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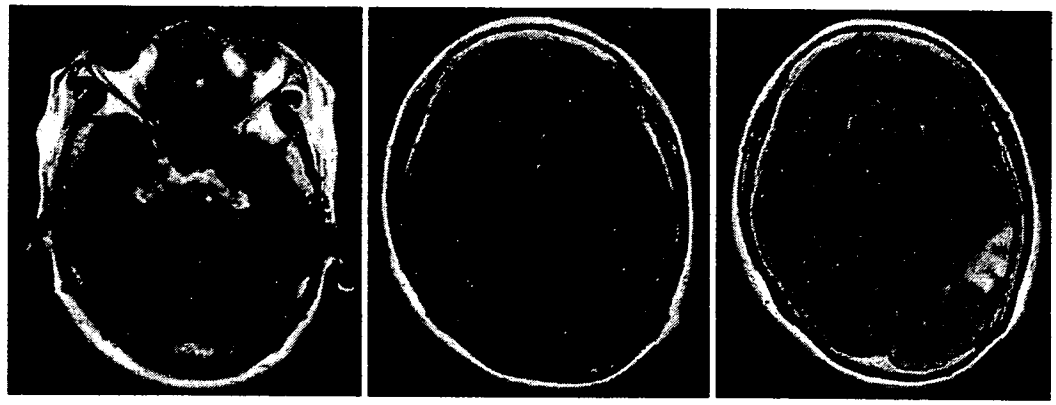

1-1 1-2 1-3

Figure 1. Magnetic resonance images on admission. FLAIR images show high-intensity lesion in the brainstem (1-1), right thalamus and caudate nucleus (1-2) at first hospitalization, and high-intensity lesion in the cortex and subcortical white matter of the left temporal lobe at second hospitalization (1-3).

(CSF) of a patient with NSD. Our results provide important clues to the pathogenesis of NSD and may contribute to the formulation of more effective preventative NSD therapies.

Patient and Methods

Patient

At first hospitalization

A 59-year-old woman had a sore throat and a fever in late August 2005. Four days later, she visited a local hospital. She was diagnosed with acute tonsilitis, admitted to a hospital and treated with antibiotic therapy in early September 2005. The day after admission, she became drowsy and she was transferred to our hospital. She had a history of acute hepatitis B viral infection. She had a temperature of 36.6℃, a pulse of 78/min, and a blood pressure of 148/65 mm Hg. She had erythematous plaques on both legs. On neurological examination, her consciousness level was semicoma and she presented with right pupillary dilatation and delayed light reflex. The deep tendon reflexes of all four limbs were hyperactive except for the bilateral Achilles tendon reflexes. Laboratory evaluation revealed increased numbers of peripheral blood leukocytes and neutrophils: white blood cell (WBC) count, $15.1\times10^3/\mu l$ (normal range: $3.3\times10^3\sim7.9\times10^3/\mu l$) and neutrophil cell count, $13.9\times10^3/\mu l$ (normal range $1.5\times10^3\sim5.9\times10^3/\mu l$). Her serum C reactive protein (CRP) level was 18.8 mg/dl (normal <0.20 mg/dl). CSF examination showed 341 cells/mm^3 (mononuclear cells, 298; neutrophilic cells, 43) and a total protein concentration of 172 mg/dl. A culture of a CSF sample was negative for bacteria, tuberculosis and fungi. Antibodies against herpes simplex virus were absent and PCR analysis also showed no herpes simplex virus. A brain MRI scan showed increased signal intensities on T2-weighted and fluid-attenuated inversion recovery (FLAIR) images in the brainstem (Fig. 1-1), right thalamus and caudate nucleus (Fig. 1-2). The electroencephalogram showed slow basic rhythm and diffuse θ activity. After admission she was treated with an intravenous infusion of antibiotics and acyclovir. Subsequently, the disturbance of consciousness became progressively worse and mechanical respiratory management was required two days after admission. She suffered a generalized tonic seizure and was treated with phenytoin. The seizures were difficult to control, however, and required treatment with the anesthetic agent propofol. Because a brain MRI scan showed increased signal intensities on T2-weighted and FLAIR images in various subcortical brain structures, a diagnosis of acute disseminated encephalomyelitis (ADEM) was suspected. Thus, four days after admission intravenous dexamethasone (12 mg/day for 5 days) was administered for 4 days and then, ten days later, methylprednisolone (1,000 mg/day for 3 days) was administered for three days. Her condition gradually improved and she did not require respiratory management. However four weeks after admission a brain MRI scan showed an abnormal signal intensity lesion in the periventricular white matter of the left parietal lobe and expansion of the brainstem lesion. Then her symptoms and abnormal brain MRI findings gradually improved and she was discharged from the hospital without any sequelae in early November 2005.

At second hospitalization

The patient had a sore throat and a fever in mid-January 2006. Five days later, she consulted an otolaryngologist and was diagnosed with acute tonsilitis. She was treated with an intravenous infusion of antibiotics. Five days later she suffered a sudden, generalized tonic seizure during infusion and was referred to our department. She had a temperature of 37.8℃, a pulse of 95/min, and a blood pressure of 153/83 mm Hg. Her throat was reddish and the palatal tonsil was swelling with velaque. Erythematous plaques were apparent on her cheek, forearms and legs. On neurological examination, she was disoriented and could not remember her name

and birthday correctly. The deep tendon reflexes of all four limbs were hyperactive predominantly in left upper and lower limbs. She presented with bilateral Hoffman reflexes and spasticity of the lower limbs. Laboratory tests revealed increased numbers of peripheral blood leukocytes and neutrophils: WBC count, $13.7\times10^3/\mu l$ and neutrophil cell count, $11.5\times10^3/\mu l$. Her serum CRP level was elevated at 11.3 mg/dl (normal <0.20 mg/dl). The serum rheumatoid factor and antibodies including antinuclear, anti-SS-A, anti-SS-B, anti-DNA, anti-Sm, and anti-RNP antibodies, and the perinuclear anti-neutrophil cytoplasmic antibody (P-ANCA), and the cytoplasmic anti-neutrophil cytoplasmic antibody (C-ANCA) were all absent. Human leukocyte antigen (HLA) typing showed B-54 and CW1. CSF examination showed 108 cells/mm^3 (mononuclear cells, 91; neutrophilic cells, 17), a total protein concentration of 41 mg/dl. A culture of the CSF sample was negative for bacteria, tuberculosis and fungi. Antibodies against herpes simplex virus, varicella zoster virus and toxoplasma were negative. PCR analysis also showed no herpes simplex virus. A brain MRI revealed increased signal intensity on T2-weighted and FLAIR images in the cortex and subcortical white matter of the left temporal lobe (Fig. 1-3). ^{99m}Tc-HMPAO SPECT performed on the third day of hospitalization revealed hyperperfusion in the left temporal lobe. An electroencephalogram showed diffuse slow activity with small spikes and sharp waves in the left temporal region. There were no ocular lesions such as uveitis, episcleritis and conjunctivitis. Neither oral aphthae nor genital ulcers were obserbed. We performed a malignancy survey including a whole-body CT, an examination by gastrointestinal endoscopy, a bone marrow aspiration study, and a gynecological consultation, all of which showed negative results. After admission she was treated with an intravenous infusion of antibiotics and acyclovir. Her consciousness was progressively disturbed and she suffered frequent generalized tonic seizures; therefore, at ten days after admission she required propofol treatment and mechanical respiratory management. A skin biopsy of the erythema on her right forearm was performed. Histological examination showed dense dermal infiltration of neutrophils with no signs of vasculitis, and as a result she was diagnosed with Sweet's disease. Corticosteroid therapy was initiated with an intravenous administration of methylprednisolone (1,000 mg/day for 3 days) from the tenth day of admission, followed by 50 mg of prednisolone administrated orally. Her symptoms gradually improved by the end of January 2006 she no longer required mechanical ventilation. However, she continued suffering from a slight fever, and elevated levels of CRP and WBCs without signs of infection and presented with aphasia. As a result, she was treated with a second intravenous administration of methylprednisolone (1,000 mg/day for 3 days) in early Feburary 2006. Subsequently, her symptoms and laboratory data improved, and she was discharged from the hospital without any sequelae about three weeks later.

Methods

Analysis of levels of cytokines and chemokines

We measured the levels of cytokines (i.e., IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α) and chemokines (i.e., CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, CXCL8/IL-8, CXCL10/IP-10 and GM-CSF) in 10 CSF samples from the patient throughout the clinical course. We also measured the levels of those cytokines and chemokines in CSF samples from the control subjects. The control subjects for cytokines were 21 noninfected patients with neurological disorders (epilepsy, 8; psychomotor delay, 5; psychogenic response, 5; functional headache, 1; myopathy, 1; agenesis of corpus callosum, 1) and the control subjects for chemokines were 10 noninfected subjects with neurological disorders (functional headache, 3; Parkinson disease, 1; normal pressure hydrocephalus, 2; spinocerebellar degenetration, 2; amyotrophic lateral sclerosis, 2). CSF samples were obtained from them on routine analysis and they all had normal CSF cell counts. All upper values of control subjects are expressed as mean + 3SD.

Determination of cytokine levels

The levels of IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 in CSF were measured with a cytometric bead array (CBA) kit (BD PharMingen, San Diego, CA) as previously described (6-8), with the exception that data analysis was performed using GraphPad Prism software (GraphPad Prism Software, San Diego, CA). The lower detection limits for IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 were 7.1 pg/mL, 2.8 pg/mL, 2.6 pg/mL, 2.6 pg/mL, 2.5 pg/mL, and 2.8 pg/mL, respectively.

Determination of chemokine levels

The levels of CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, CXCL8/IL-8, and GM-CSF were measured using ELISA kits (Endogen, Woburn, MA, USA), and the concentration of CXCL10/IP-10 was measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) on the basis of the quantitative sandwich enzyme immunoassay technique, as previously described (9). The sensitivity of these assays was 10 pg/mL.

Statistical analysis

The Spearman rank correlation was calculated to assess the correlation between the levels of cytokines and total CSF cell counts, and the levels of chemokines and total CSF cell counts.

Results

Clinical course (Fig. 2)

Clinical manifestations and brain MRI findings correlated

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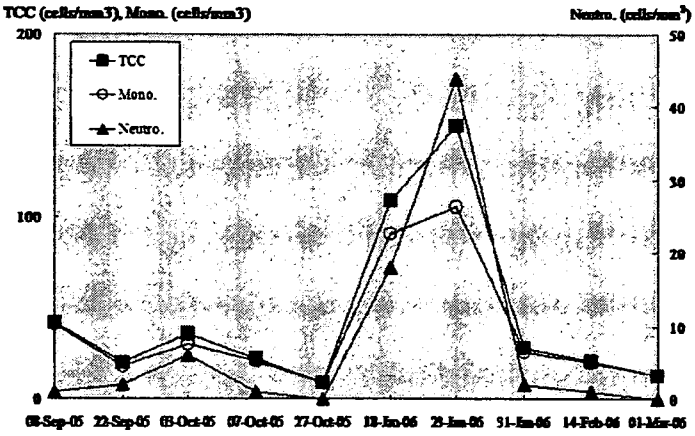

Figure 2. Cell counts [total cell count (TCC), mononuclear cell count (Mono.), neutrophilic cell count (Neutro.), cells/mm^3] in CSF.

Table 1. The Levels (pg/mL) of Cytokines (1-1), Chemokines (1-2) and Total Cell Count [(TCC), Cells/mm^3] in CSF

(1-1)

Date	8-Sep 2005	22-Sep 2005	3-Oct 2005	7-Oct 2005	27-Oct 2005	18-Jan 2006	23-Jan 2006	31-Jan 2006	14-Feb 2006	1-Mar 2006	*P value*
IL-6 (<12.1)*	209	14.5	255.8	12.3	9.3	2417.2	1329.4	182.7	13.6	12.6	<0.01
IL-4 (<14.3)*	7	<2.5	6	5	<2.5	17.6	13.2	4.4	<2.5	<2.5	<0.01
IL-2 (<5.5)*	2.7	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	ns
IFN-γ (<60.3)*	22.6	12.5	29.1	<7.1	<7.1	134.6	463.7	58	<7.1	<7.1	<0.01
TNF-α (<7.2)*	2.8	<2.8	<2.8	<2.8	<2.8	<2.8	<2.8	<2.8	<2.8	<2.8	ns
IL-10 (<7.2)*	4	3.3	4.6	<2.8	<2.8	5.9	5.3	2.9	<2.8	<2.8	<0.01
TCC	42	20	36	22	9	109	150	28	21	13	

(1-2)

Date	8-Sep 2005	22-Sep 2005	3-Oct 2005	7-Oct 2005	27-Oct 2005	18-Jan 2006	23-Jan 2006	31-Jan 2006	14-Feb 2006	1-Mar 2006	*P value*
MCP-1 (<1380)*	348.5	740.8	1344.6	730.6	962.5	1403.2	1217.2	689.9	1097.5	1235.1	ns
IL-8 (<55.23)*	198.5	146.2	283.6	96	86.5	449.4	441.4	264	50.9	80.7	<0.01
MIP-1α (<10.05)*	24.4	18.5	25.2	17.5	17.5	23.7	49.6	26.7	14.5	25.9	ns
RANTES (<7.22)*	40	56	48	26.7	21.3	13.3	50.7	37.3	42.7	50.7	ns
IP-10 (<579.2)*	1880.5	632.1	2417.8	1511.8	605.8	3076.2	3176.3	2639	358.2	932.2	<0.01
TCC	42	20	36	22	9	109	150	28	21	13	

* the levels of CSF cytokines and chemokines of the control subjects (< mean + 3SD)

well with CSF cell counts. The disease activity was divided into active and inactive phases. September 8, 2005, October 3, 2005, January 18, 2006, and January 23, 2006 correspond to the active phases.

Cytokine levels (Table 1-1, Fig. 3)

The levels of IL-6 and IFN-γ in CSF were statistically correlated with total CSF cell counts ($p < 0.01$). The elevations of these cytokines were markedly higher than the lev-

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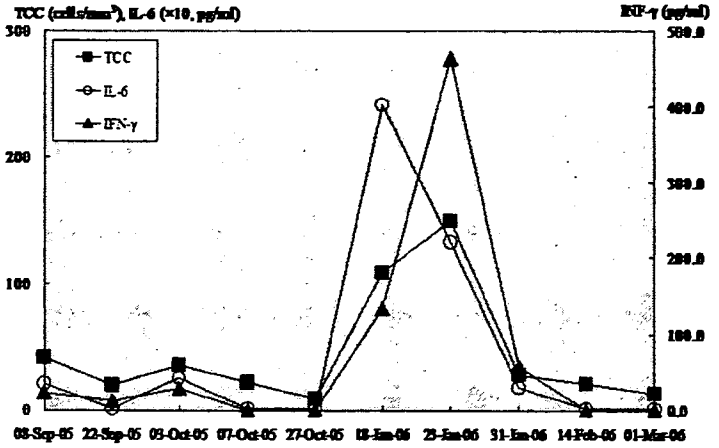

Figure 3. Levels of IL-6, IFN-γ (pg/mL) and total cell count [(TCC), cells/mm^3] in CSF.

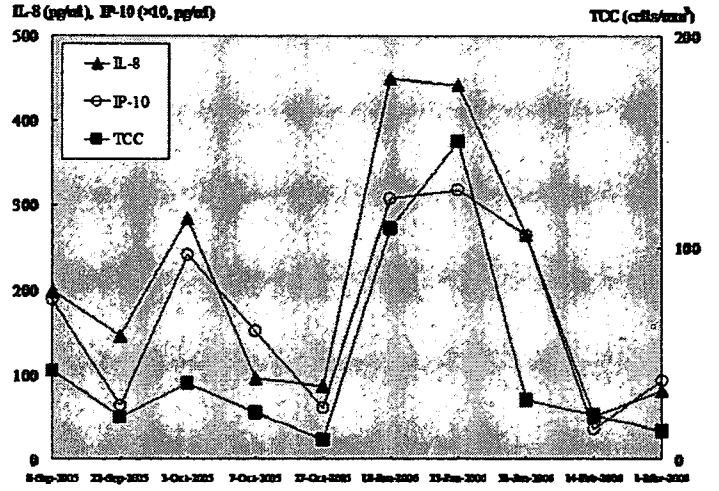

Figure 4. Levels of IL-8, IP 10 (pg/mL) and total cell count [(TCC), cells/mm^3] in CSF.

els in 21 uninfected subjects with neurological disorders. The levels of IL-4 and IL-10 in CSF were also statistically correlated with total CSF cell counts. However, the elevations of these cytokines were almost within normal ranges of control subjects. The levels of IL-2 and TNF-α in CSF were equal to or below the detection limits. The levels of CSF cytokines of the control subjects are shown in Table 1-1.

Chemokine levels (Table 1-2, Fig. 4)

The levels of IL-8 and IP-10 in CSF were statistically correlated with total CSF cell counts ($p < 0.01$). The elevations of these chemokines were markedly higher than the levels in 10 uninfected subjects with neurological disorders. The levels of other chemokines in CSF also showed various changes during the follow-up period; however, there was no significant correlation between these levels and total CSF cell counts. The levels of GM-CSF in all of the CSF samples were below the detection limits. The levels of CSF chemokines of the control subjects are shown in Table 1-2.

Correlations between level of IL-8 in CSF and neutrophilic cell counts in peripheral blood and CSF

The level of IL-8 in CSF correlated with the neutrophilic cell count in CSF (Fig. 5-1). The level of IL-8 in CSF also correlated with the peripheral neutrophilic cell count except for during the active phase (October 3, 2005) at the first time hospitalization (Fig. 5-2).

Discussion

The patient's symptoms are compatible with probable NSD consistent with the criteria advocated by Hisanaga et al and the Neuro-Sweet Disease Study Group (2). The present patient's clinical features are summarized according to the following findings: 1. She presented with recurrent encephalomeningitis with subsequent acute pharyngitis and tonsilitis. 2. She had erythematous plaques on her cheek, forearms and legs. A histological examination of the skin biopsy revealed predominant neutrophilic infiltration of the dermis, spared epidermis, and the absence of leukocytoclastic vasculitis. 3. On HLA typing, B-54 and CW1 were positive, but B-51

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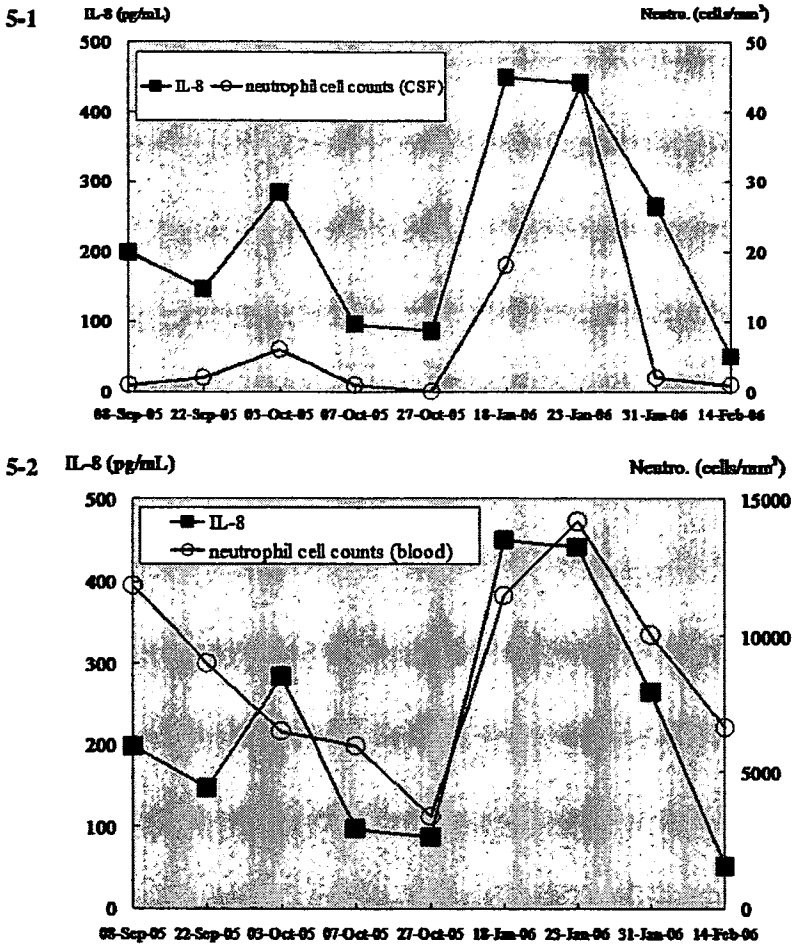

Figure 5. Correlation between level of IL-8 (pg/mL) in CSF and neutrophilic cell counts [(Neutro.), cells/mm³] in CSF (5-1) and peripheral blood (5-2).

was negative. 4. Antibiotics and antiviral therapy were not effective, but systemic glucocorticoids were so effective that the neurologic symptoms and laboratory findings markedly improved. 5. She did not display cutaneous vasculitis and thrombosis, which are seen in Behçet's disease. 6. Abnormal signal intensities on MRI were demonstrated in various CNS regions without site predilection.

The cytokines and chemokines in CSF that correlated well with the clinical state and total CSF cell counts were IL-6, IFN-γ, IL-8 and IP-10. CD4+ helper T (Th) cells can be divided into the Th1 and Th2 subtypes according to their cytokine secretion patterns (10-12). IFN-γ and IP-10, the levels of which increased in our patient, are Th1-type cytokines. Coincidentally, Th1-type cytokines have previously been implicated as mediators of the pathogenesis of Sweet's disease (13, 14). Our data suggest an important role of Th1 cells in the pathogenesis of NSD which is Sweet disease with CNS involvement. The levels of IL-4 and IL-10, which are Th2-type cytokines, were also statistically correlated with total CSF cell counts. However, the elevations of these cytokines were almost within normal ranges of control subjects. It is known that these cytokines in turn cause a decrease in the release of the Th1-type cytokines, thereby regulating the inflammatory response. We thought that the elevations of these cytokines were induced by the elevations of the Th1-type cytokines. IFN-γ causes overexpression of adhesion molecules, responsible for neutrophilic adherence and diapedesis (13). There are multiple reports that suggest that neutrophil chemotactic dysfunction may be the basis of Sweet disease (15-17). In this study, the level of IL-8, a specific neutrophil chemoattractant, correlated with the neutrophil cell count in CSF indicating that NSD may also result from neutrophil chemotactic dysfunction. The level of GM-CSF, a neutrophil chemoattractant similar to IL-8, was below the detection limits. We were therefore unable to show any correlation for this chemokine. The increases in the levels of cytokines and chemokines in the CSF of the patient at the second hospitalization were generally higher than those at the first hospitalization. This finding may be attributed to the delay of the systemic glucocorticoid therapy at the second hospitalization.

Recently, the differences between NSD and NBD have been discussed (2, 18, 19). The present patient did not fulfill the criteria of BD (20) and the HLA type (Cw1 and B54) and histology of a skin biopsy from our patient corresponded to NSD, but not to NBD (2). There are several reports of BD that demonstrate an elevation in the levels of Th1-type cytokines in the serum of patients in the active

phase (21, 22) and in turn suggest that IL-8 could be a serological marker of disease activity (23, 24). In addition, elevated levels of IFN-γ and IL-6 in CSF are detectable in patients in the active phase of NBD (25, 26). Our cytokine data suggested that there are common aspect of pathogenesis between NSD and NBD.

Therapy with systemic glucocorticoids is usually effective in improving the neurologic symptoms in patients with NSD; however, like our patient, some patients occasionally experience recurrent episodes of neurological manifestations after glucocorticoid therapy is discontinued (1). Preventive therapies have not been established, but our study demonstrates that the levels of the Th1 cytokines, IL-6 and IL-8 in CSF are important markers of disease activity in patients with NSD. It is known that the treatment with IFN-β reduces the amount of Th1 proinflammatory cytokines and shifts the immune response toward a Th2 profile (27). Therefore, this treatment might have the potential to prevent the reccurence of NSD. We believe that these results provide useful information for clarifying the pathogenesis of NSD, which may contribute to the development of future therapeutic strategies.

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PAPER

Proteomic analysis of autoantibodies in neuropsychiatric systemic lupus erythematosus patient with white matter hyperintensities on brain MRI

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The pathogenesis of neuropsychiatric systemic lupus erythematosus (NPSLE) may be related to autoantibody-mediated neural dysfunction, vasculopathy and coagulopathy. We encountered an NPSLE patient whose brain showed characteristic diffuse symmetrical hyperintensity lesions in the cerebral white matter, cerebellum and middle cerebellar peduncles on T2-weighted magnetic resonance (MR) images. In this study, we investigated all the antigens that reacted strongly with autoantibodies in this patient's serum by two-dimensional electrophoresis (2DE), followed by western blotting (WB) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) using rat brain proteins as the antigen source. As a result, we identified four antigens as beta-actin, alpha-internexin, 60 kDa heat-shock protein (Hsp60) and glial fibrillary acidic protein (GFAP). There are several reports on the detection of anti-endothelial cell antibodies (AECAs) in an SLE patients. Recently, one of the antigens reacting with AECAs in SLE patient's sera has been identified as human Hsp60. We speculated that the abnormal findings on brain MR images of our patient may be due to impairment of microcirculation associated with vascular endothelial cell injury mediated by the antibody against Hsp60. This proteomic analysis is a useful tool for identifying autoantigens in autoimmune diseases involving autoantibodies. *Lupus* (2008) **17**, 16–20.

Key words: endothelial cell; 60 kDa heat shock protein (Hsp60); neuropsychiatric systemic lupus erythematosus (NPSLE); proteome; white matter hyperintensity (WMH)

Introduction

Patients with neuropsychiatric systemic lupus erythematosus (NPSLE) frequently show various abnormal findings including white matter hyperintensities (WMHs) on T2-weighted brain magnetic resonance (MR) images.[1–3] White matter hyperintensities appear to represent asymptomatic cerebral small vessel disease (SVD).[4] There is accumulating evidence that WMHs are associated with several impairments such as cognitive deficits.[5–7] The pathogenesis of cerebral SVD is poorly understood, but endothelial activation and dysfunction may play a causal role.[4]

Here, we report the case of an NPSLE patient, whose brain MRI showed characteristic WMHs on T2-weighted and fluid-attenuated inversion recovery (FLAIR) images. We examined the reactivity of his serum antibodies against rat brain antigens using the two-dimensional immunoblotting method and identified the antigens that reacted with these autoantibodies by the proteomic method.

Materials and methods

Patient and serum samples

Serum samples were collected from an untreated 69-year old male patient with NPSLE. His clinical features are summarized as follows:

1. He showed slowly progressive polyneuropathy predominantly in the lower limbs and subsequent

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encephalopathy one year after the onset of polyneuropathy.

2. Proteinuria was detected by urinalysis and membranous nephropathy was demonstrated by renal biopsy.
3. Our patient presented delusion and hallucination without insight. Disorientation of time and place were noted. His recent memory was impaired, as he was unable to recall any of three objects after 5 min. He showed impairment of complex attention [disability of digit span (backward)]. Our patient's neurological symptoms were cognitive dysfunction, psychosis and polyneuropathy, as determined on the basis of American College of Rheumatology (ACR) Nomenclature on the NPSLE.[8]
4. Laboratory tests revealed the presence of several autoantibodies [anti-nuclear antibody, anti-DNA antibody, anti-Sm antibody, anti-RNP antibody and lupus anti-coagulant (dRVVT 1.31: normal <1.3)]; hyperglobulinemia [IgG (3448 mg/dL, normal 890–1850 mg/dL)]; decreases in the levels of complements [CH50 (16.9 CH50U/mL, normal 23–46 CH50U/mL) and C4 (2 mg/dL, normal 12–30 mg/dL)], white blood cell count (3620/μl, normal 3400–9200/μl) and lymphocyte cell count (1340/μl, normal 646–4177/μl); and coagulation-fibrinolysis abnormalities [increases in the levels of fibrinogen/fibrin degradation products (FDP) (11.3 μg/mL, normal ≤4.0 μg/mL), D-dimer (1.5 μg/mL, normal ≤1.0 μg/mL) and alpha2-plasmin inhibitor-plasmin complex (PIC) (1.4 μg/mL, normal ≤0.8 μg/mL) and decreases in the value of the thrombotest (48%, normal 70–150%), fibrinogen level (150 mg/dL, normal 150–350 mg/dL), anti-thrombin III activity (71%, normal 80–130%), protein C activity (58%, normal 64–146%), protein C antigen level (61%, normal 70–150%) and protein S activity (52%, normal 60–150%)].
5. Brain MRI showed characteristic diffuse symmetrical hyperintensity lesions in the cerebral white matter, cerebellum and middle cerebellar peduncles on T2-weighted and FLAIR images (Figure 1). Diffusion-weighted images (DWIs) showed high intensities in the bilateral middle cerebellar peduncles with decreased apparent diffusion coefficient (ADC) values. These findings on DWIs and the ADC map suggest that the lesions represent cytotoxic edema caused by ischemic changes.
6. The findings of a nerve conduction study revealed sensory motor axonal degeneration predominantly in the lower limb.

The above-mentioned findings fulfilled the ACR criteria on SLE.[9] He had no risk factors for atherosclerosis, such as hypertension, hyperlipidemia and diabetes mellitus.

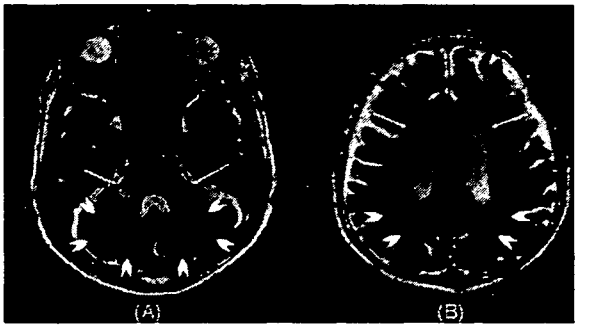

Figure 1 T2-weighted brain MR images (SE, TR/TE: 4080/100 ms). Brain MRI showed symmetrical hyperintensity lesions in bilateral cerebellar hemispheres (arrowheads), middle cerebellar peduncles (arrows) (A), periventricular white matter (arrows) and (B), deep white matter (arrowheads).

Preparation of tissue proteins

Under ether anesthesia, adult Sprague-Dawley rats were sacrificed. The cerebrums were immediately removed and frozen in dry-ice powder. The frozen brain tissue was homogenized with a tissue homogenizer in lysis solution, consisting of 20 mM Tris, 7M urea, 2M thiourea, 4% CHAPS, 10 mM 1,4-dithioerythritol (DTT), 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride containing a cocktail of protease inhibitors (Calbiochem, San Diego, CA, USA). The homogenate was centrifuged at 150 000 × g for 45 min and the supernatant was used in all experiments. Protein concentration was determined by Bio-Rad Protein assay based on the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

Two-dimensional electrophoresis

The samples were dissolved in destreak rehydration solution (GE Healthcare, Buckinghamshire, UK) and loaded by in-gel rehydration into 7-cm long immobilized pH gradient dry strips (GE Healthcare, Buckinghamshire, UK). Up to 250 μg of extracted proteins was applied to the dry strips for Western blotting (WB). Isoelectric focusing was conducted at 20°C for 24 000 Vh at a maximum of 5000 V using a horizontal electrophoresis system, Multiphor III (GE Healthcare, Buckinghamshire, UK). Before separation in the second dimension, the IPG strips were equilibrated for 15 min in a buffer containing 2% SDS, 6M Urea, 30% v/v glycerol, 0.001% BPB and 50 mM Tris-HCl (pH 8.8) under reducing conditions with 65 mM DTT, followed by incubation for 15 min in the same buffer

under alkylating conditions with 140 mM iodoacetamide. Equilibrated IPG strips were transferred to a 12.5% polyacrylamide gel and run at 15 mA/gel. After the electrophoresis, the SDS-PAGE gels were stained with Coomassie Brilliant Blue (GelCode Blue Stain Reagent, Pierce) or used for protein transfer onto polyvinylidine difluoride (PVDF) membranes.

Immunoblotting

Separated proteins were electrophoretically transferred to a PVDF membrane at 50 volts for 3 h using a buffer transfer tank with cold equipment. The PVDF membrane was incubated in blocking solution (5% skim milk in 1 × TBST; 1 × TBS containing 0.1% Tween 20) overnight in a cold room and then reacted with the patient's serum diluted (1:1000) in 1% skim milk in 1 × TBST for 1 h at room temperature. The PVDF membrane was washed five times with 1 × TBST and reacted with peroxidase-conjugated goat anti-human Ig (A + G + M) antibodies (P.A.R.I.S, France) diluted (1:1000) with 1% skim milk in 1 × TBST for 1 h at room temperature. After six washes, the membrane was incubated with the ECL reagent for 1 min and then exposed to an x-ray film for 15–300 s.

Gel digestion and mass spectrometry

The target spot was excised from the gel and subjected to trypsin digestion and peptide fragments were analysed using a nanoscale capillary LC system (LV-VP, Shimadzu) and an ion trap tandem mass spectrometer (LCQ Advantage Max, Thermo Electron). Proteins were identified from MS/MS spectra using protein identification software (X calibur TM, Thermo Finnigan and MASCOT Search, Matrix Science).

Determination of anti-60 kDa heat shock protein antibodies

We determined by enzyme-linked immunosorbent assay (ELISA) the titers of anti-Hsp60 antibodies in sera from our patient, patients with NPSLE (n = 5; age range, 22–58; mean age, 42.4) without abnormal WMHs and healthy controls without abnormal WMHs (n = 7; age range, 17–67; mean age, 43.1). We carried out ELISA to analyse the reactivities of autoantibodies against human Hsp60, which were measured using an ELISA kit (Stressgen, Ann Arbor, MI, USA). Sera diluted 1:1000 in a dilution buffer were added to a precoated ready-to-use recombinant human Hsp60 immunoassay plate and then incubated for 2 h at room temperature (RT). After four washes, peroxidase-conjugated anti-human IgG, A or M was added to each well and then incubated for 1 h at RT. After four washes, a stabilized tetrametylbenzidine substrate was added to each well and then incubated for 15 min at RT. The reaction was stopped by adding acid stop solution and the plate was read at 450 nm on a microplate reader. The OD of control wells without Hsp60 was subtracted from the OD of Hsp60-coated wells. Serial dilutions of serum samples of healthy blood donors having high antibody levels against the tested Hsp60 were used as standards.

Results

Screening and identification of target proteins that reacted with autoantibodies in patient's serum

We detected nine spots (pI 4.2–124 kDa, pI 5.15–kDa, pI 5.3–53 kDa, pI 5.4–53 kDa, pI 5.5–53 kDa, pI 5.25–57 kDa, pI 5.4–57 kDa, pI 5.15–63 kDa, pI 8.0–35 kDa) that strongly reacted with autoantibodies in patient's serum on 2DE-WB (Figure 2). Five among the nine spots that matched proteins on 2-DE gels were analysed by LC-MS/MS. These immunoreactive proteins were identified as beta-actin (pI 5.15–46 kDa), alpha-internexin (pI 5.15–63 kDa), Hsp60 (pI 5.25–57 kDa and pI 5.4–57 kDa) and glial fibrillary acidic protein (GFAP) (pI 5.3–53 kDa) (Table 1).

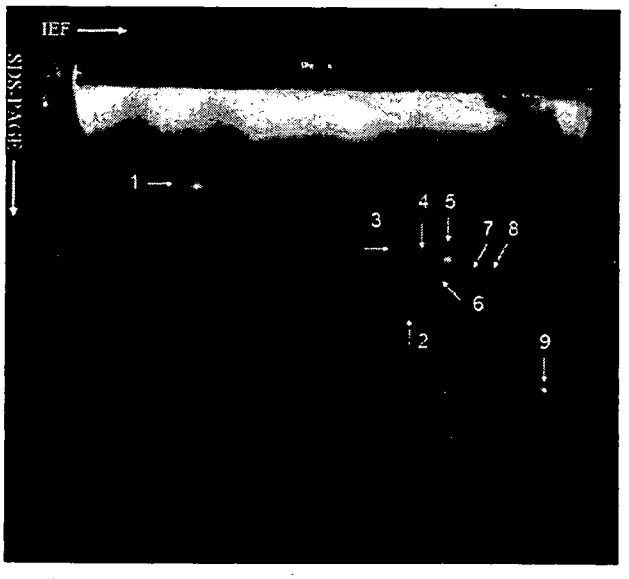

Figure 2 Two-dimensional electrophoresis (2DE) and western blotting (WB). Nine spots strongly reacted with autoantibodies in patient's serum on 2DE-WB. Five spots (No. 2–6) were analysed using mass spectrometry. No. 2: Beta actin; No. 3: Alpha-internexin; No. 4 and 5: 60 kD heat-shock protein (Hsp60); No. 6: Glial fibrillary acidic protein (GFAP); No. 1, 7, 8 and 9: no identification was made.

Table 1 Autoantigens identified using mass spectrometry

Spot Number[a]	*Protein name*	*Mascot score*	*Number of peptides*	*Coverage%*	*Observed M.W.(kDa) /pI*	*Calculated M.W.(kDa) /pI*
2	Beta-actin	421	16	41	46/5.15	42/5.29
3	Alpha-Inx[b]	203	5	12	63/5.15	56/5.20
4	Hsp 60[c]	112	3	6	57/5.25	61/5.91
5	Hsp 60	112	3	8	57/5.4	61/5.91
6	GFAP[d]	113	2	2	53/5.3	50/5.35

[a]Spot number corresponds to the number shown in Figure 2.
[b]Alpha-Inx, Alpha-internexin.
[c]Hsp 60, 60 kD heat-shock protein.
[d]GFAP, Glial fibrillary acidic protein.

Detection of anti-Hsp60 antibodies in our patient and controls

The titer of the anti-Hsp60 antibody in our patient was 133.6 ng/mL. The mean titer of this antibody in five NPSLE patients without WMHs on brain MR images was 19.72 (SD 10.65; range 9.0–32.2) ng/mL. The mean titer of this antibody in the seven healthy controls without WMHs on brain MR images was 15.36 (SD 11.85; range 5.7–39.5) ng/mL.

Discussion

In this study, we detected some autoantigenic proteins reacting with autoantibodies in a serum sample from a patient with NPSLE using the proteomic approach and we identified four autoantigens, namely, beta-actin, alpha-internexin, Hsp60 and GFAP. There are some previous studies demonstrating the association of autoantibodies in serum and cerebrospinal fluid (CSF) with central nervous system involvement in patients with NPSLE.[10–12] Anti-endothelial cell antibodies (AECAs) have been detected in SLE patients.[13,14] Recently, one of the antigens that reacted with AECAs in a SLE patient's sera has been identified as human Hsp60.[15] Human Hsp60 is a molecular chaperone that participates in the folding of mitochondrial proteins and facilitates proteolytic degradation of misfolded or denatured proteins.[16] However, it has also been reported that an enhanced expression of this protein on endothelial cells has been noted and antibodies against human Hsp60 induce endothelial cell toxicity.[15,17,18]

Our patient's brain MR images showed characteristic cerebral WMHs, which appear to represent cerebral SVD. The pathogenesis of cerebral SVD is poorly understood, but endothelial activation and dysfunction may play a causal role.[4] It has been reported that the anti-Hsp60 antibody is present in most patients with coronary artery disease that its titer correlates with disease severity[19] and that it may contribute to the initiation or amplification of vascular endothelial cell damage in atherosclerosis, which is considered a crucial event.[20] Our patient's laboratory findings showed a slightly high level of lupus anticoagulant and some coagulation-fibrinolysis abnormalities. It has been reported that the anti-Hsp60 antibody bind to endothelial cells and induce a thrombotic cascade following endothelial cell apoptosis in SLE patients with the anti-phospholipid antibody.[15] In this study, we determined by ELISA the titer of the anti-Hsp60 antibody in sera from our patient and controls without WMHs on brain MR images. The titer of this antibody in serum from our patient was markedly higher than the mean +2SD of NPSLE patients without WMH or that of healthy controls without WMHs. Thus, the abnormal WMH lesions on brain MR images in our patient may be at least partially due to the impairment of microcirculation associated with vascular endothelial cell dysfunction mediated by the antibody against Hsp60. Further studies using a large series of controls are required to clarify the relationship between the anti-Hsp60 antibody and WMHs on brain MR images.

On the other hand, the clinical significance of the anti-GFAP antibody in NPSLE remains controversial. There is a report showing that the anti-GFAP antibody is specific for NPSLE.[2] Another report suggested that GFAP might be a useful marker in the diagnosis and monitoring of NPSLE, because GFAP level increases in the CSF of NPSLE.[21] However, Valesini *et al.*[22] have reported that the presence of the anti-GFAP antibody in sera of SLE patients showed no significant correlation with neurologic or psychiatric morbidity. Further study will be necessary to clarify the association between the anti-GFAP antibody and NPSLE.

Previously, there were several reports, which described that the autoantibodies against beta-actin and alpha-internexin were detected from non-neurological diseases or healthy controls.[23,24] Therefore, we thought that these autoantibodies were not specifically related to NPSLE and are parts of the natural autoantibody repertoire.

In this study, we detected several autoantibodies from our NPSLE patient and identified the autoantigens that they reacted. Several autoantibodies are generated in systemic autoimmune diseases, and an understanding of the interaction among these

autoantibodies will help clarify their pathogenesis. The proteomic analysis used in our study is a very useful tool for identifying several autoantigens reacting with autoantibodies at one time.

Acknowledgements

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Subacute Encephalopathy: Clinical Features, Laboratory Data, Neuroimaging, and Outcomes

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We sought to clarify the clinical, laboratory, neuroradiologic, and neurophysiologic features of the "subacute" subtype of encephalopathy. We retrospectively identified nine patients with subacute encephalopathy out of 97 patients diagnosed as manifesting acute encephalopathy. Neurologic symptoms, clinical course, laboratory data, neuroradiologic and electroencephalographic findings, and outcomes were reviewed through medical records. The median age of patients was 44 months (range, 28-156 months). The initial neurologic sign was a brief seizure in 4, a prolonged seizure in 3, delirious behavior in 1, and a loss of consciousness in 1. Loss of consciousness the next day was subtle in 4, and mild in 5. However, a worsening of consciousness was observed 3-7 days after onset. Laboratory data were unremarkable, and electroencephalography during the early phase found abnormalities in 4 of 7 patients. Magnetic resonance imaging revealed no abnormalities during the early phase, and mild cortical atrophy during the late phase. All but one patient had various degrees of neurologic sequelae. Subacute encephalopathy was characterized by a delayed worsening of neurologic symptoms, mild cortical atrophy on late magnetic resonance imaging, and poor neurologic outcomes. Recognition of this type of acute encephalopathy is important, and a method to promote early diagnosis is desirable.
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Okumura A, Kidokoro H, Itomi K, Maruyama K, Kubota T, Kondo Y, Itomi S, Uemura N, Natsume J, Watanabe K, Morishima T. Subacute encephalopathy: Clinical features, laboratory data, neuroimaging, and outcomes. Pediatr Neurol 2008;38:111-117.

Introduction

Rapid deterioration in association with convulsions during a febrile illness is a common clinical manifestation of acute encephalopathy. Several new subtypes of acute encephalopathy were proposed based on their clinical, neuroradiologic, and laboratory findings. Acute necrotizing encephalopathy, as proposed by Mizuguchi, is characterized by symmetric lesions in the thalami and other brain regions [1]. There were several reports on mild subtypes of acute encephalopathy associated with transient splenial or white-matter lesions [2-4]. Subcortical white-matter lesions on diffusion-weighted images are characteristic in children with encephalopathy with prolonged seizures [5].

A unique subtype of acute encephalopathy, characterized by a relatively slow worsening of neurologic signs, has attracted the attention of pediatric neurologists in Japan [6-13]. According to these previous reports, the common neurologic sign at the outset is a seizure, and especially a prolonged one. The next day, patients may appear relatively well. Consciousness seems almost recovered, but slightly reduced responsiveness, an appearance of absent-mindedness, or subtle disorientation may be observed by parents or caregivers. Deterioration of consciousness, clustered seizures, and involuntary movements appear 3-7 days after the first seizure. Most patients have moderate to severe cognitive impairment. However, the clinical, laboratory, neuroradiologic, and neurophysiologic features of this subtype of encephalopathy are not yet fully understood. The aim of this study was to clarify the clinical, laboratory, neuroradiologic, and neurophysiologic features of the "subacute" subtype of encephalopathy, characterized by a relatively slow progression of neurologic symptoms.

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Table 1. Patient characteristics, clinical manifestations, and outcomes

Patient	Age (Months)	Sex	Prodromal Infection*	Initial Neurologic Signs	LOC on Next Day
1	45	F	URI (9)	LOC alone	Mild
2	156	M	NSFI (2)	Brief SZ	Subtle
3	28	M	Enterocolitis‡ (6)	Prolonged SZ	Mild
4	32	M	NSFI (0)	Delirious behavior	Subtle
5	33	F	URI (1)	Brief SZ	Subtle
6	37	M	NSFI (0)	Prolonged SZ	Mild
7	44	F	Influenza A (0)	Prolonged SZ	Mild
8	60	M	Influenza A (0)	Brief SZ	Mild
9	44	F	Influenza A (0)	Brief SZ	Subtle

* Numbers in parentheses indicate interval between prodromal illness and onset of encephalopathy.
† Numbers in parentheses indicate days after onset.
‡ Verotoxin-producing *Escherichia coli* were isolated from stool.
§ Oral tendency was also observed 20 days after onset.

Abbreviations:
F = Female
LOC = Loss of consciousness
M = Male
NSFI = Nonspecific febrile illness
SZ = Seizure
URI = Upper respiratory infection

Patients and Methods

We reviewed the hospital records of patients with acute encephalopathy who were admitted to the Department of Pediatrics in Nagoya University Hospital (Nagoya, Aichi, Japan) and its affiliated 12 hospitals between January 1998 and March 2005. We identified 97 patients with acute encephalopathy, as characterized by decreased consciousness with or without other neurologic signs lasting for >24 hours in children with infectious symptoms. We carefully excluded patients with sustained decreased consciousness after a febrile seizure, or those with delirious behavior without obvious reduced consciousness.

We assessed the detailed clinical course of each patient on the basis of medical records. We paid attention to initial neurologic signs, and the severity and time course of decreased consciousness. Nine (9%) of 97 patients fulfilled the following conditions: (1) mildly decreased consciousness on the day after onset, (2) deterioration of consciousness a few days after onset, and (3) no other cause of encephalopathy such as electrolyte derangement, metabolic abnormalities, or worsening of systemic diseases. These nine patients were the subjects of this study.

In this study, the initial neurologic signs were divided into the following four items: prolonged seizure, brief seizure, delirious behavior, and decreased consciousness alone. A prolonged seizure was defined as lasting for >20 minutes. A brief seizure was defined as those <20 minutes, irrespective of the number of seizures. Delirious behavior was defined as disoriented and incoherent action or speech lasting for >30 minutes. It may include visual hallucinations, irritability, fearful responses, and sensory misperception. Coma was defined as a condition in which a patient could not be aroused by maximal painful stimulation. This is consistent with a score of 3-5 in the Glasgow Coma Scale-Modified for Children or a score of 100-300 on the Japan Coma Scale. Semicoma was defined as a condition in which a patient could be aroused by painful stimulation. The loss of consciousness on the day after onset was milder than in most of these patients. Thus, we defined mild loss of consciousness as the condition in which a patient tended to be asleep but could be aroused without painful stimulation, and subtle loss of consciousness as the condition in which a patient remained awake but lacked spontaneity, or seemed absent-minded or slightly disoriented.

Laboratory data were also assessed through medical records. The following values were investigated: aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatinine kinase, glucose, ammonia, and cell counts and protein in cerebrospinal fluid. All findings of cranial computed tomography and magnetic resonance imaging were evaluated retrospectively by 17 pediatric neurologists who were unaware of patients' detailed clinical courses. Single-photon emission tomography and electroencephalograms were interpreted by pediatric neurologists in each hospital.

All patients were followed by pediatric neurologists for at least 1 year. The severity of cognitive impairment was defined as mild when intelligence or developmental quotient was between 50-70, moderate when it was between 30-50, and severe when it was <30. The severity of motor impairment was defined as mild when a patient could walk with or without support, moderate when a patient could seat oneself without support, and severe when a patient could not seat oneself.

Results

Patient Characteristics

Patient characteristics are summarized in Table 1. Their median age was 44 months (range, 28-156 months). No patient was <2 years of age. No patient had a family history of febrile seizures, epilepsy, or other neurologic disorders. One patient (patient 4) manifested mild cognitive impairment and focal epilepsy of unknown origin. Three (patients 7, 8, and 9) had a past history of febrile seizures. The remaining 5 patients had no history of neurologic disorders.

The pathogen of prodromal infection was identified in 4 patients. Influenza A infection was virologically proven in 3 patients. Verotoxin-producing *Escherichia coli* were isolated in a stool sample from patient 3. In this patient, there were no clinical or laboratory findings suggesting an association with hemolytic-uremic syndrome. Prodromal illness involved upper respiratory infection in 2 patients, and nonspecific febrile illness without respiratory, urinary,

Table 1. Continued

Most Severe LOC†	Sz During Subacute Phase†	Behavioral Abnormalities†	Cognitive Impairment	Motor Impairment
Coma (3)	None	Stereotypic movement (3)	None	None
Coma (4)	Brief SZ (6)	None	Moderate	Mild
Semicoma (4)	None	Stereotypic movement (4)	Severe	None
Semicoma (7)	Clustered brief SZs (5)	None	Severe	Moderate
Coma (3)	Clustered brief SZs (4)	Oral automatic movement (6)§	Moderate	Mild
Coma (3)	Prolonged SZ (7)	Oral tendency (18)	Severe	Mild
Coma (3)	None	Stereotypic movement (2)	Moderate	Mild
Coma (4)	None	Delirious behavior (3)	Mild	Mild
Coma (4)	Brief SZ (5)	None	None	Mild

or gastrointestinal signs in 3 patients. The interval between prodromal illness and onset of encephalopathy was <2 days in 7 patients. Theophylline had not been used in any of these patients before the onset of encephalopathy, whereas acetaminophen had been administered in 2 patients (patients 2 and 7).

Neurologic Signs and Clinical Courses

Neurologic signs and clinical courses are also listed in Table 1. The initial neurologic sign was a brief seizure in 4 patients, a prolonged seizure in 3, delirious behavior in 1, and loss of consciousness alone in 1. The severity of loss of consciousness on the next day of onset was subtle in 4 patients and mild in 5. However, worsening of consciousness was observed 3-7 days after onset. The most severe loss of consciousness was coma in 7 patients, and semicoma in 2.

During the subacute phase, seizures or behavioral abnormalities were present in all patients. Seizures were observed in 5 patients. A prolonged seizure was observed in 1 patient, clustered brief seizures in 2, and a single brief seizure in 2. Behavioral abnormalities were recognized in 6 patients. Stereotypic movements, such as purposeless hand movements, were recognized in 3 patients, oral tendency in 1, smacking-like oral automatic movements followed by an oral tendency in 1, and delirious behavior such as meaningless speech in 1.

Laboratory Data

In all but one patient, laboratory tests on admission revealed normal levels of aspartate aminotransferase, alanine aminotransferase, and creatinine kinase. One patient (patient 9) had mildly elevated levels of aspartate aminotransferase and alanine aminotransferase. Lactate dehydrogenase was mildly to moderately elevated in most patients on admission. These values reached peak levels on days 3-10 from the onset of encephalopathy. Marked elevations of aspartate aminotransferase (>200 IU/L) and alanine aminotransferase (>200 IU/L) were observed in 3 patients (patients 7-9), elevations of lactate dehydrogenase (>1000 IU/L) were observed in 3 (patients 5, 7, and 8), and elevations of creatinine kinase (>1000 IU/L) were observed in 2 (patients 2 and 6). Hyperammonemia, hypoglycemia, and metabolic or respiratory acidosis were not recognized in any patients. Cerebrospinal fluid analyses revealed mild pleocytosis with mildly increased protein levels in 3 patients (patients 1, 2, and 9).

Neuroradiologic Findings

Neuroradiologic findings are listed in Table 2. During the acute phase, computed tomography was performed in 6 patients, magnetic resonance imaging in 1, and both computed tomography and magnetic resonance imaging in 1. No abnormal findings were observed in these 8 patients.

During the subacute phase, when the worsening of consciousness had occurred, computed tomography was performed in 2 patients, magnetic resonance imaging in 3, and both computed tomography and magnetic resonance imaging in 3. No neuroradiologic examination was performed in 1 patient during this phase. Computed tomography demonstrated mild blurring of gray-white matter differentiation in the bilateral frontal areas of 2 patients (Fig 1). Magnetic resonance imaging revealed mild high

Table 2. Neuroradiologic findings

Patient	CT			MRI			SPECT
	Acute Phase	Subacute Phase	Late Phase	Acute Phase	Subacute Phase	Late Phase	
1	Normal (0)	Not performed	Not performed	Normal (1)	Normal (8)	Mild CA (40)	Not performed
2	Not done	Normal (7)	Normal (17)	Not performed	Normal (8)	Mild CA (33)	Hypoperfusion in bilateral F areas (11)
3	Normal (0)	Blurring of GWD in bilateral F areas (5)	Not performed	Not performed	HIA in bilateral F areas on T2WI/ FLAIR (14)	Mild CA (39)	Hypoperfusion in bilateral F-T areas (13)
4	Not done	Not performed	Not performed	Not performed	Normal (12)	Mild CA (34)	Diffuse hypoperfusion (20)
5	Normal (0)	Normal (4)	Not performed	Not performed	HIA in bilateral F areas on T2WI/ FLAIR (9)	Mild CA (23)	Hypoperfusion in bilateral F areas (22)
6	Normal (0)	Not performed	Not performed	Not performed	HIA in left T-P-O areas on DWI (9)	Mild CA (31)	Hypoperfusion in left T-P-O areas (16)
7	Normal (0)	Blurring of GWD in bilateral F areas (3)	Not performed	Not performed	Not performed	Mild CA (35)	Not performed
8	Normal (0)	Not performed	Mild CA (15)	Not performed	Not performed	Mild CA (21)	Not performed
9	Normal (0)	Normal (10)	Not performed	Normal (3)	Not performed	Normal (20)	Not performed

Numbers in parentheses indicate days after onset.

Abbreviations:
CA = Cortical atrophy
DWI = Diffusion-weighted images
F = Frontal
FLAIR = Fluid-attenuated inversion-recovery images
GWD = Gray-white matter differentiation
HIA = High-intensity area
O = Occipital
P = Parietal
T = Temporal
T2WI = T_2-weighted images

intensities in the bilateral frontal areas on T_2-weighted and fluid-attenuated inversion-recovery images in 2 patients (Fig 1), and marked high intensities in the left temporo-parieto-occipital area on diffusion-weighted images in 1 patient (Fig 2). No abnormalities were seen in 4 patients.

During the late phase, magnetic resonance imaging was performed in all 9 patients, and computed tomography was performed in 2 patients. Magnetic resonance imaging demonstrated mild cortical atrophy with widening of the extracerebral space and ventricles in all but one patient (Figs 1, 2). In 3 (patients 1, 2, and 8), previous neuroradiologic examinations did not indicate abnormal findings. In one patient (patient 9), neuroradiologic abnormalities were not observed throughout the clinical course.

Single-photon emission tomography was performed during the subacute or late phase in 5 patients. Four patients manifested marked hypoperfusion in the bilateral frontal areas (Fig 1), and one exhibited marked hypoperfusion in the left temporo-parieto-occipital area (Fig 2).

Electroencephalogram Findings

Electroencephalograms were recorded during the acute phase in 7 patients. Marked, generalized slowing was observed in 3 patients (patients 1, 5, and 9), and a slow basic rhythm in 1 patient (patient 3). However, electroencephalogram findings were unremarkable in 3 patients (patients 2, 6, and 8). An electroencephalogram during the subacute phase was performed in 8 patients, and revealed various degrees of abnormalities in all of them. Marked, generalized slowing was seen in 1 (patient 2), mild generalized slowing in 4 (patients 1, 3, 7, and 8), regional slowing in 2 (patients 5 and 6), and a slow basic rhythm in 1 (patient 4). Electroencephalograms during the late phase were performed in 6 patients (patients 1-6). Abnormalities in background activities were recognized in all of them, and paroxysmal discharges were observed in 3 (patients 3-5).

Treatments and Outcomes

Methylprednisolone pulse therapy was performed in 1 patient, intravenous dexamethasone therapy in 1, intravenous immunoglobulin therapy in 3, glycerol therapy in 3, and mannitol therapy in 1. In regard to anticonvulsants, diazepam was used in 6 patients, phenobarbital in 5, phenytoin in 2, carbamazepine in 2, valproate in 1, clonazepam in 1, and midazolam in 1. Mechanical ventilation was not necessary in any patients.

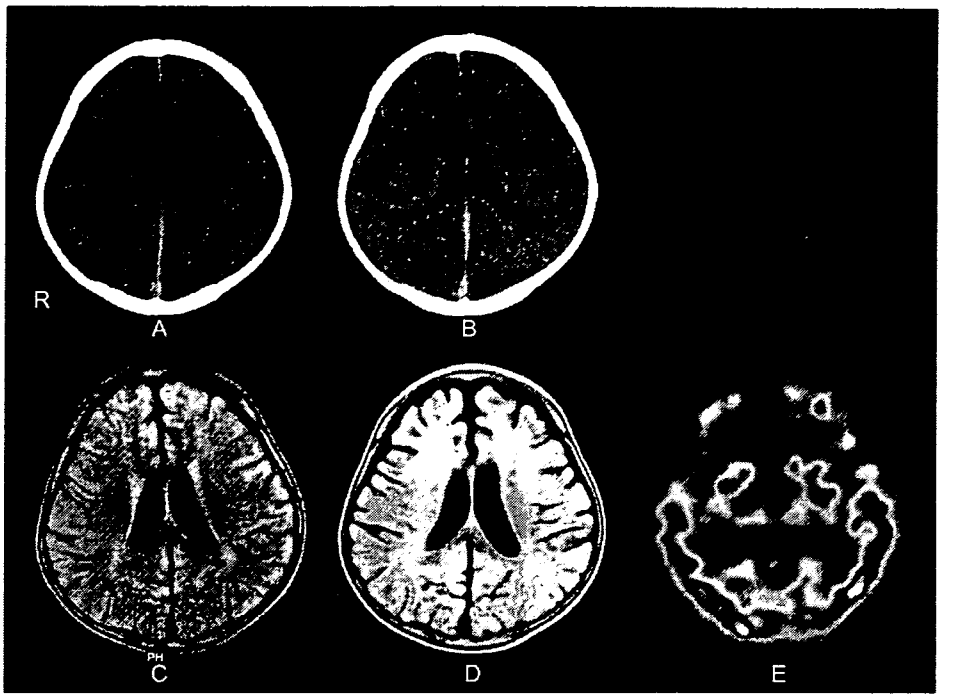

Figure 1. Neuroradiologic findings of patient 3. (A) Computed tomography on day of onset. No abnormal findings were recognized. (B) Computed tomography 5 days after onset. Mild blurring of gray-white matter differentiation was observed in the bilateral frontal areas. (C) Magnetic resonance imaging 14 days after onset (fluid-attenuated inversion recovery images, fast spin-echo, TR/TE/TI = 8000/120/1900 ms). Mild high intensities in the subcortical white matter were seen in the bilateral frontal areas. (D) Magnetic resonance imaging 39 days after onset (fluid-attenuated inversion recovery images, fast spin-echo, TR/TE/TI = 8000/120/2300 ms). Mild but diffuse cortical atrophy was observed. (E) Single-photon emission tomography 13 days after onset. Marked hypoperfusion was observed in the bilateral frontal areas.

The outcomes of patients are given in Table 1. No patients died, although all but one patient had various degrees of neurologic sequelae. Cognitive impairment was seen in 7 patients (severe in 3 patients, moderate in 3, and mild in 1), and motor impairment was seen in 7 (moderate in 1 patient, and mild in 6). In most patients, cognitive impairment was more prominent than motor impairment. The patient who had manifested mild cognitive impairment before the onset of encephalopathy (patient 4) had severe cognitive impairment with moderate motor impairment. The relationship between treatment and outcome could not be analyzed, because of the small number of patients and wide variety of treatments.

Discussion

Several authors proposed different names for acute encephalopathy syndromes with a relatively slow worsening of neurologic signs [6-13]. Although minor features are different among these syndromes, they have many points in common. Therefore, acute encephalopathy syndromes with a delayed worsening of consciousness should be integrated into one syndrome to avoid unnecessary confusion. To this end, we used the term "subacute" encephalopathy as a clinical entity to cover these syndromes, rather than devise a new name.

In the present study, we described the unique features of subacute encephalopathy. At the onset of encephalopathy, a prolonged or brief seizure was commonly observed, but some patients did not have a seizure at onset. The severity of decreased consciousness was invariably mild on the following day. Thereafter, worsening of consciousness began, and reached maximal severity 3-7 days after onset. Although neuroimaging was unremarkable at the outset, mild cortical atrophy was observed in most patients during the late phase. Most patients had moderate to severe

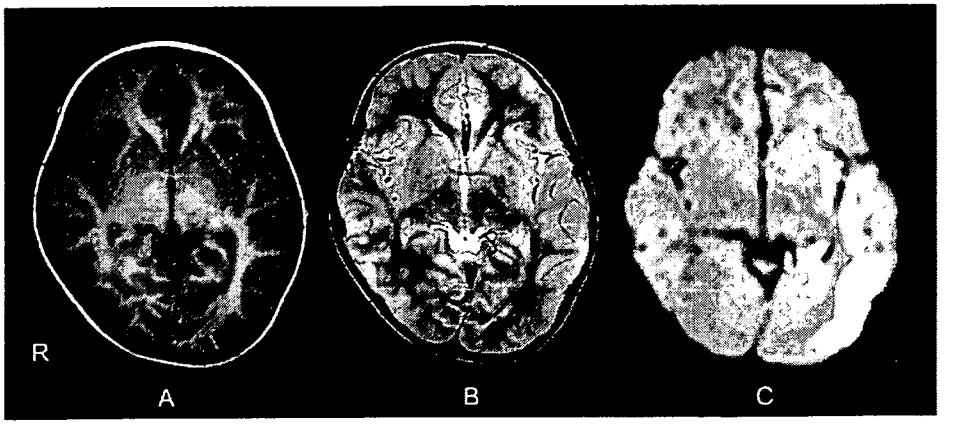

Figure 2. Neuroradiologic findings of patient 6: magnetic resonance imaging 9 days after onset. Subtle thickening of the cortex in the left temporo-parieto-occipital area was seen on T_1-weighted (A: fast spin-echo, TR/TE = 596/15 ms) and T_2-weighted (B, fast spin-echo, TR/TE = 4162/100 ms) images. Diffusion-weighted images (C: spin-echo echo-planar imaging, TR/TE = 2835/89 ms, b = 1000 sec/mm²) demonstrated marked high intensities in the same region.

cognitive impairment, whereas motor impairment was relatively mild. Although these features are similar to those in previous reports [6-13], there are some differences between the patients in previous studies and ours.

One important difference involves the initial neurologic signs. A prolonged seizure or status epilepticus as the initial neurologic sign had been emphasized in this type of encephalopathy [5,10,12]. Yamanouchi and Mizuguchi included the presence of convulsive status epilepticus in the tentative diagnostic criteria of acute infantile encephalopathy predominantly affecting the frontal lobes [13]. However, a prolonged seizure was observed at the onset in only 3 of 9 patients in our cohort. Therefore, we think that the presence of a prolonged seizure or convulsive status epilepticus should not be stressed too strongly, although it is certainly an important neurologic sign at the onset.

Another prominent difference involves the localization of brain lesions. In previous studies, magnetic resonance imaging invariably revealed the involvement of the frontal lobes. Although this was the case in most of our patients, one of them (patient 6) exhibited a unilateral temporo-parieto-occipital lesion. In this patient, regional cerebral blood flow in the frontal lobes was not decreased. The localization of brain lesions will not be limited to the frontal lobes in patients with subacute encephalopathy. Age at onset was also different between our patients and those in previous studies. In previous studies, the age of patients was $\leq$3 years [9,12,13]. On the other hand, 6 of 9 patients were aged >36 months in this study. This finding indicates that subacute encephalopathy can be present in older children as well as in infants and young children.

The most important similarity between our study and previous studies involves poor neurologic outcomes despite a relatively mild loss of consciousness during the early phase. Although no patients were comatose on the next day after onset, moderate to severe cognitive impairment was present as a neurologic sequel in the majority of our patients. This was also the case with patients in previous studies [6-8,10-13]. We consider this to be the most prominent feature of subacute encephalopathy. We must be aware of the presence of this unique type of encephalopathy.

The pathophysiology of subacute encephalopathy remains unknown. Hypoxic brain injury is unlikely, because no patients had overt evidence of profound hypoxia. There have been several studies on the role of cytokines in the development of acute encephalopathy [14-17]. However, the role of cytokines in subacute encephalopathy is uncertain. Serum and cerebrospinal fluid levels of interleukin-6 were lower in patients with acute infantile encephalopathy predominantly affecting the frontal lobes than in patients with influenza-associated encephalopathy. A relatively slow worsening of neurologic signs may be suggestive of delayed neuronal loss caused by prolonged convulsions. Takanashi et al. suggested an accumulation of glutamate because of hyperactivity of glutamatergic neurons, using magnetic resonance spectroscopy in a child with prolonged febrile seizure with encephalopathy [5]. Apoptotic changes were also revealed in patients with acute encephalopathy [18]. However, these findings have not been investigated in children with subacute encephalopathy. Further multidisciplinary studies are necessary to clarify the pathogenesis of subacute encephalopathy.

The diagnostic value of neuroimaging in subacute encephalopathy remains unclear at present. Computed tomography or magnetic resonance imaging within a few days after the onset will be useless. Takanashi et al. indicated that magnetic resonance imaging within 2 days of the onset of encephalopathy revealed no acute lesions in children with prolonged febrile seizures with encephalopathy [5]. Computed tomography or conventional magnetic resonance imaging during the subacute phase will be of limited use. Computed tomography may reveal a blurring of gray-white matter differentiation, and magnetic resonance imaging may demonstrate mildly increased intensities in the subcortical white matter on T_2-weighted and fluid-attenuated inversion-recovery images. However, these changes can be subtle and overlooked. Diffusion-weighted imaging will be useful if it is performed during an appropriate period, as stated in previous studies [5,10,12].

The early diagnosis of subacute encephalopathy is of clinical interest. However, no useful clues have been delineated for such an early diagnosis. Neuroimaging during the early phase will not be useful, as explained above. Laboratory data will not be helpful, because the abnormalities in laboratory data are non-specific and are not pathogomotic in children with subacute encephalopathy. We think that electroencephalograms may be useful in some patients, because electroencephalograms indicated abnormalities in 4 of 7 patients in whom they were performed during the acute phase. Yamanouchi et al. reported that electroencephalograms were recorded at onset in 6 of 9 patients with acute infantile encephalopathy predominantly affecting the frontal lobes, and revealed slow background activity [12]. We think that careful evaluation of consciousness will be also useful for early recognition of subacute encephalopathy. Maegaki et al. reported on acute encephalopathy with a biphasic clinical course, whose clinical features were very similar to those of subacute encephalopathy, in 6 of 16 patients with grade II or III loss of consciousness according to the Japan Coma Scale, 12 hours after onset [8]. Therefore, a combination of electroencephalograms and a precise neurologic examination focusing on responsiveness and wakefulness will be a diagnostic clue in regard to subacute encephalopathy.

In conclusion, "subacute" encephalopathy, characterized by a delayed worsening of neurologic signs, is a distinct subtype of acute encephalopathy. Early recognition is not easy, because the initial neurologic signs are varied, and laboratory data and neuroimaging are unremarkable during the acute phase. Magnetic resonance imaging during the late phase reveals mild cortical atrophy, and single-photon emission tomography indicates hypoperfusion

in the affected regions. The neurologic outcome is poor in most patients. Recognition of subacute encephalopathy is important, and a method to promote early diagnosis is desirable.

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Effects of FAK ablation on cerebellar foliation, Bergmann glia positioning and climbing fiber territory on Purkinje cells

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Abstract

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that is widely expressed in the brain, and plays key roles in various cellular processes in response to both extracellular and intracellular stimuli. Here, we explored the role of FAK in cerebellar development. In the mouse cerebellum, FAK was found to be distributed as tiny cytoplasmic aggregates in various neuronal and glial elements, including Purkinje cells (PCs), Bergmann glia (BG), parallel fiber (PF)-terminals and climbing fiber (CF)-terminals. The neuron/glia-specific ablation of FAK impaired cerebellar foliation, such as variable decreases in foliation sizes and the lack of intercrural and precentral fissures. Some of the BG cells became situated ectopically in the molecular layer. Furthermore, the FAK ablation altered the innervation territories of CFs and PFs on PCs. CF innervation regressed to the basal portion of proximal dendrites and somata, whereas ectopic spines protruded from proximal dendrites and PFs expanded their territory by innervating the ectopic spines. Furthermore, the persistence of surplus CFs innervating PC somata caused multiple innervation. When FAK was selectively ablated in PCs, diminished dendritic innervation and persistent somatic innervation by CFs were observed, whereas cerebellar foliation and cell positioning of BG were normally retained. These results suggest that FAK in various neuronal and glial elements is required for the formation of normal histoarchitecture and cytoarchitecture in the cerebellum, and for the construction of proper innervation territory and synaptic wiring in PCs.

Introduction

Proper function of the nervous system requires precise formation of neuronal circuitry during development. The histoarchitecture and cytoarchitecture of the cerebellum and the pattern of intrinsic neural connections are known in considerable detail (Altman & Bayer, 1997). The cerebellum is composed of only a few cell types, all organized in a precise fashion in distinct morphological layers, and the microcircuitry is highly specific and uniform. The cerebellum receives two excitatory afferents, the climbing fiber (CF) and the mossy fiber–parallel fiber (PF) pathway, both converging onto Purkinje cells (PCs; Palay & Chan-Palay, 1974). CFs originate in the inferior olivary nucleus and innervate proximal dendrites of PCs, whereas PFs are granule cell axons forming synapses onto distal dendrites, called spiny branchlets. PCs also receive inhibitory, γ-aminobutyric acid (GABA)ergic inputs from basket and stellate interneurons. These PC synapses are thoroughly enwrapped by Bergmann glia (BG), which situate cell bodies around PC somata, extend radial or Bergmann fibers to the pial surface, and express a variety of transmitter transporters (Yamada *et al.*, 2000). These features make the cerebellum an ideal system for studying how the histoarchitecture and cytoarchitecture develop, how different neuronal populations find and maintain their target, and what these molecular mechanisms are.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that is widely expressed in different cell types. Although integrins are known as the main extracellular regulators of FAK during focal adhesion assembly (Schaller *et al.*, 1992), FAK has recently been suggested to mediate signaling through several cell surface receptors, including platelet-derived growth factor receptor, epidermal growth factor receptor (Sieg *et al.*, 2000), netrin receptor (Li *et al.*, 2004; Liu *et al.*, 2004; Ren *et al.*, 2004; Nikolopoulos & Giancotti, 2005) and Ephs (Miao *et al.*, 2000; Cowan & Henkemeyer, 2001; Moeller *et al.*, 2006). FAK is involved in multiple cellular processes, including adhesion, spreading, migration, survival, cell cycle progression and proliferation (Parsons, 2003). FAK is also expressed in the brain (Burgaya *et al.*, 1995; Menegon *et al.*, 1999). Recent studies revealed the functional roles of FAK in cortical basement membrane assembly and/or remodeling, neural migration, dendritic morphology, axonal branching and synapse formation (Beggs *et al.*, 2003; Xie *et al.*, 2003; Rico *et al.*, 2004).

Here, we studied the cellular and subcellular distributions of FAK in the cerebellum. Furthermore, we generated neuron/glia-specific and PC-specific FAK mutant mice to investigate its roles in cerebellar

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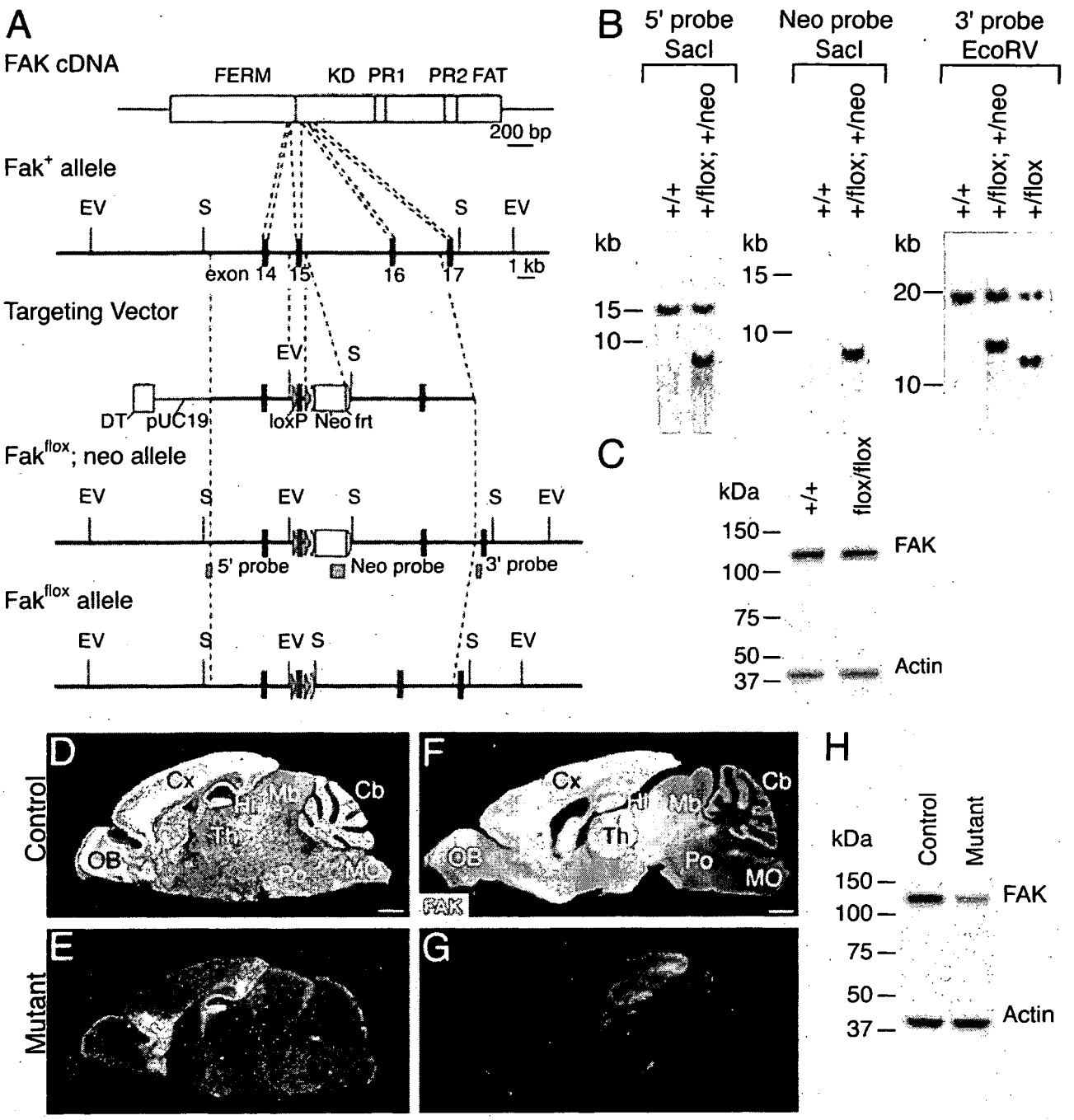

FIG. 1. Generation of neuron/glia-specific FAK mutant mice. (A) Schematic representation of FAK cDNA, *Fak* gene (Fak^{+}), targeting vector, floxed and *neo*-inserted allele (Fak^{flox};*neo*), and floxed allele (Fak^{flox}). Exon 15 encodes the first kinase domain of FAK. The Fak^{flox};*neo* allele contains two *loxP* sequences flanking exon 15 of the *Fak* gene and the *neo* gene flanked by two *frt* sequences. The *neo* gene was removed *in vivo* by crossing $Fak^{+/flox}$;+/*neo* mice with FLP66 mice carrying the Flp recombinase gene under the control of the *EF1α* promoter (Takeuchi *et al.*, 2002). DT, diphtheria toxin gene; EV, *Eco*RV; FAT, focal adhesion targeting domain; FERM, erythrocyte band 4.1-ezrin-radixin-moesin domain; KD, kinase domain; Neo, neomycin phosphotransferase gene; PR1 and PR2, proline-rich domain 1 and 2; pUC19, plasmid pUC19; S, *Sac*I. (B) Southern blot analysis of genomic DNA from $Fak^{+/+}$, $Fak^{+/flox}$;+/*neo* and $Fak^{+/flox}$ mice. Left, *Sac*I-digested DNA hybridized with 5′ probe; middle, *Sac*I-digested DNA hybridized with Neo probe; right, *Eco*RV-digested DNA hybridized with 3′ probe. (C) Immunoblot analysis of FAK and actin proteins in the whole-brain homogenates from $Fak^{+/+}$ and $Fak^{flox/flox}$ mice at P21. (D and E) *In situ* hybridization for *Fak* mRNA in parasagittal brain sections of control (D) and mutant (E) mice at P21 with an antisense oligonucleotide probe against the sequence of the exon 15 of *Fak* gene. (F and G) Immunofluorescence for FAK in parasagittal brain sections of control (F) and mutant (G) mice at P21. (H) Immunoblot analysis of FAK and actin proteins in the whole-brain homogenates from control and mutant mice at P21. Cb, cerebellum; Cx, cerebral cortex; Hi, hippocampus; Mb, midbrain; MO, medulla oblongata; OB, olfactory bulb; Po, pons; Th, thalamus. Scale bars: 1 mm (D and F).

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