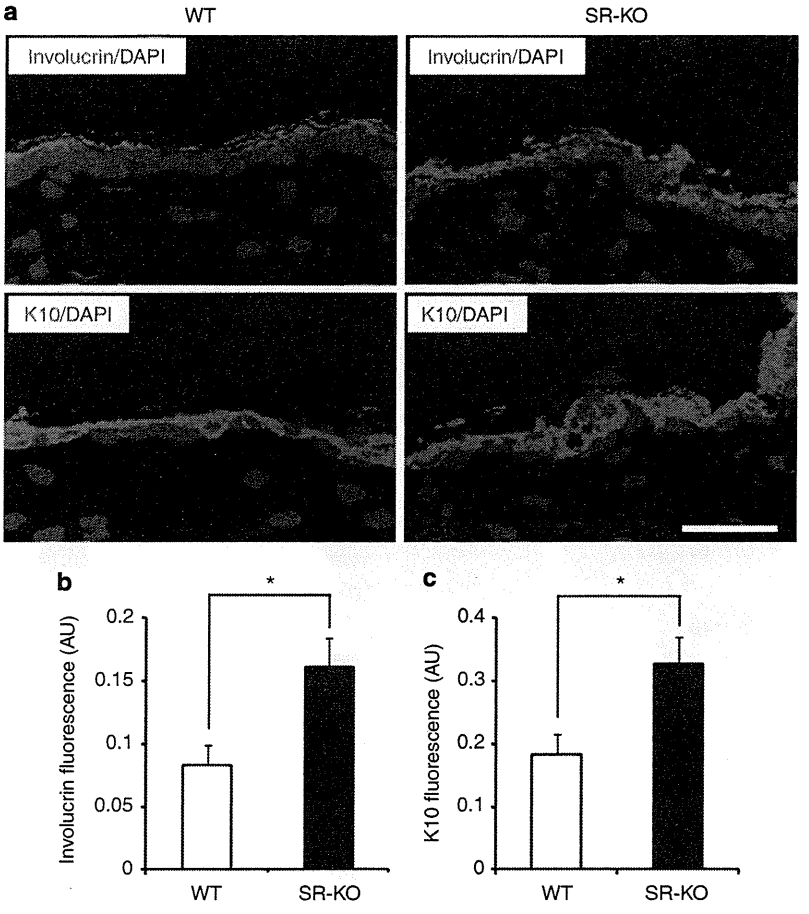

Figure 5. Expression of involucrin and keratin 10 (K10) in the epidermis of wild type (WT) and serine racemase–knockout (SR-KO) mice.
(**a**) Immunofluorescence staining of skin from WT and SR-KO mice with anti-involucrin (magenta) and anti-K10 antibodies (magenta). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Scale bar = 20 μm. (**b**, **c**) Graphs showing the immunopositivity signal intensities of involucrin (**b**) and K10 (**c**) in the epidermis of WT and SR-KO mice. The expression levels of involucrin and K10 proteins were significantly higher in SR-KO mice than in WT mice. Data are presented as mean ± SEM ($n = 5$). AU, arbitrary units. *$P < 0.05$; two-tailed Student's *t*-test.

together, the negative influence on NMDA receptor function resulting from the deficiency of D-serine in SR-KO mice may affect KC cornification.

Accordingly, an enlargement of keratohyaline granules was observed in the transition zone of the SG in the epidermis of the P5 SR-KO mice. Although there is no evidence showing a direct association between keratohyaline granules and barrier function of the skin, it is likely that the abnormally enlarged keratohyalin granules in the SG of SR-KO mice may indicate the effect of SR-KO on KC differentiation and may affect the production of filaggrin (Dale *et al.*, 1978) that is important for skin barrier (Candi *et al.*, 2005).

It is worth mentioning that our data on the recovery of barrier function are inconsistent with one previous report (Fuziwara *et al.*, 2003) in which the recovery of skin barrier after tape stripping in hairless mice was delayed by the topical application of NMDA receptor agonists, presumably through an NMDA receptor–mediated mechanism of accelerating calcium influx into KCs and consequently perturbing the secretion of lamellar bodies, and such delay was erased by NMDA receptor antagonists. This inconsistency is probably attributed to the following reasons: (1) differences in the pharmacological and genetic approaches; (2) the different types of mice at different ages that were used for analysis; and (3) the developmental deletion of SR that affects the KC differentiation and leads to a significant decrease in the number of SC layers as observed in P5 mice that may overcome the influence resulting from an increase or a decrease in calcium influx into KCs on the secretory system of lamellar granules.

There is also another inconsistent finding in SR-KO mice: the lack of diffusion of toluidine blue into the skin and the increased TEWL. This inconsistency is probably attributed to the methodological validity. Dye diffusion is appropriate for the measurement of a large magnitude of barrier disruption, whereas TEWL is sensitive for the measurement of subtle

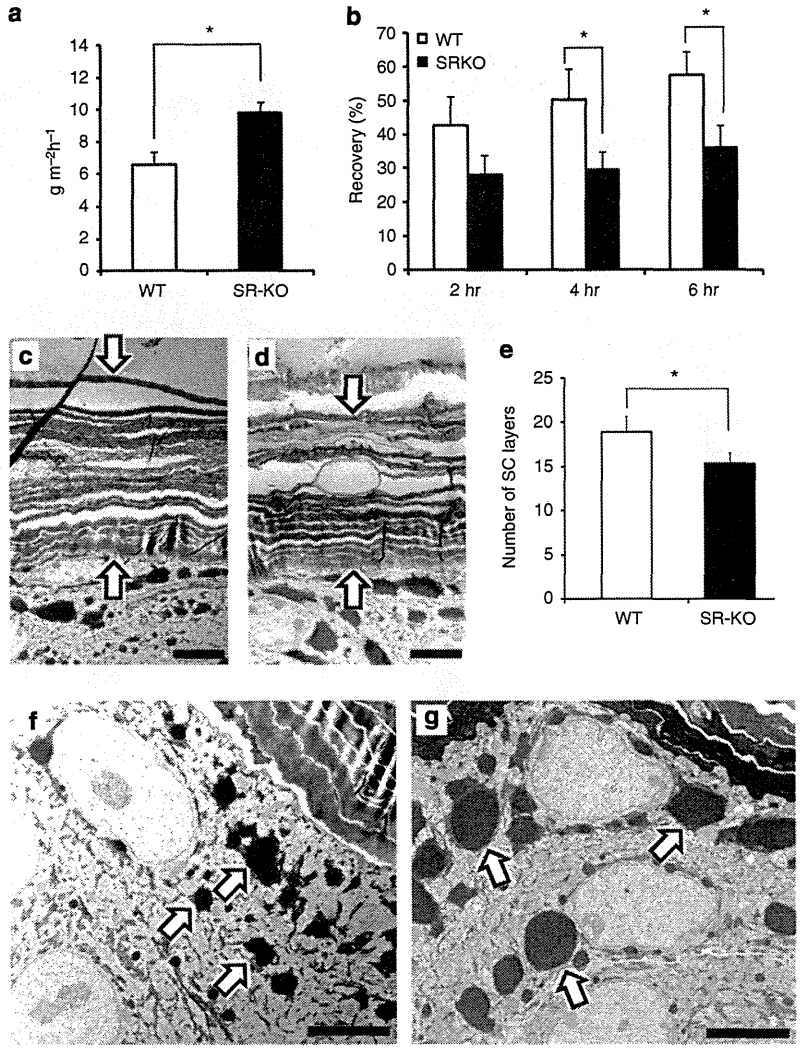

Figure 6. Barrier function and ultrastructural analysis of skin. (**a**) Transepidermal water loss (TEWL) in the dorsal skin of postnatal day 5 (P5) wild type (WT) and serine racemase–knockout (SR-KO) mice. (**b**) Barrier recovery of P5 mice 2, 4, and 6 hours after tape stripping. (**c–g**) Electron microscopy of P5 mice epidermis. Stratum corneum (SC) of (**c**) WT and (**d**) SR-KO mice. Arrows indicate the outermost and innermost layers of SC. (**e**) Bar graph showing the number of SC layers in WT and SR-KO mice. (**f**, **g**) Keratohyalin granules (arrows) in the transition zone of the stratum granulosum of the (**f**) WT and (**g**) SR-KO mice. Scale bars = 5 μm. Data are presented as mean ± SEM. **P*<0.05; two-tailed Student's *t*-test.

changes in barrier function (Indra and Leid, 2011). The effect of the deletion of SR on the skin barrier function observed in P5 mice needs to be examined in adult mice.

MATERIALS AND METHODS

Animals

Animal care and experimental protocols were carried out basically in accordance with the "Guidelines for the Care and Use of Laboratory Animals, DHEW, publication no. (NIH) 80-23, revised 1996" and approved by the Experimental Animal Committee of the University of Toyama (Authorization No. 2010-MED-61). The SR-KO mice with 100% C57BL/6 genetic background were generated as previously reported (Miya *et al.*, 2008). The WT and SR-KO mice were used for analyses in a genotype-blind manner.

Antibodies

The pET-His expression system (Novagen, Birmingham, UK) was used to produce a His-tagged fusion protein containing the full length of mouse SR amino acids. The glutathione *S*-transferase (GST) fusion protein expression system (GE Healthcare, Buckinghamshire, UK) was used to produce a GST-tagged fusion protein containing amino acid residue nos. 150–190 of the mouse SR ($SR_{150–190}$). Polyclonal antibodies against His-tagged SR were produced in rabbits and guinea pigs and were further purified using an antigen-affinity column coupled with the GST fusion protein $SR_{150–190}$. Antibodies against SR were used at a concentration of 0.5 mg ml^{-1} for western blot analysis, immunohistochemistry, and immunocytochemistry. A rabbit polyclonal anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to

loricrin, involucrin, filaggrin, and K10 were purchased from Covance (San Diego, CA).

Western blot analysis

The skin epidermis from WT and SR-KO mice was homogenized in ice-cold mammalian protein extraction reagent (Pierce, Rockford, IL). Protein extracts (100 μg) were subjected to SDS-PAGE, and separated proteins were transferred onto polyvinylidene difluoride membranes. After blocking with a solution containing 5% skim milk in phosphate-buffered saline (PBS, pH 7.4), the membranes were incubated with a rabbit anti-SR or rabbit anti-actin (1:2,000) polyclonal antibody overnight at 4 °C, then with a horseradish peroxidase–conjugated secondary antibody for 1 hour. Protein bands were detected using an ECL chemiluminescence detection system (GE Healthcare).

Immunohistochemistry

Frozen skin tissues from WT and SR-KO mice were cut into 25-μm-thick sections using a freezing microtome and were mounted on slides. The cryosections of the skin were fixed in 0.1 M phosphate buffer (PB, pH 7.4) containing 4% (w/v) paraformaldehyde for 30 minutes, rinsed in PBS, and blocked with Protein Block Serum-Free (DakoCytomation, Carpinteria, CA) for 10 minutes at room temperature. The sections were then incubated with primary antibodies (guinea pig anti-SR, rabbit anti-SR, anti-involucrin, anti-filaggrin, anti-loricrin, or anti-K10 antibodies) diluted in PBS containing 1% BSA overnight at 4 °C. After washing in PBS, the sections were incubated with Alexa Fluor 488– and Alexa Fluor 594–conjugated species-specific secondary antibodies (Invitrogen, Carlsbad, CA) for 1 hour at room temperature. The sections were then washed in PBS, counterstained with 4′,6-diamidino-2-phenylindole (Vector, Burlingame, CA), and coverslipped. Images were obtained using a confocal laser scanning microscope (Leica TCS-SP5, Leica Microsystems, Mannhein, Germany). For the quantitative analysis of immunopositivity signals, the obtained images were analyzed using the public domain Java image processing program ImageJ (National Institutes of Health, Bethesda, MD).

Culture of primary KCs

The excised skin samples from 1-day-old WT and SR-KO mice were floated on CnT-07 medium (CELLnTEC Advanced Cell Systems, Bern, Switzerland) supplemented with antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B) and 0.1% dispase II (Invitrogen), and incubated overnight at 4 °C. Epidermal sheets were then separated from the dermis with forceps and treated with TrySELECT (Invitrogen) for 30 minutes to isolate KCs. The cells were collected by centrifugation and then seeded in 3.5 cm culture dishes containing CnT-07 medium. They were then incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Western blot analysis of KC proteins (100 μg) was performed as described above.

Immunocytochemistry

Confluent or growth-phase KCs that were cultured on glass-bottomed dishes were immersed in 4% paraformaldehyde for 30 minutes for fixation, incubated for 10 minutes in PBS containing 0.1% Triton X-100 for permeabilization, and blocked with PBS containing 3% BSA for 30 minutes. Thereafter, the cells were incubated with a guinea pig anti-SR antibody overnight at 4 °C, followed by incubation with donkey anti-guinea pig IgG conjugated with Alexa Fluor 488 for 1 hour at room temperature. The cells were rinsed in PBS after each treatment. Finally, the cells were counterstained with 4′,6-diamidino-2-phenylindole. Images were obtained using a fluorescence microscope.

Measurement of intracellular D-serine and L-serine in cultured KCs

Confluent KCs were further cultured in media supplemented with 10 mM L-serine for 48 hours. The concentration of intracellular D-serine and L-serine was analyzed by two-dimensional HPLC (Miyoshi *et al.*, 2011) as described in Supplementary Materials and Methods online.

Quantitative real-time PCR analysis

RNA was prepared from the epidermis of the back skin, then reverse transcribed and subjected to quantitative real-time PCR as described in Supplementary Materials and Methods online.

Cutaneous barrier function

TEWL was measured on the back skin of P5 mice using an evaporimeter (VapoMeter SWL2g; Delfin Technologies, Kuopio, Finland). In the assay of barrier recovery, epidermal barriers of P5 mice were disrupted by tape stripping until the TEWL reached 30–40 g m^{-2} h^{-1}. In each animal, the percentage of recovery was calculated by the following formula: (TEWL immediately after barrier disruption – TEWL at indicated time point)/(TEWL immediately after barrier disruption – baseline TEWL) × 100%.

Electron microscopy

The back skin from P5 mice was minced into 1-mm-thick blocks and fixed immediately in 0.1 M cacodylate buffer (pH 7.4) containing 2% paraformaldehyde and 2% glutaraldehyde overnight at 4 °C. The samples were then washed with 0.1 M cacodylate buffer and followed by postfixation with 2% osmium tetroxide in 0.1 M cacodylate buffer at 4 °C for 3 hours. For observation of lamellar membrane and lamellar bodies, mouse skin was postfixed with 2.5% ruthenium tetroxide in 0.1 M cacodylate buffer. The blocks were ultrathin, sectioned at 70 nm, and were examined using a JEM-1400 electron microscope (JEOL, Tokyo, Japan).

The number of SC layers was determined from osmium postfixed samples. Three points were selected at random and the number of SC layers was counted.

Statistical analyses

All values are presented as mean ± SEM. The statistical significance of difference between WT and SR-KO mice was determined by two-tailed Student's *t*-test. Values of $P<0.05$ were considered statistically significant. For quantitative real-time–PCR data, *P*-values were corrected for type I errors using the Benjamini–Hochberg method.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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短　報

伝染性単核球症に続発し脳脊髄液に抗グルタミン酸受容体δ2抗体をみとめた急性小脳失調症

村上　秀友[1)3)]* 飯島　昭二[2)]　河村　満[3)]
高橋　幸利[4)]　市川　博雄[1)]

要旨：症例は18歳の女性である．伝染性単核球症（IM）で入院し病状は軽快傾向にあったが，第4病日に歩行時のふらつき，めまい，悪心が急性に出現した．神経学的所見では四肢体幹の小脳性運動失調をみとめた．脳脊髄液検査，頭部画像所見や神経伝導検査に異常はなく，急性小脳失調症（ACA）と診断し，ステロイドパルス療法をおこない数日で軽快した．本例は脳脊髄液の抗グルタミン酸受容体δ2（GluRδ2）抗体が陽性であり，IM後のACAとの関連について考察した．

（臨床神経 2013;53:555-558）
Key words：伝染性単核球症，急性小脳失調症，Epstein-Barrウイルス，抗グルタミン酸受容体δ2抗体

はじめに

先行感染やワクチン接種後に急性に小脳性運動失調を発症する急性小脳失調症（acute cerebellar ataxia; ACA）の病態は未解明で，従来の多くの症例報告で病態を特定しかねていた．今回，われわれは伝染性単核球症（infectious mononucleosis; IM）にひき続き発症したACA例を経験し，脳脊髄液で抗グルタミン酸受容体δ2（GluRδ2）抗体が陽性であったことから発症機序について考察した．

症　例

症例：18歳の女性
主訴：歩行時のふらつき，めまい，悪心
既往歴・家族歴：特記すべきことなし．

現病歴：2011年5月中旬に咽頭痛，上腹部痛および食欲低下が出現し増悪した．6日後に近医を受診し，身体所見で白苔をともなう扁桃肥大，頸部リンパ節腫大，血液検査で異型リンパ球の出現や肝障害をみとめ，IMがうたがわれ対症療法が開始された．上腹部痛と食欲低下がいちじるしいため発症8日目（第1病日）に消化器内科に入院した．入院時に身体所見で扁桃肥大，肝脾腫を，血液検査で末梢血への異形リンパ球の出現と肝機能障害をみとめ，血清抗体価によりEB（Epstein-Barr）ウイルス初感染のIMと診断された．入院後は対症療法がおこなわれ各症状は軽快傾向にあったが，第4病日に歩行時のふらつき感やめまいが急性に出現し，悪心も増悪したため，第5病日に神経内科を受診した．

一般身体所見（第5病日）：特記すべき異常をみとめず．

神経学的所見（第5病日）：意識は清明で，高次脳機能，脳神経，運動系，感覚系に特記すべき異常をみとめず，髄膜刺激徴候もみとめなかった．協調運動系は指鼻試験，反復拮抗運動，踵膝試験で四肢の小脳性運動失調が著明で，体幹失調のため座位や立位の保持も困難であった．

検査所見：入院時の血液検査では，白血球数6,100/μl，うち19％が異形リンパ球であった．入院時の生化学・免疫学的検査では，GOT 331 U/l，GPT 355 U/l，LDH 655 U/lと上昇していた．EBウイルス関連の抗体価については，抗EBNA抗体は10倍，抗VCA-IgG抗体は2,560倍，抗VCA-IgM抗体は320倍であった．第11病日の血清では抗ガングリオシド抗体および抗GluRδ2抗体は陰性であった．脳脊髄液検査では，第6病日において細胞数1/μl，糖56 mg/dl，タンパク質46 mg/dlと異常をみとめず，EBウイルスDNAのPCR法および抗VCA（IgG，IgM）抗体も陰性であった．第11病日の検体では抗GluRδ2抗体が陽性であった．頭部MRI（T_1，T_2，拡散強調像）では異常をみとめず，末梢神経伝導検査（右正中，尺骨，腓骨，脛骨の各神経の運動・感覚神経伝導検査，F波，ならびに腓腹神経の感覚神経伝導検査）でも異常をみとめなかった．

経過（Fig. 1）：経過，現症，検査所見よりIMにともなう

*Corresponding author: 昭和大学藤が丘病院脳神経内科〔〒227-8501　神奈川県横浜市青葉区藤が丘1-30〕
1) 昭和大学藤が丘病院脳神経内科
2) 済生会神奈川県病院神経内科
3) 昭和大学医学部内科学講座神経内科学部門
4) 静岡てんかん・神経医療センター小児科
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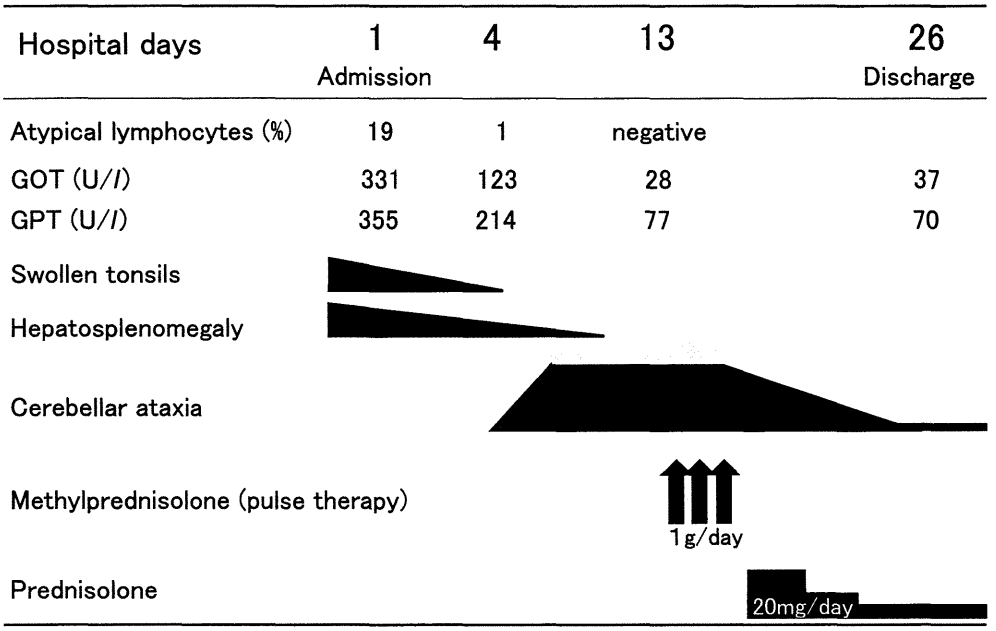

Fig. 1　Clinical course after admission.
The patient was admitted because of infectious mononucleosis. After the admission her condition improved. But on hospital day 4, she suddenly developed cerebellar ataxia in the trunk and four limbs. We diagnosed acute cerebellar ataxia and performed methylprednisolone pulse therapy. After this therapy, her cerebellar ataxia improved over a few days.

Table 1　Previously reported cases presenting cerebellar ataxia accompanied with anti-GluRδ2 antibody.

Case	Age (years)	Sex	Antecedent disease	Duration of the illness	Anti-GluRδ2 antibody		Abnormal MRI findings in the cerebellum
					Serum	CSF	
Sugiyama et al.[4] (2004)	3	M	Diarrhea, vomiting	More than 16 months	+	+	−
Usui et al.[5] (2011)	13	F	Vaccination (MR)	More than 9 months	+	+	−
Shimokaze et al.[6] (2007)	13	M	Unknown	Less than 3 weeks	+	−	+
Kubota et al.[7] (2008)	4	F	Vaccination (DPT)	20 months	−	+	−
Ichikawa et al.[8] (2009)	2	F	Respiratory infection	More than 9 months	+	unknown	−
Shiihara et al.[9] (2007)	1	M	Respiratory infection, Varicella	2 months	+	+	−
Present case	18	F	Epstein-Barr virus infection	3 weeks	−	+	−

CSF: cerebrospinal fluid, MR: measles and rubella, DPT: diphtheria, pertussis and tetanus.

ACAと診断し，第13病日からメチルプレドニゾロンパルス療法（1,000 mg/日を3日間，連日静注）をおこない，引き続いてプレドニゾロンを20 mg/日より漸減投与した．その結果，第18病日から症状は急速に改善し，第26病日に独歩で退院した．

考　察

ACAはワクチン接種や先行感染を機に突然の小脳症状が出現し，自然治癒傾向があると考えられている．またACAは，発症の誘因に各種の感染症やワクチン接種があること，症状が遷延する症例もあること，脳脊髄液や画像などの検査所見は症例毎に様々であることなど，病像が一様ではないことも指摘されている[1]．本例のようにEBウイルス感染がACA発症の誘因になりえること[1]が知られ症例報告も散見されるが，その発症機序はEBウイルスの直接浸潤説[2]や免疫介在説[3]が想定されているが不明である．本例では脳脊髄液中のEBウイルスDNAや抗VCA（IgM，IgG）抗体が陰性であり，EBウイルスの髄腔内への直接浸潤を示唆する根拠はえられなかったが，脳脊髄液に抗GluRδ2抗体をみとめた点が既報告にはない初の知見である．

抗GluRδ2抗体を検出したEBウイルス感染あるいはIM後のACA症例は過去に報告がないが，同抗体を検出した小脳障害例は過去に6例[4)~9)]報告されている（Table 1）．いず

れも若年例で何らかの先行感染あるいはワクチン接種後に発症している点は本例と類似するが，症状の持続期間や血清および脳脊髄液中の抗体検出パターンは一定していない．さらに，抗GluRδ2抗体が小脳障害の結果として産生されるのか，小脳障害の要因であるのかについては結論がえられてはいないが，GluRδ2サブユニットがPurkinje細胞に発現し小脳機能に関与している[10]ことから，Purkinje細胞の障害と同抗体の産生には関連性が示唆される．抗GluRδ2抗体が小脳障害の結果産生されるとする説を支持するのは急性期に一過性に血清に同抗体を検出したShimokazeら[6]の報告で，炎症により小脳組織が障害され，GluRδ2サブユニットが遊離し，抗原提示された結果，2次的に同抗体が血清に出現したと考察している．Shimokazeらの症例[6]は本例および他の既報告5例[4)5)7)-9)]とことなり頭部MRIで小脳実質内に異常所見をともなっている点，本例および他の既報告4例[4)5)7)9)]とことなり脳脊髄液には同抗体をみとめない点が相違するため，本例とは病態がことなると思われる．一方，臼井ら[5]，Kubotaら[7]，Shiiharaら[9]は先行感染などを誘因とする免疫学的機序を介して誘導された抗GluRδ2抗体が小脳障害をきたしたと考察している．これらの3症例で同抗体が症状の持続期間にわたり検出されたことは，同抗体が主な病態を形成していたことを支持する．本例では経時的な抗GluRδ2抗体の検索をおこなえず，脳脊髄液中のみで同抗体が産生された理由を明確に説明しがたいが，ステロイド治療によりすみやかに治癒したことも考慮するとEBウイルス感染を契機とした免疫機序により同抗体が髄腔内で誘導されACAを発症した可能性が考えられる．ACA発症と先行感染および抗GluRδ2抗体との関連性を解明するために今後の症例蓄積が期待される．

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※本論文に関連し，開示すべきCOI状態にある企業，組織，団体はいずれも有りません．

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Abstract

A case of acute cerebellar ataxia following infectious mononucleosis accompanied by intrathecal anti-glutamate receptor δ2 antibody

Hidetomo Murakami, M.D.[1)3)], Shoji Iijima, M.D.[2)], Mitsuru Kawamura, M.D.[3)], Yukitoshi Takahashi, M.D.[4)] and Hiroo Ichikawa, M.D.[1)]

[1)]Department of Neurology, Showa University Fujigaoka Hospital
[2)]Department of Neurology, Saiseikai Kanagawaken Hospital
[3)]Department of Neurology, School of Medicine, Showa University
[4)]Department of Pediatrics, National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders

An 18-year-old woman was admitted because of sore throat and pain in the epigastric region. On admission, she presented with swollen tonsils and hepatosplenomegaly. Blood examinations revealed the presence of atypical lymphocytes, liver damage and anti-VCA IgM and IgG antibodies. These findings led to diagnosis of infectious mononucleosis. After admission, her condition improved, but on hospital day 4, she suddenly developed cerebellar ataxia in the trunk and four limbs. Cranial MRI findings were normal. Cerebrospinal fluid (CSF) collected on hospital day 6 showed normal cell counts and normal concentrations of protein and glucose. EB virus DNA and anti-VCA IgM and IgG antibodies were negative and glutamate receptor δ2 antibody was positive in CSF collected on hospital day 11. We diagnosed acute cerebellar ataxia (ACA) and performed methylprednisolone pulse therapy. After this therapy, her cerebellar ataxia improved over a few days. This is the first reported case of ACA after EB virus infection presenting with glutamate receptor δ2 antibody in CSF. The glutamate receptor δ2 subunit is expressed on cerebellar Purkinje cells. Therefore, the presence of the antibody may be associated with cerebellar dysfunction. In the present case, secondary immune reactions after EB virus infection may have produced the antibody.

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Key words: infectious mononucleosis, acute cerebellar ataxia, Epstein-Barr virus, anti-glutamate receptor δ2 antibody

＝原著論文＝

難治 epileptic spasms を有する症例における ACTH 療法反復施行の検討

池上真理子[1,2] 高橋 幸利[1] 池田 浩子[1] 今井 克美[1] 大谷 英之[1]
久保田裕子[1] 重松 秀夫[1] 高山留美子[1] 最上友紀子[1]

要旨

【目的】ACTH 療法反復施行の有効例の特徴を見出す.

【方法】難治 epileptic spasms を有する症例で，初回 ACTH 療法が無効あるいは初回 ACTH 療法有効後再発した症例 25 例において，ACTH 療法反復施行後，全てんかん発作が 2 カ月以上抑制された場合を短期抑制効果ありとし，Kaplan-Meier 法を用いた長期効果の検討を行った.

【結果】短期効果は，2 回目施行時に epileptic spasms のみの症例では 76.5%で有効，複数の発作型をもつ症例では有効例はなかった.

長期効果では，複数発作型をもつ群と比較して epileptic spasms のみをもつ群で有意に発作消失期間が長く，treatment-lag が 2 カ月以内の症例では長期効果が優れていた.

【結論】ACTH 療法反復施行では短期効果・長期効果ともに発作型の影響が大きいと考えられた.

見出し語 epileptic spasms，ACTH 療法，West 症候群，発作予後，有効性

はじめに

West 症候群は hypsarrythmia，epileptic spasms，精神運動発達遅滞を有するてんかん性脳症の 1 つで，早期の発作コントロールが発達予後に関与するとされている．West 症候群の治療には，vitamin B_6，抗てんかん薬，ACTH 療法，ガンマグロブリン療法などがあるが，発作予後は必ずしも良好とはいえない．ACTH 療法の有効性は 1958 年 Sorel らによって報告され[1]，日本てんかん学会の治療ガイドラインでも最も有効な治療法であると記載され，発症後早期の使用が推奨されている．ACTH 療法の短期効果は，無作為化対照試験では 42～87%で発作を抑制する[2)～7)]．しかし，ACTH 療法は再発例も多く，ACTH の副作用として，高血圧，電解質異常，易感染性，肥満，易刺激性，視床下部－下垂体機能不全，副腎皮質機能不全，肥大型心筋症，消化管潰瘍，大脳退縮，硬膜下血腫などがあり，脳発達に及ぼす影響なども懸念され，近年では短期隔日投与[8)]や少量投与[9)]など，総投与量抑制を目指す報告が多い．ACTH 療法後の再発例では，抗てんかん薬やガンマグロブリン療法などが試されるが，発作予後は芳しくない．当センターでは，各種治療に抵抗性を示す難治 epileptic spasms を有する症例で，ACTH 療法を反復施行することで発作抑制に加え発達促進がみられた症例を経験し，ACTH 療法反復施行の有効性や副作用を明らかにするために，後方視的検討を行った.

I 対象・方法

1. 症例選択基準

2002 年 10 月から 2010 年 9 月までに国立病院機構静岡てんかん・神経医療センターでの加療歴がある難治 epileptic spasms を有する症例のうち，他病院での治療も含め ACTH 療法が 2 回以上施行され，発病年齢，ACTH 投与時期，投与量，投与時発作型，脳波所見が明記されている 25 症例につき，診療録を元に後方視的に検討した（表 1）．ACTH 療法 3 回目施行は 3 例あった（表 2）．発作型は，ビデオ脳波同時記録により判定した．Epileptic spasm は，瞬間的な全身の強直で，四肢の屈曲あるいは伸展，この両者の混合を示すものとした[10)]．Spasm を数秒の間隔をおいて数回繰り返すものをシリーズ形成 spasms（spastic cluster；SC）とし，繰り返すことがなく単発のものを，単発 spasm（single spasm；SS）とした.

2. 対象症例の特徴

明らかな基礎疾患がなく，発病前の発達が正常で，神経学的異常所見および画像所見で異常のない症例を潜因性，発病前より精神発達遅滞や他のてんかん発作などの何らかの脳障

[1]国立病院機構静岡てんかん・神経医療センター小児科
[2]東海大学医学部付属八王子病院小児科

連絡先 〒192-0032 八王子市石川町 1838
東海大学医学部付属八王子病院小児科（池上真理子）
E-mail:ikegami@is.icc.u-tokai.ac.jp

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表 1-1 25症例の臨床背景 1回目 ACTH 療法時

No.	性	病因/併存症	てんかん発病月齢	初回 ACTH 治療				
				月齢	発作型	脳波	1回投与量 mg/kg/day	合併症
1	F	不明/CP+MR	0 (3 days)	2	SC M	S-B	0.0125	—
2	M	結節性硬化症	1	4	SC	hyps	0.0070	—
3	F	不明/CP+MR	1	9	PS	S-B+Lt sp	0.0125	—
4	M	CDKL5 異常/CP+MR	1	7	SS SC	mod hyps	0.0125	CMV 感染
5	M	1p36 欠失/CP+MR	1	28	SC	multi sp+HVS	0.0125	—
6	M	不明/MR	2	4	SC	S-B	0.0125	洞性不整脈
7	F	周産期障害/MR+CP+PDD	3	6	SS	hyps	0.0125	—
8	F	異所性灰白質/MR+CP	4	5	SC	hyps	0.0150	—
9	F	皮質形成異常/CP+MR	4	6	SC	hyps	0.0128	—
10	M	不明/MR, 左不全麻痺	4	12	SC	P-O sp	0.0250	—
11	F	不明/MR	4	8	SC	hyps	0.0125	感染
12	F	Aicardi 症候群	5	6	SC	hyps	0.0100	—
13	M	周産期障害, HIE/CP+MR	5	12	TS SC	hyps	0.0125	筋緊張亢進, 高血圧
14	F	不明/皮質形成異常? MR	5	6	SC	dif sp & w	0.0125	—
15	M	不明/CP+MR	5	9	SC	hyps	0.0100	—
16	M	不明/脳形成異常? CP+MR	5	6	SC	mod hyps	0.0125	食欲亢進
17	M	21トリソミー	6	7	SC	hyps	0.0125	—
18	M	不明/MR	6	8	SC	dif sp & w	0.0150	—
19	M	周産期障害/Williams 症候群	7	8	SC	hyps	0.0100	—
20	M	周産期障害/CP+MR, 甲状腺機能低下症, 両側難聴	8	12	SC	hyps	0.0120	感染
21	F	結節性硬化症	8	11	SC	dif sp & w	0.0125	—
22	F	周産期障害/CP, CLD, MR	8	11	SC	hyps	0.0125	不機嫌, 不眠
23	F	周産期障害+脳形成異常/MR+CP	9	15	SC	hyps+focal sp & w	0.0060	感染
24	F	脳形成異常/協調運動障害	18	50	M ASC	dif sp & w+sh	0.0100	体重増加
25	F	不明/MR	18	18	SC	multi+dif HVS	0.0140	—

MR: mental retardation, CP: cerebral palsy, PDD: pervasive developmental disorders, HIE: hypoxic ischemic encephalopathy, CLD: chronic lung disease, SS: single spasm, SC: spasms in cluster, M: myoclonic seizure, TS: tonic seizure, PS: partial seizure, ASC: atypical spasms in cluster, hyps: hypsarrhythmia, multi: multifocal, S-B: suppression-burst pattern, sp: spike, mod.: modified, P: parietal, O: occipital, dif: diffuse, sp & w: spike & wave, sh: sharp wave, HVS: high voltage slow, CMV: cytomegalovirus

害を示唆する所見をもつものや原因が明らかなものを症候性として判断[11]すると25例全例が症候性であった. 全例 West 症候群と診断されており, early infantile epileptic encephalopathy with suppression burst (EIEE) から West 症候群に移行した例が2例, West 症候群から症候性部分てんかんに移行した例が5例, 症候性全般てんかんに移行した例が2例, 未決定てんかんに移行した例が1例であった. 男児12例, 女児13例で, てんかん発病年齢は日齢3～1歳6カ月 (中央値0歳5カ月), 発病から初回 ACTH 投与開始までの treatment-lag は0～32カ月 (中央値3カ月), ACTH 投与時年齢は初回2カ月～4歳2カ月 (中央値0歳8カ月), 2回目8カ月～4歳11カ月 (中央値1歳4カ月), 3回目1歳3カ月～6歳10カ月 (中央値1歳11カ月) であった. ACTH 投与量や投与期間の決定は各主治医の判断により, ACTH 投与量は初回 0.0060～0.0250 mg/kg/day, 2回目は 0.0100～0.0200 mg/kg/day, 3回目は 0.0120～0.0200 mg/kg/day であった.

最終 ACTH 投与からの観察期間は2カ月から7年10カ月であった.

3. 有効性の判断

ACTH 投与後に全発作が2カ月以上消失した場合を有効として短期効果を検討, その後の再発経過を Kaplan-Meier 法により Mantel-Cox test を用いて長期効果として検討した.

表 1-2　25 症例の臨床背景　2 回目 ACTH 療法時

No.	月齢	発作型	脳波	1 回投与量 mg/kg/day	効果	発作消失期間	合併症
1	12	SC	hyps	0.0125	有効	6 カ月	感染
2	8	SC	hyps	0.0120	無効	—	—
3	12	SC	hyps	0.0125	無効	—	—
4	16	SS SC	hyps	0.0150	無効	—	—
5	33	SS	multi sp	0.0200	有効	8 カ月	強直発作，不機嫌，不眠，軽度高血圧
6	20	SC SS	dif polysp	0.0200	無効	—	洞機能不全
7	9	PS SS	multi sp & w＋hyps	0.0125	無効	—	—
8	15	SC	multi sp & w	0.0200	再発	9 カ月	—
9	9	SC	dif sp & w	0.0120	再発	3 カ月	低 K 血症，不機嫌，体重増加，過敏性
10	18	SC	multi sp＋dif. sp & w	0.0200	有効	3 カ月	耳下腺腫脹
11	18	SC	sp & w burst (Lt>Rt)	0.0200	再発	5 カ月	—
12	56	SC	dif sp & w	0.0100	無効	—	—
13	27	TS SC	multi sp	0.0110	無効	—	筋緊張亢進
14	22	SC	dif sp & w	0.0100	再発	1 カ月	—
15	12	SC	Rt P-O ～ dif polysp & w	0.0100	無効	—	—
16	9	SC	Lt sp	0.0125	有効	3 カ月	食欲亢進
17	12	SS	dif sp & w	0.0125	有効	14 カ月	軽度高血圧，不機嫌
18	28	SC SS	dif sh & s	0.0200	再発	6 カ月	体重増加，活気低下
19	15	SC	hyps	0.0125	再発	5 カ月	高血圧，洞性不整脈，イレウス
20	16	SC	hyps	0.0120	再発	3 カ月	—
21	12	SC M	multi sp	0.0200	無効	—	—
22	13	SC SS	multi sp & w	0.0200	無効	—	洞性徐脈，脳退縮
23	19	SS	hyps＋sp & w	0.0200	再発	4 カ月	—
24	59	PS SC	multi sp & w	0.0140→0.018	無効	—	体重増加
25	29	SC	multi sp＋HVS	0.0200	再発	6 カ月	—

SS:single spasm, SC:spasms in cluster, M:myoclonic seizure, TS:tonic seizure, PS:partial seizure, hyps:hypsarrhythmia, sp:spike, dif:diffuse, multi:multifocal, polysp:polyspike, P:parietal, O:occipital, sp & w:spike & wave, sh:sharp wave, sh & s:sharp & slow wave, HVS:high voltage slow

表 2　ACTH 療法 3 回目施行例

No.	月齢	発作型	脳波	1 回投与量 mg/kg/day	効果	発作消失期間	合併症
9	15	SC	dif polysp	0.0120	再発	13 カ月	—
20	23	SC SS	hyps＋Rt F sp & w	0.0150→0.0200	再発	6 カ月	—
25	60	SC M PS TS	multi sp＋dif HVS	0.0130	無効	—	強直発作一過性に増加

SS:single spasm, SC:spasms in cluster, M:myoclonic seizure, TS:tonic seizure, PS:partial seizure, hyps:hypsarrhythmia, dif:diffuse, multi:multiple focal abnormality, F:frontal, sp:spike, sp & w:spike & wave, HVS:high voltage slow

Ⅱ 結　　果

1 回目 ACTH 療法施行時には SS，SC 含め epileptic spasms のみをもつ症例が 20 例（80.0%），ミオクロニー発作（myoclonic seizure;M）や強直発作（tonic seizure;TS），部分発作（partial seizure;PS）を併せもつ症例が 5 例（20.0%）であるが，2 回目施行時には epileptic spasms のみの症例が 68.0%と減少し，複数発作型をもつ症例が増加していた（図 1-A）．脳波所見は 2 回目施行時には hypsarrhythmia をもつ症例が減少した（図 1-B）．

初回 ACTH 療法は 25 例中 12 例（48.0%）で短期効果が認められた．

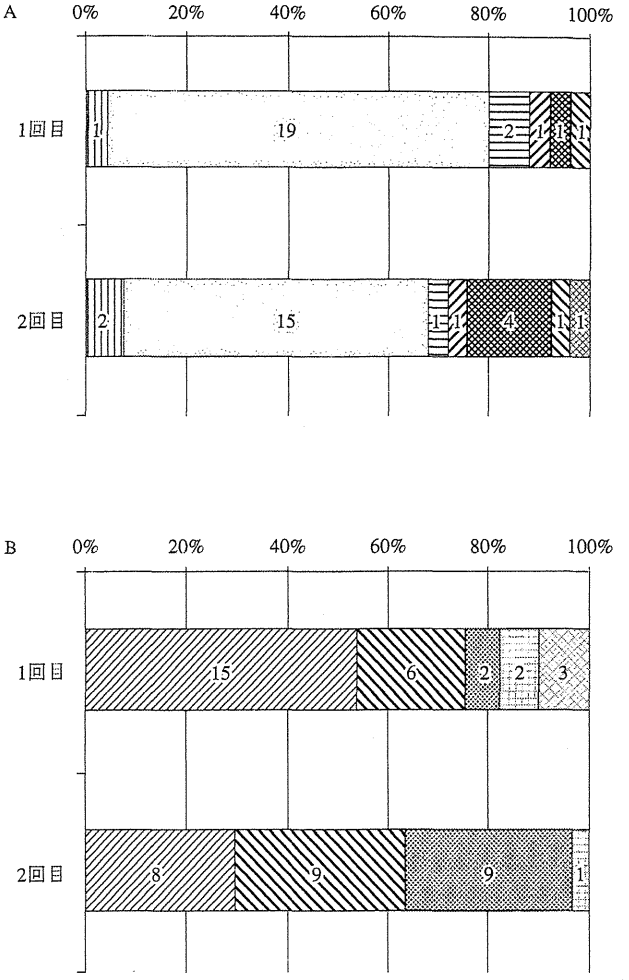

図1 ACTH療法時の発作型・脳波所見

A：ACTH療法時の発作型

B：ACTH療法時脳波所見

▥：SS, ⬚：SC, ▤：SC+M, ▨：SC+TS, ▩：SS + SC, ▧：SC+PS, ▩：SS+PS

▨：hyps, ▧：diffuse, ▩：multi, ▤：focal, ▨：SB

SS:single spasm, SC:spasms in cluster, M:myoclonic seizure, TS:tonic seizure, PS:partial seizure

hyps:hypsarrythmia, diffuse:diffuse interictal discharge, multi:multifocal interictal discharge, focal:focal interictal discharge, SB:suppression-burst pattern

1. 2回目投与の短期効果

2回目ACTH療法は25例中13例（52.0%）で有効であった．病因別にみると，出生前因子をもつ9例中4例（44.4%），出生時因子をもつ6例中3例（50.0%），原因不明の10例中5例（50.0%）で有効で，有意な差はみられなかった．短期効果で1回目無効例は13例であったが，そのうち7例は2回目投与が有効，ACTHを増量して有効となったものが5例あった．しかし，1回目より2回目ACTH投与量を増量した13例中5例は無効であった．

発作型別効果では，2回目投与時にSSのみ，SCのみ，SS+SC含めて，epileptic spasmsのみの症例では17例中13例（76.5%）で有効，複数の発作型をもつ症例では有効例はなく，ACTH投与後に新たな発作型が出現した例が1例みられた．

発病年齢別効果では，3カ月以下発病の症例では7例中2例（28.6%），4カ月以上8カ月未満の症例では12例中8例（66.7%），8カ月以上の症例では6例中3例（50.0%）で有効であり，比較的年長の1歳6カ月時発病でも有効例が1例あった．4カ月以上で発病した例では発病年齢と短期効果に明らかな差はなく，3カ月以下での発病では有効率が低い傾向があったが有意差はなかった．

Treatment-lagについては，2カ月以下の症例では11例中8例（72.7%），3カ月以上5カ月未満の症例は7例中2例（28.6%），5カ月以上の症例は7例中3例（42.9%）で有効であり，treatment-lagが2カ月以下で有効率が高い傾向にあったが，有意差はなかった．

2回目ACTH投与時の年齢と短期効果の関係では，2回目が12カ月以下の症例で9例中4例（44.4%），13カ月以上24カ月未満の症例で10例中6例（60.0%），24カ月以上の症例で6例中3例（50.0%）が有効であり，有意差はみられなかった．

2回目ACTH投与時にhypsarrhythmiaをもつ症例では7例中4例（57.1%），その他の高度脳波異常をもつ症例では18例中9例（50.0%）で有効であった．今回の検討においてはACTH反復投与の短期効果には脳波所見による差がみられなかった．

2. 2回目投与の長期効果

最終観察時に発作抑制されている症例は25例中5例（20.0%）あり，そのうち最長は14カ月の発作抑制期間を得ている．2回目ACTH投与時にepileptic spasmsのみをもつ症例と複数の発作型をもつ症例で長期効果をKaplan-Meier法で比較すると，平均発作消失期間はそれぞれ4.5カ月，0.1カ月と，複数の発作型を有する症例で明らかに再発が早い傾向が見られた（$p=0.0381$）（図2-A）．複数の発作型をもつ症例は全例短期効果判定中の2カ月以内に再発した．てんかん発病年齢で2回目ACTH後の平均発作消失期間を比較すると，生後3カ月以下の発病症例で2.29カ月，4カ月以上8カ月未満発病で4.88カ月，8カ月以降発病例で2.22カ月と有意差はなかった（$p=0.5412$）．最終観察時有効例は，4カ月以上8カ月未満に発病した3例，3カ月以下に発病した2例で，West症候群の一般的発病年齢より高い8カ月以上の発病年齢の症例では，有効例がなかった（図2-B）．2回目ACTH投与時の年齢で平均発作消失期間を比較すると，12カ月以下で5.00カ月，13カ月以上24カ月未満で3.28カ月，24カ月以上で3.39カ月であった．12カ月以下の群では早期に再発しなければ発作消失期間が長いと考えられる（図2-C）．発病から初回ACTH療法施行までのtreatment-lagで2回目ACTH投与後の平均発作消失期間を比較すると，2カ月以下で5.86カ月，3カ月以上5カ月未満で1.33カ月，5カ月以上で3.06カ月で

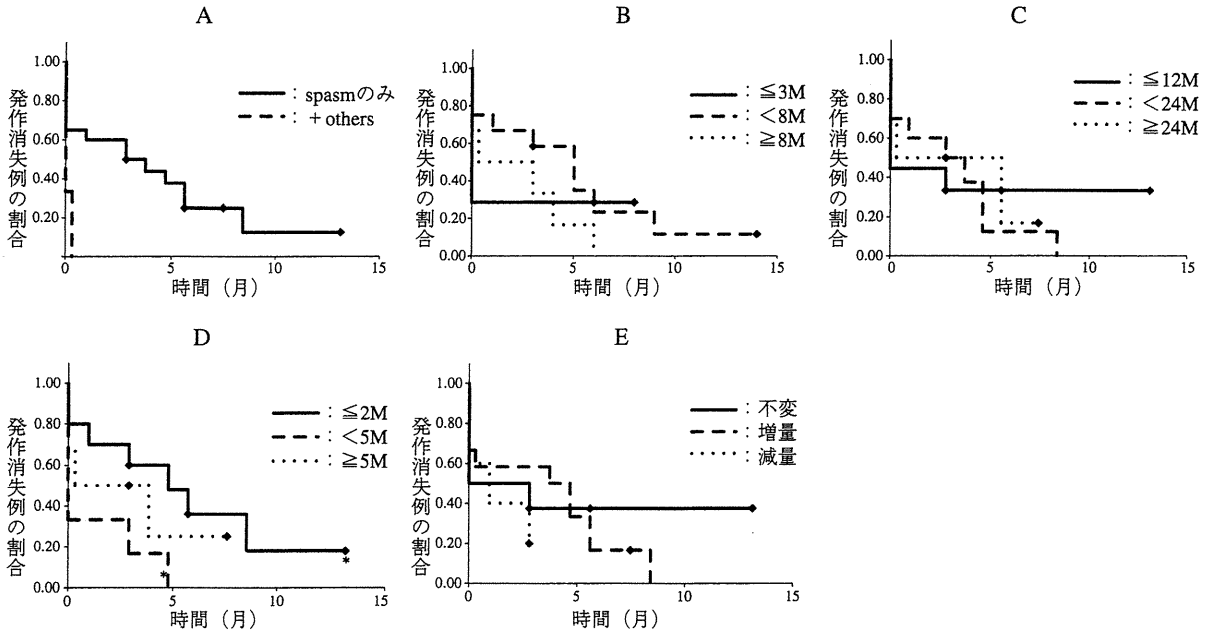

図2　2回目ACTH投与後の発作抑制例の割合

A：発作型と発作抑制例の割合推移
◆：最終観察時発作抑制例，spasmのみ：epileptic spasmsのみをもつ症例群，＋others：epileptic spasmに加えて他の発作型を有する症例群（Mantel-Cox test，p=0.0381）

B：てんかん発病年齢とACTH2回目投与後の発作抑制例の割合の推移
◆：最終観察時発作抑制例，≦3M：3カ月齢以内での発病群，<8M：4～7カ月齢での発病群，≧8M：8カ月齢以上での発病群（Mantel-Cox test，p=0.5412）

C：ACTH2回目投与時年齢と2回目投与後の発作抑制例の割合の推移
◆：最終観察時発作抑制例，≦12M：12カ月齢以内の2回目投与群，<：13～23カ月齢の2回目投与群，≧24M：24カ月齢以上での2回目投与群，（Mantel-Cox test p=0.8842）

D：Treatment-lagとACTH2回目投与後の発作抑制例の割合の推移
◆：最終観察時発作抑制例，≦2M：treatment-lagが2カ月以内の症例群，<5M：treatment-lagが3～4カ月の症例群，≧5M：treatment-lagが5カ月以上の症例群（＊Mantel-Cox test p=0.0092）

E：ACTH投与量の変化とACTH2回目投与後の発作抑制例の割合の推移
◆：最終観察時発作抑制例，不変：1回目投与量と2回目投与量が同量の群，増量：2回目投与量を増量した群，減量：2回目投与量を減量した群，（Mantel-Cox test，p=0.2126）

あった（図2-D）．Treatment-lagが3カ月以上5カ月未満の群と比較すると2カ月以下の群では発作消失期間は有意に長かった（p=0.0092）．3カ月以上5カ月未満では最終観察時の発作抑制例はなかった．2回目ACTHの投与量においては，0.0125 mg/kg/day以下の群とそれ以上の群では再発経過に明らかな差はみられなかったが（p=0.7673），平均発作消失期間は2回目投与量を減量した群では1.4カ月，不変の群では5.63カ月と減量した群が短い傾向にあった（図2-E）．脳波所見では，hypsarrhythmiaをもつ症例ともたない症例で2回目ACTH投与後の平均発作消失期間を比較すると，再発経過に差はみられなかった（p=0.6060）．

3．2回目投与の有害事象

25例中9例に何らかの合併症が起こった．感染症4例（15%）不整脈，高血圧などの循環器症状が2例（8%）などであるが，ACTH療法中断に至る重篤な有害事象はみられなかった．

4．3回目投与の効果

3回以上ACTH療法を施行している3例中2例はepileptic spasmsのみをもつ症例で，6カ月，1年1カ月の長期の発作消失期間が得られた（表2）．

Ⅲ　考　　察

ACTHの難治てんかん発作に対する効果は1950年にKleinらによって報告された[12]．その後1958年Sorelらにより infantile spasms症例における有効性が報告され[1]，以降West症候群における治療の中心となっている．Mackayらのreview[2)13)]によると，infantile spasmsにおけるACTH療法の（初回）発作抑制効果は42～87%，hypsarrhythmiaの消失も同様に42～87%と報告されている．Valproate sodiumは22～73%，vitamin B_6は13～29%，topiramateは45%，zonisamideは33～36%の発作抑制効果とされている．ACTH療法は強力な治療といえるが，至適投与法は確立されず，長

期効果は確立できていないとされている[2)14)]．現存の治療法の中で強力な治療とされるACTH療法は，短期効果の無効例が0～70%程度あり，短期有効であってもその後の再発例は30～60%程度あることから[2)]，初回ACTH無効あるいは再発例の治療法の確立が望まれる．

今回われわれは，初回ACTH無効あるいは初回ACTH後再発症例の治療選択肢としてのACTH療法反復施行について，25症例で短期効果，Kaplan-Meier法を用いた長期効果検討を行った．ACTH療法反復施行の発作抑制効果についてはこれまで報告がほとんどないが，松崎らの報告[15)]における短期発作抑制効果は1回目71%，2回目47%，3回目50%，4回目33%，5回目0%とされている．我々の症候性West症候群での検討では2カ月後の短期効果で52.0%，長期効果では25例中5例（20.0%）で発作抑制できており，初回ACTH無効例の治療効果としては十分検討に値するものと考える．われわれの検討では，treatment-lagが2カ月以下，2回目ACTH投与時にepileptic spasmsのみの症例で長期発作抑制が期待できることが分かった．このような症例を選択していくことで，ACTH療法反復施行の有効率が向上する可能性がある．

近年日本においては，副作用の軽減のためACTH投与量はより少ない方向に向かっているが，今回の検討において，松崎らの報告と同様に，初回ACTH療法が無効であっても投与量を増量することで2回目施行に効果を得られた症例があり，ACTH療法反復施行においては，十分な説明同意のもと，投与量を増量することも，発作抑制効果を高める可能性があるため考慮すべきである．本検討においては不機嫌や洞性徐脈，一過性脳退縮，内服でコントロール可能な高血圧などは認めたが，頭蓋内出血や重症感染症など重篤な合併症は認めなかった．今後も多数例での検討を行って，より安全なACTH療法反復施行を確立したい．

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