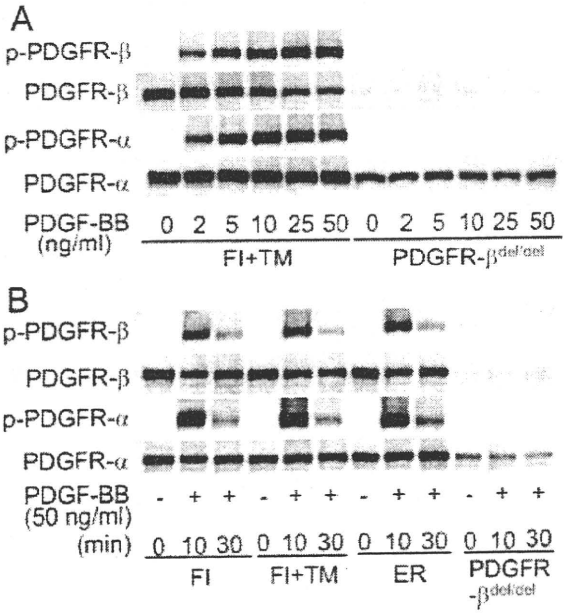


Fig. 5. In vitro induction of *PDGFR-β* gene deletion in primary cultured neurons. *Cre-ER*$^{TM+/-}$*/PDGFR-β*$^{flox/flox}$ neurons were treated with 1 μM 4-OH-TM for 48 hr from 3 to 5 DIV to delete the *PDGFR-β* gene (*PDGFR-β*$^{del/del}$ neurons). For control analyses, three types of neurons were prepared: *PDGFR-β*$^{flox/flox}$ neurons (Fl), 4-OH-TM- treated *PDGFR-β*$^{flox/flox}$ neurons (Fl + TM) and untreated *Cre-ER*$^{TM+/-}$*/PDGFR-β*$^{flox/flox}$ neurons (ER). (**A**) Different concentrations of PDGF-BB (0–50 ng/ml) were applied to neurons at 6 DIV, and the cultures were incubated for 10 min. Immunoblot was performed using antibodies against total and phosphorylated forms of PDGFR-α and -β. (**B**) Cultured neurons were stimulated by PDGF-BB (50 ng/ml) for 0, 10 and 30 min at 6 DIV. The cells were lysed and analyzed using the same methods as in (A).

## Elimination of the *PDGFR-β* Gene Enhances $H_2O_2$-Mediated Cytotoxicity

PDGF-induced neuroprotection was examined by an MTT assay on *PDGFR-β*$^{del/del}$ neurons, using *PDGFR-β*$^{flox/flox}$, 4-OH-TM-treated *PDGFR-β*$^{flox/flox}$, and untreated *Cre-ER*$^{TM+/-}$*/PDGFR-β*$^{flox/flox}$ neurons as controls. All three types of control neurons showed comparable levels of cell viability to each other in resting conditions and under the oxidative stress, and responded similarly to PDGF-BB-mediated cytoprotection (Fig. 6). These results confirmed that *Cre-ER*$^{TM+/-}$ transgene and 4-OH-TM treatments did not affect cell viability in the MTT assay.

The viability of *PDGFR-β*$^{del/del}$ neurons was significantly lower than that of all three types of control neurons, both in resting and oxidatively stressed conditions (Fig. 6). PDGF-BB treatment significantly attenuated $H_2O_2$-mediated cytotoxicity in *PDGFR-β*$^{del/del}$ neurons in the presence of $H_2O_2$ concentrations of 5 and 10, but not in the presence of 20 μM $H_2O_2$. However, the protective effects of PDGF-BB were apparently weaker in

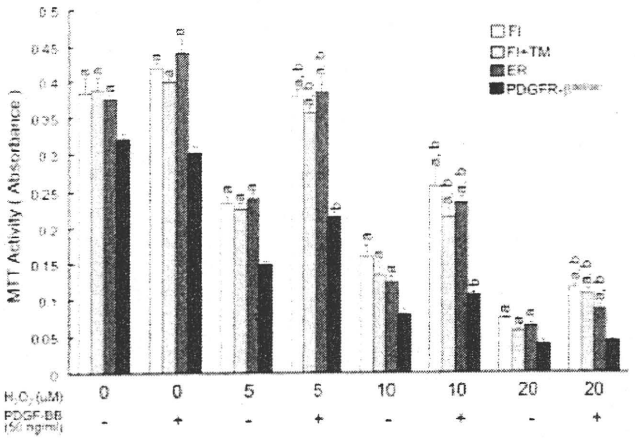


Fig. 6. *PDGFR-β*$^{del/del}$ neurons were vulnerable to $H_2O_2$-induced cytotoxicity and were less responsible to PDGF-B-mediated neuroprotection. *PDGFR-β*$^{del/del}$ neurons and three types of control neurons, *PDGFR-β*$^{flox/flox}$ (Fl) neurons, 4-OH-TM-treated *PDGFR-β*$^{flox/flox}$ (Fl + TM) neurons and untreated *Cre-ER*$^{TM+/-}$*/PDGFR-β*$^{flox/flox}$ (ER) neurons, were analyzed. Neurons were pretreated with or without 50 ng/ml PDGF-BB for 24 hr, and then exposed to different concentrations of $H_2O_2$ (5, 10, and 20 μM) for 24 hr. Cell viability was determined by MTT assay. The data represent the means ± SEM (n = 4, n indicates the number of wells of a plate for each experimental condition). [a] $p < 0.01$ vs. *PDGFR-β*$^{del/del}$ within the same treatment; [b] $p < 0.01$ vs. the same $H_2O_2$ exposure without PDGF-BB pretreatment in cells of the same genotype with or without 4-OH-TM.

*PDGFR-β*$^{del/del}$ neurons than in all three types of control neurons.

## PDGF-B-Induced Activation of PI3-K/Akt and MAPK Pathways is Decreased After *PDGFR-β* Gene Deletion

The levels of phosphorylation of signaling molecules were characterized in PDGF-BB-stimulated *PDGFR-β*$^{del/del}$ neurons. The dose-dependency of phosphorylation was examined at 10 min after PDGF-BB stimulation from 2 to 50 ng/ml using 4-OH-TM-treated *PDGFR-β*$^{flox/flox}$ neurons as a control (Fig. 7A1-D1). The kinetics of phosphorylation was examined at 10 and 30 min after 50 ng/ml PDGF-BB stimulation, using *PDGFR-β*$^{flox/flox}$, 4-OH-TM-treated *PDGFR-β*$^{flox/flox}$, and untreated *Cre-ER*$^{TM+/-}$*/PDGFR-β*$^{flox/flox}$ neurons as controls (Fig. 7A2-D2). In these two types of analyses, the levels of phosphorylated proteins were equivalent in resting conditions between *PDGFR-β*$^{del/del}$ neurons and control neurons for all proteins examined (Fig. 7A-D).

In the dose-dependency studies, the phosphorylation of Akt, ERK, JNK and p38 was highly induced after PDGF-BB stimulation from 2 to 50 ng/ml in control neurons (Fig. 7A1-D1). In the same experiment, induced phosphorylation of all these molecules examined was lower in *PDGFR-β*$^{del/del}$ neurons than in control neurons (Fig. 7A1-D1). It was note worthy that the induction of Akt and p38 phosphorylation was very little (Fig. 7A1, D1), comparing to that of ERK and JNK

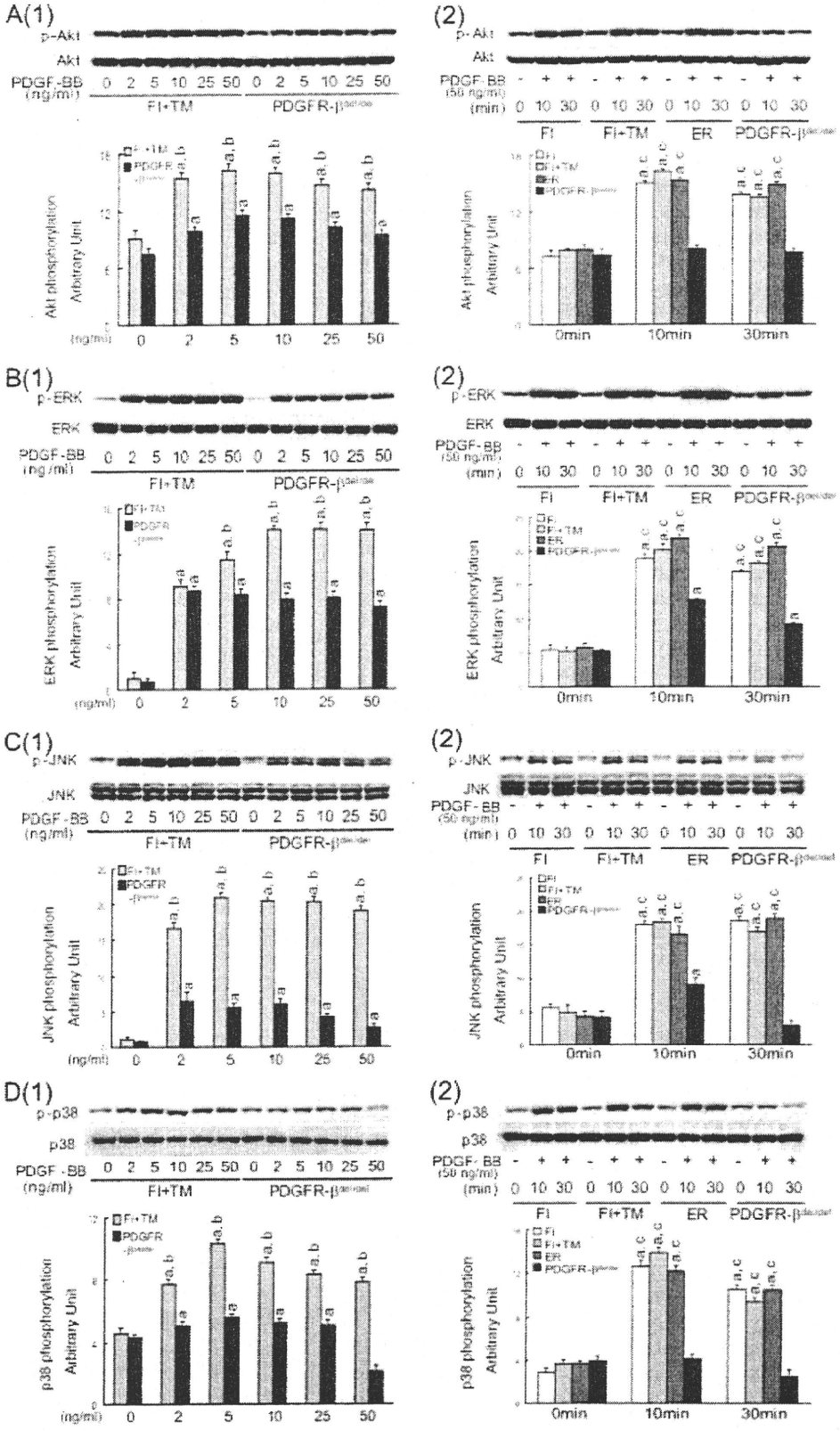


Fig. 7. PDGF-B-induced phosphorylation of PI3K and MAPK pathway was significantly lower in *PDGFR-β*$^{del/del}$ neurons than in control neurons. *PDGFR-β*$^{del/del}$ neurons and different types of control neurons, *PDGFR-β*$^{flox/flox}$ (Fl) neurons, 4-OH-TM-treated *PDGFR-β*$^{flox/flox}$ (Fl + TM) neurons and 4-OH-TM-untreated *Cre-ER*$^{TM+/-}$*/PDGFR-β*$^{flox/flox}$ (ER) neurons, were analyzed in dose effect and kinetic studies. (**1**) Cultured neurons were stimulated by PDGF-BB at the indicated concentrations for 10 min at 6 DIV. Immunoblot was performed using antibodies against total and phosphorylated forms of Akt (**A**), ERK (**B**), JNK (**C**), and p38 (**D**). (**2**) Cultured neurons were stimulated by 50 ng/ml PDGF-BB for 0, 10 and 30 min at 6 DIV. These cells were lysed and analyzed using same methods as described in (1). Data represent means ± SEM (n = 3, from three independent cultures). [a] $p < 0.01$ vs. PDGF-BB untreated base line in cells of the same genotype with or without 4-OH-TM. [b] $p < 0.01$ vs. *PDGFR-β*$^{del/del}$ with the same doses of PDGF-BB treatment. [c] $p < 0.01$ vs. *PDGFR-β*$^{del/del}$ at the same time points after PDGF-BB treatment.

phosphorylation in *PDGFR-β$^{del/del}$* neurons after PDGF-BB stimulation (Fig. 7B1, C1).

In the kinetic studies, the phosphorylation of Akt, ERK, JNK, and p38 was highly induced at 10 and 30 min after PDGF-BB stimulation in control neurons, and levels of the phosphorylation were at comparable levels among three types of control neurons (Fig. 7A2–D2). In contrast, the phosphorylation of Akt and p38 was not induced in *PDGFR-β$^{del/del}$* neurons (Fig. 7A2, D2). Induced phosphorylation of ERK and JNK in *PDGFR-β$^{del/del}$* neurons was significantly lower than that in control neurons (Fig. 7B2, C2).

## DISCUSSION

Oxidative stress is importantly involved in the pathogenesis of number of CNS diseases, however, data were limited on the mechanism in which PDGF protects neurons from oxidative stress. In the present study, we demonstrated the neuroprotective effects of PDGF using primary cultured neurons subjected to $H_2O_2$-induced cytotoxicity that has been frequently used to induce oxidative stress in various cell types (Satoh et al., 1996; Numakawa et al., 2006).

We demonstrated the neuroprotective effects of PDGF-BB in the wild-type neurons that were exposed to $H_2O_2$, through the analyses of MTT assays, LDH cytotoxicity assays and PI staining experiments. PDGF-AA also showed similar neuroprotective effects, but to a lesser extent than PDGF-BB, in the MTT assay. It was suggested that two types of PDGFR additively mediate the neuroprotective effects of PDGF. The degree of cytotoxicity and rescue after PDGF was different depending on the detection method. The percentage of PI-positive cells was higher than that of active caspase-3-positive cells at each $H_2O_2$ concentration, because PI staining labels dead cells irrespective of the type of cell death, and active caspase-3 is present only in cells undergoing apoptotic cell death. Caspase-3 has been reported to be activated by $H_2O_2$ as a final effector of apoptotic cell death in vitro (Matsura et al., 1999). In the present study, PDGF-BB pretreatment suppressed the increase in caspase-3 activation after $H_2O_2$ treatment, and the antiapoptotic effects were, at least partly, shown to underlie PDGF-mediated neuroprotection.

Despite the growing understanding of PDGF signaling, the distinctive function of PDGFR-α or PDGFR-β cannot be readily analyzed and differentiated because of their functional redundancy or compensation of the receptors with respect to one another (Wu et al., 2008). In the present study, Cre-loxP system induced the substantial suppression of PDGFR-β expression. 4OH-TM that was used to induce gene deletion did not affect the survival and the signaling of the control neurons. These allowed us to analyze the distinctive role of *PDGFR-β* in PDGF-mediated neuroprotection. In *PDGFR-β$^{del/del}$* neurons, the expression and phosphorylation of *PDGFR-α* were decreased compared with control neurons. This showed that PDGFR-β is involved in the activation of PDGFR-α in PDGF-BB-stimulated neurons.

In the wild type neurons, PDGFR-β, but not PDGFR-α, was down-regulated at 10–60 min after PDGF-BB treatment. This difference between the two receptors might correspond to the fact that the trafficking of the two receptors after activation was differently regulated as demonstrated in the embryonic fibroblasts isolated from the knockout mouse of T-cell protein tyrosine phosphatase (Karlsson et al., 2006). The ubiquitination of PDGFR has been shown to be involved in the down-regulation after receptor activation in fibroblasts (Haglund et al., 2003; Lennartsson et al., 2006). The raft/caveolin-mediated endocytosis per se, an important initial step of PDGFR down-regulation, was approved to be involved in the signal transduction and cellular response after PDGF stimulation in fibroblasts (De Donatis et al., 2008). Accordingly, the down-regulation of PDGFR-β detected in our study may reflect the fact that the activated PDGFR-β elaborated signals that could be associated with the process of receptor down-regulation. However, the mechanism and functional relevance of the PDGFR down-regulation are largely unknown in neurons.

In MTT assay, the survival of *PDGFR-β$^{del/del}$* neurons was significantly lower than that of control neurons in both basal and $H_2O_2$-exposed conditions, in which the neurons were not stimulated by PDGF. Also, the degree of protection induced by applied PDGF-BB was much less in *PDGFR-β$^{del/del}$* neurons than in control neurons. PDGFs were synthesized in neurons (Yeh et al., 1991; Sasahara et al., 1995). Taken these data together, it was suggested that PDGFR-β mediates autocrine- and exogenously applied-PDGF signals, and is involved in the survival of neurons under basal and stressed conditions. Furthermore, the enforcement of PDGFR-α signaling could be partly responsible for this function of PDGFR-β, because PDGFR-α signaling was decreased in *PDGFR-β$^{del/del}$* neurons.

PDGF-BB highly induced the phosphorylation of Akt, ERK, JNK and p38 in wild-type neurons. Comparing to these, the phosphorylation induced by PDGF-AA was less in all examined molecules; in particular, PDGF-AA-induced phosphorylation of Akt was minimum in wild-type neurons. PDGFR-β has been reported to be expressed in neurons but not in glial cells, and PDGFR-α in neurons and glial cells (Smits et al., 1991; Heldin and Westermark, 1999). Our cultured cells consisted mainly of neurons, and the glial components were limited. Taken all together into consideration, we concluded that the induced phosphorylation of Akt, ERK, JNK and p38 in wild-type neurons after PDGF stimulation mainly derived from neurons but not from glial cells.

In *PDGFR-β$^{del/del}$* neurons, the PDGF-BB-induced phosphorylation of all signaling molecules examined occurred at lower levels than in control neurons. Among them, the induction of Akt and p38 phosphorylation was specifically less than that of ERK and JNK phosphorylation. This indicated that PDGFR-α protein, detected by

Western blot, seems to have mediated the phosphorylation of JNK and ERK, but was not sufficient to phosphorylate Akt and p38 in *PDGFR-β*$^{del/del}$ neurons. Considering data from wild-type and *PDGFR-β*$^{del/del}$ neurons, it could be deduced that PDGFR-β is crucially important for the activation of Akt pathway, and that both types of PDGFR could efficiently activate MAPK pathways.

Using specific inhibitors, we demonstrated that PI3-K/Akt and MAPK pathways mediate the neuroprotective effects of PDGF against $H_2O_2$-induced neurotoxicity, although it is often reported that PI3-K/Akt and MAPK pathways can function bi-directionally to mediate both cell death and survival (Philpott and Facci, 2008). In our study, the inhibitors of PI3-kinase and JNK completely abrogated the PDGF-mediated neuroprotection in dose-dependent manner. ERK has been reported to mediate $H_2O_2$-induced cytotoxicity (Stanciu et al., 2000; Ruffels et al., 2004), however, MEK inhibitor partly inhibited PDGF-mediated neuroprotection and indicated the role of ERK in cytoprotective effects of PDGF. The effects of the p38 inhibitor were partial, and the involvement of p38 might be less than that of the other molecules. Downstream of the Akt and MAPK pathways, number of molecules including phosphorylated NF-κB, CREB and GSK-3β are listed to stimulate the transcription of antiapoptotic genes and mediate neuroprotective signaling against oxidative stress (Du and Montminy, 1998; Rojo et al., 2008; Venugopalan and Olanow, 2008). However, in our system, the survival cascades downstream of Akt and MAPK pathways after PDGF stimulation remain to be determined.

In our present study, PDGF-BB exerted more potent neuroprotective effects than PDGF-AA, as has been reported (Pietz et al., 1996; Iihara et al., 1997). In addition to the additive effects of activated two PDGFRs after PDGF-BB stimulation, our present data, more specifically, suggested that Akt that was preferentially activated by PDGFR-β might convey the potent neuroprotective effects of PDGF-BB in primary cultured neuron. In support of this notion, hypoxic stress induced the activation of PDGFR-β, and the subsequent activation of Akt was assumed to mediate the survival of cultured RN46A neuronal cells and brain stem neurons in rats (Simakajornboon et al., 2001; Zhang et al., 2003). Akt is one of the well-characterized and potent survival signals in neurons, and vanadyl, a tyrosine phosphatase inhibitor, effectively decreased the degree of ischemia-induced cerebral injury through the potentiation of the PI3-K/Akt pathway (Brazil et al., 2004; Shioda et al., 2007). Accordingly, currently characterized survival signaling at the downstream of PDGFR suggested that the activation of PDGFR-β that is widely expressed in CNS neurons could be an important therapeutic strategy for diseases of the CNS, including stroke and neurodegenerative diseases in which the PI3-K/Akt pathway has been assumed to be involved in the pathogenesis (Ishii et al., 2006; Choi et al., 2006; Caraci et al., 2008).

In the present study, we demonstrated that PDGF protects neurons from $H_2O_2$-induced cytotoxicity, and that PI-3K/Akt and MAPK pathways are involved in the protective effects. In addition, through the combined PDGF-AA, -BB stimulations and *PDGFR-β* deletion analyses, two types of PDGFRs were shown to convey neuroprotective effects of PDGFs with overlapping signaling. Furthermore, PDGFR-β was indicated to be specifically potent for the activation of the PI3-K/Akt pathway and was suggested to be important in the neuroprotection. Further studies are required on the molecular mechanism to prevent oxidative stress-induced neuronal cell damage for the prevention and care of the CNS diseases.

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Editorial

# Acute encephalopathy/encephalitis in childhood: A relatively common and potentially devastating clinical syndrome

Acute encephalopathy/encephalitis in childhood is a life-threatening disease that can result in sudden death or the development of neurological sequelae. Moreover, it is not a rare disease in childhood. Therefore, pediatric neurologists in clinical settings should recognize the significance of acute encephalopathy/encephalitis and attempt to understand its pathogenesis, clinical symptoms, diagnosis, and treatment.

About 10 years ago, influenza-associated encephalopathy was identified as an important subtype of acute encephalopathy/encephalitis in childhood. Many cases of influenza-associated encephalopathy in childhood have been reported in Japan and East Asia, and recently, some cases have been reported in Europe and the USA as well. The mortality rate associated with this form of encephalopathy was at one time as high as 30%. Research on influenza-associated encephalopathy revealed that affected children exhibit hypercytokinemia and activation of nuclear factor-κB (NF-κB)—an intracellular transcription factor—within peripheral blood mononuclear cells. Therefore, anti-proinflammatory cytokine therapy such as methylprednisolone pulse therapy and intravenous immunoglobulin therapy has been recommended for treating encephalopathy. In recent years, the mortality rate associated with encephalopathy has been reduced to 10%. However, further research has indicated that the pathogenesis of influenza-associated encephalopathy is multifactorial. Recently, acute encephalopathy with biphasic seizures and late reduced diffusion (AESD) has been identified as a subtype of influenza-associated encephalopathy. AESD is induced not only by influenza virus but also by other pathogens. The clinical course is characterized by an initial seizure or a cluster of seizures lasting for >30 min within 1 day after the onset of fever, then mild continuous disturbance of consciousness or hemiparesis after the initial prolonged seizure followed by clustered seizures. On days 3–7, lesions can be detected in the subcortical white matter by diffusion-weighted magnetic resonance imaging (MRI). Although the mortality rate due to AESD is not high, associated neurological sequelae are frequently observed. Hypercytokinemia is not the primary pathogenetic mechanism of AESD. Strategies should be developed to elucidate the pathogenesis of AESD and to find effective treatments.

The special features of acute encephalopathy are herein described. Tanuma et al. [1] measured the cerebrospinal fluid (CSF) levels of tau protein—a marker of axonal damage—in children with AESD, after having clarified the kinetics of this protein in normal controls. They found that affected children exhibit axonal damage, and that in some of these children elevated CSF tau protein levels were detected before abnormal MRI findings were obtained. Their study provides a basis for the development of early diagnostic and therapeutic strategies for AESD. Komatsu et al. [2] reported the case of an infant with AESD who experienced a cluster of subclinical seizures. The seizures were monitored by single-channel amplitude-integrated electroencephalography (aEEG). Prolonged or clustered seizures are believed possibly causative of AESD. Neonates and infants easily and frequently experience subclinical seizures due to various diseases, including acute encephalopathy. Prolonged or clustered seizures can induce neuronal damage. Therefore, especially in children with acute encephalopathy, seizures should be quickly detected and treated with anticonvulsants. The usefulness of aEEG for continuous monitoring has been reported. Further studies of AESD will hopefully provide data that can improve its prognosis.

Tanabe et al. [3] investigated the clinical course of children with influenza treated with oseltamivir who exhibited abnormal behavior. They studied whether the abnormal behavior occurred before or after the administration of oseltamivir. Moreover, they reported the details of the abnormal behavior. These authors concluded that oseltamivir treatment is not a prerequisite for the development of abnormal behavior in children with influenza. Likewise, the US FDA has declared that the contribution of oseltamivir to abnormal behavior

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has not been established. The study by Tanabe et al. [3] will ease the anxiety of physicians and parents of children with influenza.

I hope that this special issue will be useful for clinical physicians and researchers dealing with acute encephalopathy/encephalitis in childhood, and that the data reported herein will improve the outcome of children affected with this potentially devastating condition.

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Original article

# Serum and cerebrospinal fluid cytokine concentrations in subacute sclerosing panencephalitis

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**Abstract**

Subacute sclerosing panencephalitis (SSPE) is a neurodegenerative disease due to persistent measles virus infection. Its immunopathogenesis is unknown. Tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-6, IL-10 and IL-4 concentrations were measured in cerebrospinal fluid (CSF) and serum samples from 30 SSPE patients and 19 control subjects by cytometric bead array. CSF and serum IFN-γ, IL-12 and IL-18 levels were measured in 18 SSPE patients by ELISA. Serum IL-4 and IL-10 ($p < 0.001$), CSF IL-4 ($p < 0.001$) and IL-6 ($p = 0.049$) concentrations were lower, and serum IL-2 concentrations, higher ($p = 0.001$) in SSPE patients. Serum TNF-α and IL-6, CSF TNF-α, IL-10, and IL-2 concentrations were not different between SSPE and control groups. Serum IFN-γ levels were higher in stage I and II than stage III patients ($p < 0.05$), whereas there was no difference between stages in terms of other cytokines. The levels of Th2-type cytokines: IL-4, IL-6 and IL-10 were suppressed in our SSPE cases. This finding, along with relatively elevated IFN-γ and IL-2 levels, may suggest more active effector T cells compared to regulatory T cells (Treg), especially induced Treg, in early disease. High serum IL-2 concentrations might indicate peripheral Th1 activation. Discrepancies between various reports in the literature should be examined in view of the ages, stage and treatments of the patients studied. The interplay of various cytokines or cellular systems which may vary over time and between patients. Studies of treatment measures favoring the preservation of the early inflammatory response may be of interest in SSPE.


*Keywords:* Subacute sclerosing panencephalitis; Viral; Immune; Th1; Th2; Cytokines

## 1. Introduction

Subacute sclerosing panencephalitis (SSPE) is a neurodegenerative disease of children and young adults due to persistent measles virus (MV) infection. Factors related to host and virus appear involved in its pathogenesis. Among host factors, the immaturity of the immune system, altered cellular immunity especially in T lymphocytes and T helper (Th) subtypes, presence of high-titer anti-MV antibodies, suppression of some cytokines: IL-12, IFN-γ TNF-α, IL-1β, and elevation of IL-10, IL-6, IFN-β, have been suggested. Most patients with SSPE exhibit decreased MV-specific Th1 cytokine and preserved Th2 cytokine synthesis [1–4]. We measured IFN-γ IL-12, IL-18, TNF-α, IL-2 and IL-6 as markers for Th1-responses and IL-4 and IL-10 as markers for Th2-responses in SSPE in comparison with control subjects.

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## 2. Material and methods

### 2.1. SSPE patients

Paired serum and cerebrospinal fluid (CSF) samples were collected from 48 patients diagnosed with SSPE. The diagnosis was based on three criteria (1) typical clinical manifestations, (2) EEG pattern of high voltage periodic complexes, and (3) measles antibody titers in the serum and CSF detected by complement fixation test ($\geq 1/8$) or ELISA (>1.1 IU/ml). All CSF and serum samples were obtained at the time of diagnosis and before treatment, and frozen at −70 °C until tested.

We separated the patients in two groups because of lack of sufficient amount samples. One group of patients ($n = 30$, 23 males, 7 females, 3.5–12 years; median 7 years old) was tested for TNF-α, IL-2, IL-6, IL-10 and IL-4 concentrations in CSF and serum by cytometric bead array. Another group ($n = 18$; 16 males, 2 females, aged 2.5–11 years, median 5 years) was tested for IFN-γ, IL-12 and IL-18 in CSF and serum by sandwich ELISA. All CSF and serum samples were initially analyzed for protein, glucose, and measles antibodies.

The clinical data of the patients were reviewed for clinical staging (stage I to III) and neurological disability index. The course of the disease was classified as subacute, rapidly progressive or slowly progressive according to the rate of change in the neurologic disability index [5].

### 2.2. Control subjects

Control samples for serum and CSF cytokine levels were obtained from 19 Japanese children with non-degenerative neurological disorders such as epilepsy, psychomotor delay, psychosis (10 males and nine females, aged 3–12 years; median 7 years). All had normal CSF cell counts and biochemistry.

The study was approved by the institutional ethics committee of Dr. Sami Ulus Children's Hospital. Informed consent was obtained from parents of SSPE and control patients.

### 2.3. Assays of cytokines

The concentrations of serum and CSF TNF-α, IL-2, IL-4, IL-6, and IL-10 were measured with a cytometric bead array (CBA) kit (BD PharMingen, San Diego, CA) according to the manufacturer's manual, as previously described [6], with modification of the data analysis using GraphPad Prism software (GraphPad Prism Software, San Diego, CA) [7]. The lower detection limits for TNF-α, IL-2, IL-4, IL-6, and IL-10 were 2.8, 2.6, 2.6, 2.5, and 2.8 pg/ml, respectively.

Concentrations of IFN-γ, IL-12 and IL-18 were measured by a sandwich ELISA using the Human IFN-γ ELISA Kit [CytElisa™, Maryland, USA]; Human IL-12 (p70) ELISA kit [CytElisa™, Maryland, USA] and Human IL-18 ELISA Kit [BenderMedSystems, Vienna, Austria]: the sensitivity ranges were: IFN-γ, 15.6–1000 pg/ml; IL-12, 15.6–1000 pg/ml and IL-18, 55–582 pg/ml according to manufacturer's instructions. The inter-assay variations were 10.9% for IFN-γ, 10.9% for IL-12 and 12.9% for IL-18.

Cytokine index was calculated using the following equation:

[Cytokine]CSF/[Cytokine]Serum

: [Albumin]CSF/[Albumin]Serum

### 2.4. Statistical analysis

Data were analyzed using SPSS 9.05 for Microsoft Office. The Mann–Whitney $U$ test was used for comparison between patients, and Wilcoxon Signed Ranks Test, for comparisons between the serum and CSF measurements of each patient. Correlations between variables were calculated using Spearman's and Pearson's Correlation Coefficients.

## 3. Results

Serum IL-4 and IL-10 ($p < 0.001$), CSF IL-4 ($p < 0.001$) and IL-6 ($p = 0.049$) concentrations were lower, and serum IL-2 concentrations, higher ($p = 0.001$) in SSPE than controls. There were no differences in serum TNF-α, IL-6 or CSF IFN-γ, TNF-α, IL-10 and IL-2 concentrations (Table 1). No correlation was observed between cytokine and measles IgG levels in serum or CSF.

Serum IFN-γ levels were higher than CSF IFN-γ ($p < 0.001$) (Table 2). Serum IFN-γ was higher in stage I or II ($81.3 \pm 49.5$) than stage III patients ($42.6 \pm 34$) ($p < 0.05$). The IFN-γ index was higher in patients older than 5 years ($n = 7$) ($178.3 \pm 94.6$) compared to those younger ($n = 11$) ($87.5 \pm 80.4$) ($p < 0.05$). Levels of IL-12 and IL-18 were not associated with any particular feature in terms of age, CSF measles antibody titers, clinical course, stage, neurological disability index (NDI). A rapidly progressive course was found in 10 cases, subacute course in 6, and slow course in 2 patients. Most patients were in stage III ($n = 11$), and others, in stage II ($n = 4$) or I ($n = 2$). There was no difference of cytokine values between patients with progressive course and those with subacute or slow course.

Correlations between serum and CSF levels were examined. Among serum levels, only IFN-γ and IL-12 correlated with each other ($r = 0.48$, $p < 0.05$). Serum IFN-γ correlated with CSF IFN-γ ($r = 0.50$, $p < 0.05$) and CSF IL-18 ($r = 0.47$, $p < 0.05$). Serum IL-12 correlated with CSF IFN-γ, IL-12 and IL-18 ($r = 0.77$ and

Table 1
Mean cytokine levels in patient and control groups.

| | Patients ($n = 30$) | Controls ($n = 19$) | $p$ Value |
|---|---|---|---|
| *TNF-α (pg/ml)* | | | |
| Serum | 6 ± 7.5 (2.8–41.3) | 3.76 ± 2.21 | >0.05 |
| CSF | 4.3 ± 2 (2.8–9.4) | 3.8 ± 1.36 | >0.05 |
| *IL-2 (pg/ml)* | | | |
| Serum | **3.7 ± 2.5 (2.6–12.1)** | **2.06 ± 1.3** | **0.001** |
| CSF | 3.5 ± 1.8 (2.6–9.9) | 2.44 ± 1.06 | >0.05 |
| *IL-6 (pg/ml)* | | | |
| Serum | 20.7 ± 90.6 (2.5–491.6) | 6.83 ± 7.94 | >0.05 |
| CSF | **4.2 ± 3.1 (2.5–18.8)** | **5.11 ± 2.33** | **0.049** |
| *IL-4 (pg/ml)* | | | |
| Serum | **2.6 ± 0 (2.6–2.6)** | **6.21 ± 4.87** | **<0.001** |
| CSF | **2.6 ± (2.6–3.1)** | **6.11 ± 2.85** | **<0.001** |
| *IL-10 (pg/ml)* | | | |
| Serum | **4.1 ± 1.5 (2.8–9.2)** | **7.85 ± 3.23** | **<0.001** |
| CSF | 3.5 ± 1.1 (2.8–7.5) | 3.47 ± 1.34 | >0.05 |

Significant values are marked in bold characters.

$p < 0.01$, $r = 0.90$ and $p < 0.01$, $r = 0.75$ and $p < 0.01$, respectively). CSF IFN-γ correlated with CSF IL-12 ($r = 0.91$, $p < 0.01$) and CSF IL-18 ($r = 0.71$ and $p < 0.01$). CSF IL-12 correlated with CSF IL-18 ($r = 0.78$ and $p < 0.01$). The IFN-γ index correlated with IL-12 ($r = 0.75$, $p < 0.01$) and IL-18 ($r = 0.73$, $p < 0.01$) indexes, but not with clinical stage, course or neurological disability. No correlations were found between CSF cytokine levels and CSF measles antibody titers or protein concentrations.

## 4. Discussion

Cytokine studies in SSPE produced variable results in the literature, including low IL-12 production by mononuclear cells, elevated serum IL-1β, IL-6, TNF-α and IL-10 levels, elevated CSF IL-10 and TNF-α and unchanged CSF IL-4, IL-18 and IFN-γ levels. Intracellular IL-4 and TNF-α showed no particular pattern [1,8–11]. IFN-γ mediates the elimination of MV from neurons; it induces macrophage activation, Th-cell differentiation and MHC expression, thereby enhancing the antigen-presenting capabilities of dendritic cells and macrophages and promoting killing of intracellular pathogens. The combination of IL-12 and IL-18 generates maximal IFN-γ production by macrophages and dendritic cells. These three cytokines can therefore be considered as the IFN-γ system [12,13]. According to our observations, these cytokines correlate with each other, and the IFN-γ system appears intact in SSPE. Serum IFN-γ levels were higher in early stage patients, and IFN-γ indexes, in those older than 5 years. The capacity of cytokine production might vary with age, and the frequency of SSPE in children who had primary measles infection before 2 years old might be related to insufficient IFN-γ synthesis contributing to MV persistence.

Table 2
IFN-γ, IL-12, and IL-18 index in SSPE patients ($n = 18$).

| | |
|---|---|
| *IFN-γ (pg/ml)-ELISA* | |
| Serum | 57.6 ± 43.8 (11.0–166.0) |
| CSF | 19.9 ± 15.2 (9.0–63.0) |
| **IFN-γ index** | **122.8 ± 95.0** |
| *IL-12 (pg/ml)-ELISA* | |
| Serum | 26.2 ± 28.6 (9.0–136.0) |
| CSF | 27.6 ± 28.2 (12.0–128.0) |
| **IL-12 index** | **320.3 ± 286.0** |
| *IL-18 (pg/ml)-ELISA* | |
| Serum | 92.7 ± 58.8 (40.0–240.0) |
| CSF | 73.0 ± 31.7 (40.0–160.0) |
| **IL-18 index** | **259.6 ± 179.0** |

* $p < 0.001$ compared to CSF.

High serum IL-2 concentrations and suppressed Th2-type cytokines (IL-4, IL-6 and IL-10) found in our study might reflect the activation of peripheral Th1 cells during inflammation and the counterbalance between Th1- and Th2-types of immune response. On the other hand, certain studies found preserved IL-10 production by mononuclear cells in vitro or in the CSF of SSPE patients [1,11]. This discrepancy might be due to the stage of the disease at the time of sampling: effector T cells might be more active, and regulatory T cells (Treg), relatively suppressed in early stages of the disease, as were most of our patients. Hara et al. studied cytokine productions by peripheral blood mononuclear cells in response to live measles virus in 15 SSPE patients [1]. Although most patients had a defect in production of IFN-γ, they noted that four patients did produce significant amounts of IFN-γ (three in stage II and one in early stage III). Of eleven patients with defective IFN-γ production, seven were in stage III and IV. On the other hand, they observed IL-10 values were heterogeneous in despite of presence in all cases. We suppose that before the onset of SSPE, induced Treg and especially Tregulatory1 (Tr1) cells may take part in the pathogenesis. Tr1 cells are generated from naive T cells in the presence of IL-10; they produce high levels of IL-10 and inhibit some T cell responses through IL-10 production in vivo [14,15]. Their suppressive activity is higher on Th1 than on Th2 cells [16]. Tr1 cells play a role in limiting pathologies associated with a high antigenic load such as infections [17]. Because natural Treg cells monitor the immune activation, immune-mediated damage takes place in host tissue when they are spent [18,19]. This phenomenon, "Tr1 exhausting", might underlie the decreased serum IL-10 levels observed in our SSPE patients, associated with the ending of latency and the beginning of inflammation.

Ichiyama reported that fever in SSPE patients might be related to elevated serum IL-6 and IL-10, and myoclonic jerks, elevated CSF IL-6 levels [7]. The absence

of fever may account for the low IL-6 levels in our SSPE group. In that study, there was no information about concomitant infections or therapy during sampling. On the contrary of other studies, we compared the patients with age-matched controls as in Ichiyama's study [1,7,8,10], and used further parameters such as cytokine indices and NDI for comparison [1,7,8]. We think that taking into consideration the stage and course of the disease while comparing the patients with each others that was not done before is also unique to our study [1,7,8].

We investigated two potent IFN-inducers: IL-12 and IL-18, as markers for Th1-responses in SSPE. Our study shows correlations between IFN-γ, IL-12 and IL-18 especially in the CSF. Saruhan-Direskeneli et al. reported no difference of serum and CSF IFN-γ and IL-18 levels between SSPE and neurological control subjects [10]. Serum levels being susceptible to peripheral activation of the IFN-γ system due to various non-specific immune stimuli, cytokine indices appear to be more accurate measurements: they can be elevated while serum or CSF cytokine levels are not (4 patients).

Immunological status in SSPE may be related to clinical stage and the network of various cytokines or cellular systems, which vary over time and between patients. This might be the one of the underlying reasons for clinical heterogeneity. In this study, only a certain part of the cytokine network has been evaluated: further studies examining other cytokines and regulating cellular systems may contribute to clarify this hypothesis.

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# Prostaglandin $E_2$ suppresses $\beta_1$-integrin expression via E-prostanoid receptor in human monocytes/macrophages

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ABSTRACT

$\beta_1$-Integrins mediate cell attachment to different extracellular matrix proteins, intracellular proteins, and intercellular adhesions. Recently, it has been reported that prostaglandin $E_2$ ($PGE_2$) has anti-inflammatory properties such as inhibition of the expression of adhesion molecules or production of chemokines. However, the effect of $PGE_2$ on the expression of $\beta_1$-integrin remains unknown. In this study, we investigated the effects of $PGE_2$ on the expression of $\beta_1$-integrin in the human monocytic cell line THP-1 and in $CD14^+$ monocytes/macrophages in human peripheral blood. For this, we examined the role of four subtypes of $PGE_2$ receptors and E-prostanoid (EP) receptors on $PGE_2$-mediated inhibition. We found that $PGE_2$ significantly inhibited the expression of $\beta_1$-integrin, mainly through $EP_4$ receptors in THP-1 cells and $CD14^+$ monocytes/macrophages in human peripheral blood. We suggest that $PGE_2$ has anti-inflammatory effects, leading to the inhibited expression of $\beta_1$-integrin in human monocytes/macrophages, and that the $EP_4$ receptor may play an important role in $PGE_2$-mediated inhibition.



## 1. Introduction

During inflammation, leukocytes interact with proteins of the extracellular matrix after migration through vascular endothelia to the site of tissue injury or infection. These interactions are mediated through integrins, which exist as heterodimers of noncovalently associated α and β subunits [1]. It has already been reported that 13 different integrins are expressed in the vascular system during developmental or postnatal angiogenesis: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_8\beta_1$, $\alpha_9\beta_1$, $\alpha V\beta_1$, $\alpha V\beta_3$, $\alpha V\beta_5$, and $\alpha V\beta_8$ [2]. $\beta_1$-Integrins are thought to play an important role in inflammation-associated cell–cell interactions, atherosclerosis, and myocardial infarction by promoting the migration and adhesion of vascular smooth muscle and endothelial cells, modulation of matrix synthesis, and tissue repair remodeling [3,4]. We previously reported that plasma prostaglandin $E_2$ ($PGE_2$) levels were markedly elevated during the acute stage of Kawasaki disease, and $PGE_2$ induced the expression of activated $\beta_1$-integrin in human coronary arterial endothelial cells [5,6]. $PGE_2$ contributes to dilation of coronary arteries and increased vascular permeability, and acts in a complex manner via four subtypes of receptor ($EP_1$, $EP_2$, $EP_3$, and $EP_4$) that belong to the G-protein-coupled receptor family [7]. The action of $PGE_2$ varies among different receptor subtypes in the same cell and among different cells with the same receptor subtypes [7,8]. It is commonly recognized that $PGE_2$ may have various functions mediated via individual EP receptors in different kinds of cells. But $PGE_2$ may have both inflammatory and anti-inflammatory functions in the same cells via different co-expressed EP receptors. Indeed, $PGE_2$ has been reported to have inhibitory effects on interleukin-18 (IL-18)-induced expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1, CD54) or CD86 (B7.2) [9]. We focused on the inhibitory effects of $PGE_2$ on human monocytes/macrophages in inflammation. We investigated whether the effect of $PGE_2$ on $\beta_1$-integrin expression in human monocytes/macrophages plays an important role in inflammatory disease. In this study, we examined the expression of EP receptors and investigated the inhibitory effects of $PGE_2$ through EP receptors for $\beta_1$-integrin expression in human monocytes/macrophages.

## 2. Materials and methods

### 2.1. Cell culture and stimulation condition

THP-1 cells, a human monocytic leukemia cell line, were obtained from the American Type Culture Collection, and were maintained at 37 °C under humidified 5% $CO_2$ as stationary cultures.

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The cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood of six healthy volunteers after obtaining their informed consent. We obtained the PBMCs by Lymphoprep (Nycomed Pharma AS Diagnostics; Oslo, Norway) gradient centrifugation and washing. Purification of individual cell subpopulations was achieved with a high gradient MiniMACS purification system (Miltenyi; Sunnyvale, CA, USA). $CD14^+$ monocytes/macrophages were depleted of non-monocytes (negative selection) with a Monocytes Isolation Kit II (Miltenyi). The purity of the isolated cells was determined using fluorescein isothiocyanate-conjugated CD14 monoclonal antibody (Becton–Dickinson Bioscience; San Diego, CA, USA) and flow cytometric analysis (FACS Calibur; Becton–Dickinson Bioscience). We collected the highly-purified human peripheral monocytes/macrophages (more than 80%, data not shown).

The cells were exposed to 0.1, 1, 10, 100, or 1000 ng/ml $PGE_2$ (Sigma–Aldrich; Oakville, Ontario, Canada), 10 μM $EP_1$ (ONO-DI-004), $EP_2$ (ONO-AE1-259-01), $EP_3$ (ONO-AE-248), or $EP_4$ (ONO-AE1-329) agonists, provided by Ono Pharmaceutical Co. (Osaka, Japan) for 24 h. Some samples were pretreated with 10 μM of an $EP_2$ antagonist (AH 6809) (Cayman Chemical; Ann Arbor, MI, USA), or an $EP_1$ (ONO-8713), $EP_3$ (ONO-AE3-240), or $EP_4$ (ONO-AE3-208) antagonist, provided by Ono Pharmaceutical Co., 60 min before the addition of $PGE_2$.

### *2.2. mRNA expression of EP receptors in human monocytes/macrophages analyzed by reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was prepared from PBMCs, THP-1, U937, and Jurkat cells using TRIzol reagent (Invitrogen; Carlsbad, CA, USA). RT-PCR was performed with a SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen) according to the manufacturer's instructions. The primers specific for each EP were designed as described previously [10,11]. The sequences of the primers were as follows:

$EP_1$ (322 bp), 5′-CTT GTC GGT ATC ATG GTG GTG TC-3′ (forward) and 5′-GGT TGT GCT TAG AAG TGG CTG AGG-3′ (reverse); $EP_2$ (654 bp), 5′-GCC ACG ATG CTC ATG CTC TTC GCC-3′ (forward) and 5′-CTT GTG TTC TTA ATG AAA TCC GAC-3′ (reverse); $EP_3$ (837 bp), 5′-CGC CTC AAC CAC TCC TAC ACA-3′ (forward) and 5′-GCA GAC CGA CAG CAC GCA CAT-3′ (reverse); and $EP_4$ (434 bp), 5′-TGG TAT GTG GGC TGG CTG-3′ (forward) and 5′-GAG GAC GGT GGC GAG AAT-3′ (reverse); and GAPDH (452 bp), 5′-ACC ACA GTC CAT GCC ATC AC-3′ (forward) and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (reverse).

PCR was performed using a Dice TaKaRa PCR Thermal cycler (TaKaRa; Tokyo, Japan). We used DNase (Invitrogen) before the RT-PCR to avoid genomic DNA contamination. The PCR products were electrophoresed on 2% agarose gels and, after staining with ethidium bromide, the results were visualized under ultraviolet illumination. All experiments were performed five times.

### *2.3. The expression of EP receptors in human monocytes/macrophages analyzed by Western blotting*

Whole cell lysates were obtained by the incubation of cell samples in ice-cold lysis buffer (1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) with protease inhibitors (1 μM leupeptin and 1 μM pepstatin) and centrifugation to remove debris (12,000*g* for 10 min at 4 °C). The protein concentrations of the samples were determined with Bio-Rad protein concentration reagent (Bio-Rad; Hercules, CA, USA). Samples containing 20 μg of protein were separated in denaturing 10% polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After three washes in Tris-buffered saline with Tween 20 (TBST; 40 mM Tris–HCl, pH 7.6, 300 mM NaCl, and 0.5% Tween 20), the membranes were incubated overnight with 1:200, 1:100, 1:500, and 1:500 dilution of rabbit polyclonal anti-$EP_1$, $EP_2$, $EP_3$, and $EP_4$ receptor antibodies (Cayman Chemical), respectively, in TBST containing 5% nonfat dry milk at 4 °C. We also used rabbit polyclonal anti-human β-actin antibody (1:200, AnaSpec Inc.; San Jose, CA, USA) as an internal control. After three washes in TBST, the membranes were incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 1 h at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham; Arlington Heights, IL) and analyzed by radiography. We used Jurkat cells, human T cell leukemia cell line, and PBMCs as positive controls in Western blotting. All experiments were performed three times.

### *2.4. Determination of total or activated $\beta_1$-integrin expression*

The expression of $\beta_1$-integrin was determined by flow cytometric analysis. The cells were labeled with 10 μl of phycoerythrin-labeled anti-human CD29 antibodies (BD Pharmingen; San Jose, CA) as the surface antigen for total $\beta_1$-integrin or activated $\beta_1$-integrin (clone HUTS21, BD Pharmingen) [12]. Immunofluorescence staining was analyzed using a FACS Calibur flow cytometer equipped with CellQuest software (BD Pharmingen). Ten thousand cells were analyzed for each subject in the flow cytometric studies. We compared geometric mean fluorescence intensity between cells treated with $PGE_2$ and those treated with EP receptor agonists or antagonists.

### *2.5. Statistical analysis*

All values are presented as the means ± standard deviation. Differences in the results were analyzed by means of analysis of variance, with a $p$ value less than 0.05 being taken as significant.

## 3. Results

### *3.1. Expression of EP receptors in human monocytes/macrophages*

RT-PCR of PBMCs, THP-1 cells, U937 cells, and Jurkat cells showed expression of $EP_1$ (322 bp), $EP_2$ (654 bp), $EP_3$ (837 bp), and $EP_4$ (435 bp) receptors (Fig. 1). Western blot analysis of THP-1 cells revealed expression of $EP_1$ (42 kDa), $EP_2$ (52 kDa), $EP_3$ (53 kDa), and $EP_4$ receptor (65 kDa) (Fig. 2). Also, U937 cells, from a human monocytic leukemic cell line, expressed $EP_{1-4}$ receptors the same as THP-1 cells. We used Jurkat cells and PBMCs as positive controls, because it has been previously reported that $EP_2$,

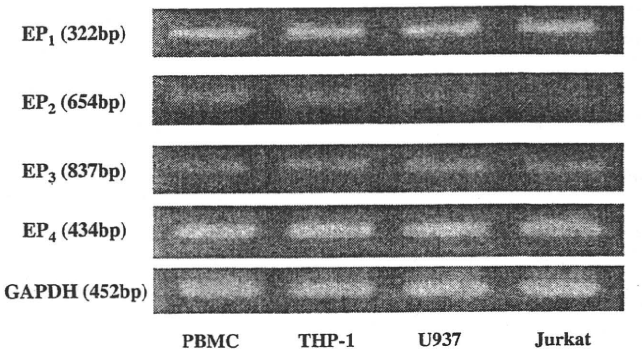


**Fig. 1.** The mRNA expression of EP subtypes measured by reverse transcription-PCR. mRNA expression of each EP receptor was observed in PBMCs, THP-1 cells, U937 cells, and Jurkat cells. Representative data are shown. Similar results were obtained in five independent experiments.

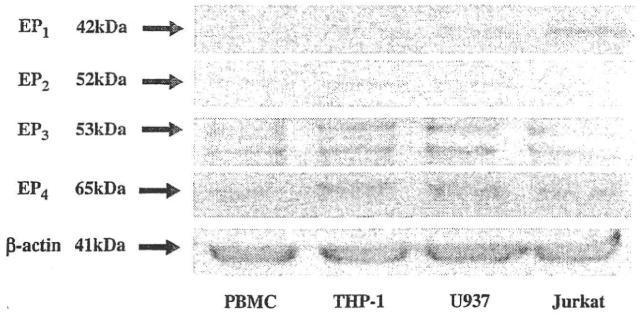


**Fig. 2.** Expression of EP subtypes measured by Western blot analysis in PBMCs, THP-1 cells, U937 cells, and Jurkat cells. $EP_1$, $EP_2$, $EP_3$, and $EP_4$ receptors were expressed in PBMCs and THP-1 cells. Representative data are shown. Similar results were obtained in three independent experiments.

$EP_3$, or $EP_4$ receptors are functionally expressed in Jurkat cells or PBMCs [13,14]. The expression levels of all subtypes were slightly higher in THP-1 cells compared with PBMCs. These results suggest that THP-1 cells and PBMCs constitutively express mRNA and protein of all four EP receptors.

### *3.2. Inhibitory effects of $PGE_2$ through EP receptors on the expression of total or activated $\beta_1$-integrin in $CD14^+$ human peripheral monocytes/macrophages and THP-1 cells*

$PGE_2$ significantly reduced total or activated $\beta_1$-integrin expression in $CD14^+$ human peripheral monocytes/macrophages (Fig. 3A and Table 1) and THP-1 cells (Fig. 3B and Table 1) at 10, 100, or 1000 ng/ml ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, respectively). The inhibitory effects on the expression of activated $\beta_1$-integrin by $PGE_2$ occurred in a dose-dependent manner. By Western blotting, we confirmed higher levels of each EP receptor expression in THP-1 cells compared with PBMCs as shown in Fig. 2. Therefore, we used THP-1 cells to characterize the function of each EP receptor in the expression of $\beta_1$-integrin. Fig. 4 and Table 2 present the effect of $EP_{1-4}$ agonists, respectively, on total or activated $\beta_1$-integrin expression in THP-1 cells. $EP_2$, $EP_3$, and $EP_4$ agonists significantly reduced expression of total $\beta_1$-integrin (Fig. 4 and Table 2). The inhibitory effect of $EP_4$ agonists was most significant among them. As for the inhibitory effects on the expression of activated $\beta_1$-integrin, only $EP_4$ agonist suppressed the levels of activated $\beta_1$-integrin expression (Fig. 4 and Table 2).

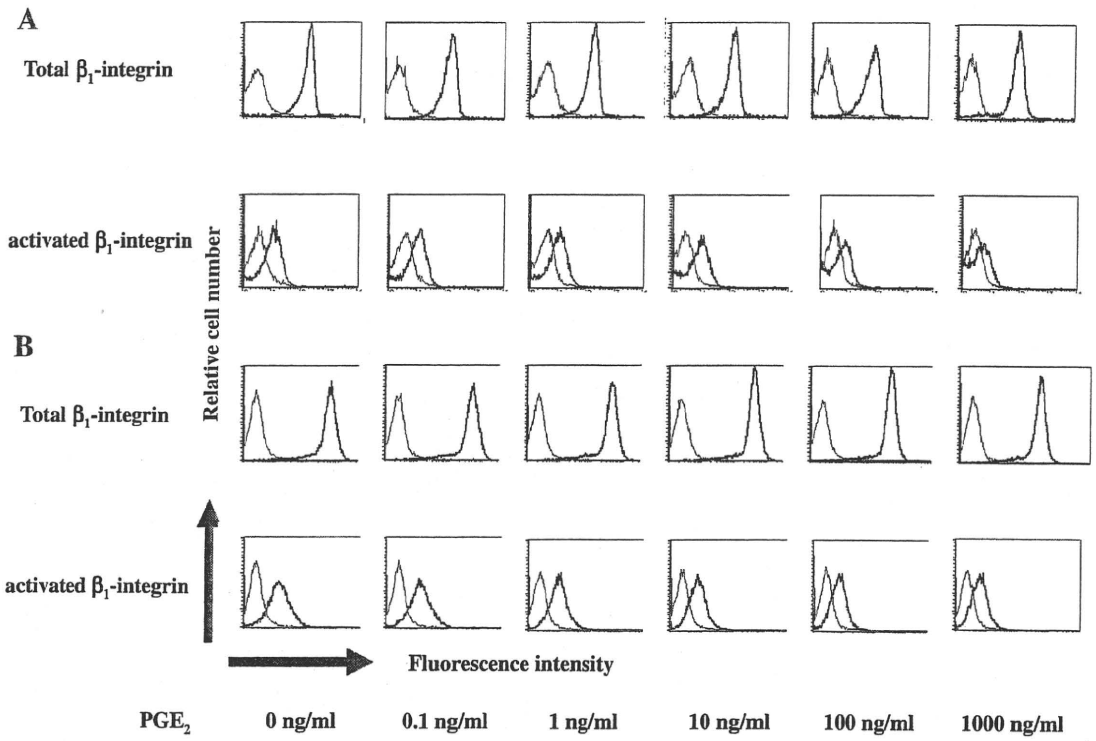


**Fig. 3.** Flow cytometric analysis demonstrated inhibitory effects of $PGE_2$ on the total and activated $\beta_1$-integrin expression in $CD14^+$ human peripheral blood monocytes/macrophages (A) and THP-1 cells (B). Cells were treated with 0, 0.1, 1, 10, 100, and 1000 ng/ml $PGE_2$ for 24 h. $PGE_2$ inhibited both total and activated $\beta_1$-integrin expression in a dose-dependent manner. Representative data are shown. Similar results were obtained in six independent experiments.

**Table 1**
Mean fluorescence intensity of both total and activated $\beta_1$-integrin expression in human $CD14^+$ monocytes/macrophages and THP-1 cells treated with $PGE_2$ (mean ± SD).

| | | 0 ng/ml | 0.1 ng/ml | 1 ng/ml | 10 ng/ml | 100 ng/ml | 1000 ng/ml |
|---|---|---|---|---|---|---|---|
| $CD14^+$ monocytes/macrophages | Total $\beta_1$-integrin | 130.7 ± 5.0 | 126.3 ± 3.1 | 106.3 ± 0.5 | 99.0 ± 7.6$^*$ | 83.6 ± 23.0$^{**}$ | 81.8 ± 6.4$^{**}$ |
| | Activated $\beta_1$-integrin | 7.97 ± 0.33 | 7.75 ± 0.22 | 7.36 ± 0.27 | 5.99 ± 1.01$^*$ | 3.64 ± 0.43$^{***}$ | 3.22 ± 0.11$^{***}$ |
| THP-1 cells | Total $\beta_1$-integrin | 377.2 ± 24.4 | 368.2 ± 27.4 | 354.2 ± 11.9 | 328.6 ± 7.3$^*$ | 279.1 ± 24.3$^{**}$ | 274.2 ± 6.3$^{***}$ |
| | Activated $\beta_1$-integrin | 13.31 ± 0.46 | 11.37 ± 1.14 | 11.01 ± 1.74 | 7.13 ± 0.07$^{***}$ | 6.54 ± 0.23$^{***}$ | 6.33 ± 0.41$^{***}$ |

$^*$ $p < 0.05$.
$^{**}$ $p < 0.01$.
$^{***}$ $p < 0.001$.

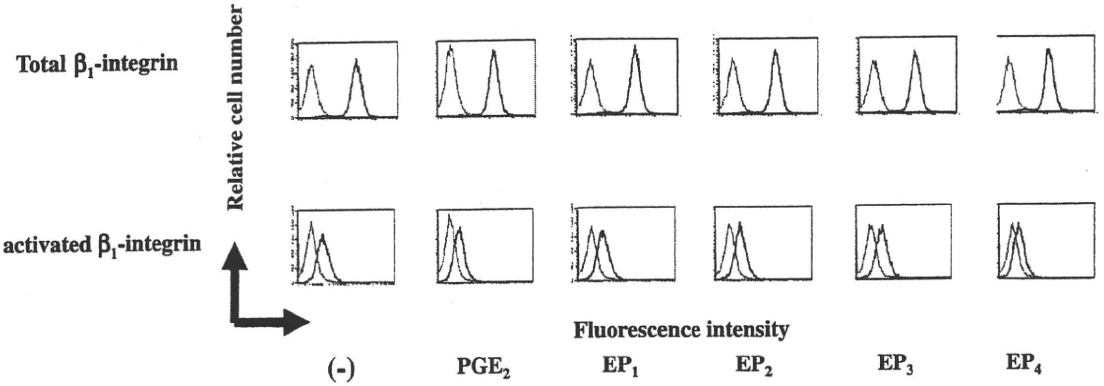


**Fig. 4.** Flow cytometric analysis demonstrated suppression of the total and activated $\beta_1$-integrin by $EP_{1-4}$ agonists in THP-1 cells. Cells were stimulated with 10 μM $EP_1$, $EP_2$, $EP_3$, and $EP_4$ agonists for 24 h. $EP_2$, $EP_3$, and $EP_4$ suppressed total $\beta_1$-integrin. Only $EP_4$ suppressed activated $\beta_1$-integrin. Representative data are shown. Similar results were obtained in six independent experiments.

**Table 2**
Mean fluorescence intensity of both total and activated $\beta_1$-integrin expression in THP-1 cells stimulated with each EP agonist (mean ± SD).

| | (-) | $PGE_2$ | $EP_1$ | $EP_2$ | $EP_3$ | $EP_4$ |
|---|---|---|---|---|---|---|
| Total $\beta_1$-integrin | 199.7 ± 5.5 | 163.0 ± 0.5* | 187.9 ± 7.0 | 159.2 ± 29.8* | 175.3 ± 7.4* | 141.8 ± 5.0** |
| Activated $\beta_1$-integrin | 9.40 ± 0.81 | 7.22 ± 0.33** | 9.51 ± 0.73 | 9.58 ± 0.42 | 9.79 ± 0.31 | 6.55 ± 0.99** |

* $p < 0.05$.
** $p < 0.01$.

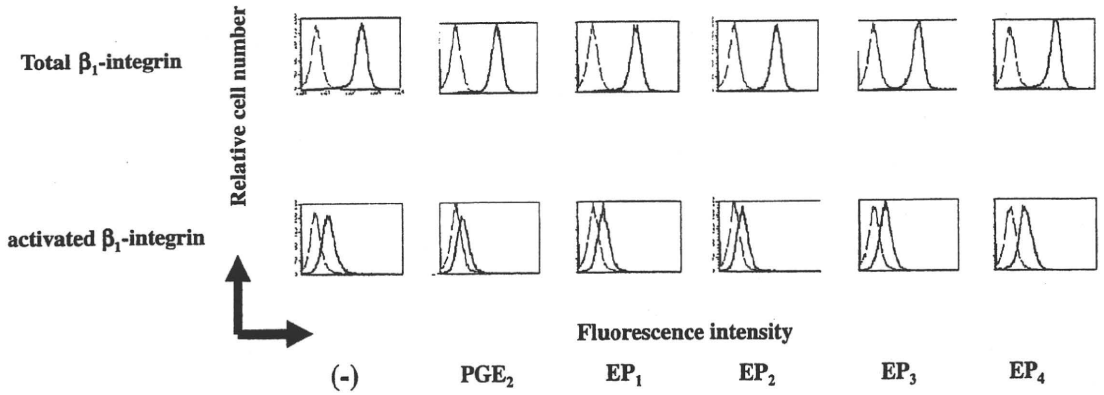


**Fig. 5.** Flow cytometric analysis demonstrated the effects of $EP_{1-4}$ antagonists on total and activated $\beta_1$-integrin expression inhibited by $PGE_2$ in THP-1 cells. Cells were pretreated with 10 μM $EP_1$, $EP_2$, $EP_3$, and $EP_4$ antagonists for 60 min and stimulated at 10 ng/ml $PGE_2$ for 24 h. $EP_3$ and $EP_4$ antagonists recovered total $\beta_1$-integrin inhibited by $PGE_2$. Only $EP_4$ antagonists recovered activated $\beta_1$-integrin inhibited by $PGE_2$. Representative data are shown. Similar results were obtained in six independent experiments.

**Table 3**
Mean fluorescence intensity in both total and activated $\beta_1$-integrin expression in THP-1 cells treated with EP antagonist (mean ± SD).

| | (-) | $PGE_2$ | $EP_1$ | $EP_2$ | $EP_3$ | $EP_4$ |
|---|---|---|---|---|---|---|
| Total $\beta_1$-integrin | 227.4 ± 16.1 | 193.1 ± 13.8** | 186.9 ± 7.7** | 199.8 ± 11.9* | 229.2 ± 11.9 | 233.5 ± 14.2 |
| Activated $\beta_1$-integrin | 12.09 ± 1.57 | 7.09 ± 0.36** | 8.26 ± 1.32* | 7.65 ± 1.68* | 9.05 ± 0.47* | 11.54 ± 2.14 |

* $p < 0.05$ (vs. control).
** $p < 0.01$ (vs. control).

Fig. 5 and Table 3 present the effects of the $EP_{1-4}$ antagonists on total or activated $\beta_1$-integrin expression in THP-1 cells. Pretreatment with the $EP_1$ and $EP_2$ antagonists did not protect against the suppression of expression levels in total $\beta_1$-integrin induced by $PGE_2$ (all $p < 0.05$), but treatment with $EP_3$ and $EP_4$ antagonists did. Pretreatment with $EP_4$ antagonist restored activated $\beta_1$-integrin expression that had been suppressed by $PGE_2$, but $EP_1$, $EP_2$, and $EP_3$ antagonists did not, as shown in Fig. 5 and Table 3.

## 4. Discussion

In this study, we demonstrated that mRNA and protein of all four subtypes of $PGE_2$ receptors are expressed in THP-1 cells and PBMCs, as analyzed by RT-PCR and Western blot. Flow cytometric analysis showed that $PGE_2$ at 10, 100, or 1000 ng/ml significantly suppressed the expression of both total and activated $\beta_1$-integrin in both THP-1 cells and $CD14^+$ monocytes/macrophages in human

peripheral blood. Furthermore, $EP_2$, $EP_3$, and $EP_4$ agonists significantly inhibited the expression of total $\beta_1$-integrin compared with non-treated THP-1 cells, but $EP_1$ agonist did not. The $EP_4$ agonist was the most effective among the agonists in this inhibition. Furthermore, only the $EP_4$ agonist significantly suppressed the expression of activated $\beta_1$-integrin with equal effectiveness to treatment with $PGE_2$. The other EP receptor agonists did not inhibit this expression. On the other hand, pretreatment with $EP_3$ or $EP_4$ antagonist prevented the $PGE_2$-induced suppression of total $\beta_1$-integrin expression, but treatment with $EP_1$ or $EP_2$ antagonists did not. In addition, only pretreatment with $EP_4$ antagonist prevented the inhibitory effect of $PGE_2$ on activated $\beta_1$-integrin expression. The $EP_1$ agonist did not decrease the expression levels of either total or activated $\beta_1$-integrin, and pretreatment with $EP_1$ antagonist did not restore the inhibition of expression in our study. We concluded that the $EP_1$ receptor is constitutively expressed in THP-1 cells, but does not contribute to suppression of either total or activated $\beta_1$-integrin expression induced by $PGE_2$. These results suggested that $PGE_2$ suppressed expression levels of both total and activated $\beta_1$-integrin mainly via $EP_4$ receptors, with some involvement in total $\beta_1$-integrin expression being mediated via $EP_2$ or $EP_3$ receptors. The $EP_4$ receptor plays an especially important role in activated $\beta_1$-integrin expression.

Some reports have shown that $PGE_2$ inhibits the expression of adhesion molecules and the production of cytokines or chemokines in human monocytes/macrophages, such as IL-18-induced CD54 and CD86 expression, lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-$\alpha$ production, CCL3/4 expression, and production of IL-8, macrophage inflammatory protein-1$\alpha$ (MIP-1$\alpha$) and $\beta$, and monocyte chemoattractant protein-1 (MCP-1) [9,17–19]. $PGE_2$ inhibits IL-18-induced CD54 and CD86 expression and LPS-induced TNF-$\alpha$ production in PBMCs via $EP_2$/$EP_4$ receptors [9,14], CCL3/4 expression in dendritic cells via $EP_2$ receptor [15], and proinflammatory cytokine-induced IL-8, MIP-1$\alpha$ and $\beta$ and MCP-1 production in human macrophages via $EP_4$ receptor [16]. Moreover, it has also been reported that $PGE_2$ inhibits trafficking of human eosinophils via $EP_2$ receptor [17]. Previous reports have shown that $EP_4$ receptor also plays an important *in vivo* role in suppression of mucosal damage from dextran sodium sulfate-induced colitis [18]. These inhibitory effects *in vivo* may involve various inflammatory cells: synovial cells, $CD4^+$ T cells, dendritic cells, etc. There are many reports that $EP_2$ and/or $EP_4$ receptor may play an important role in the anti-inflammatory effect of $PGE_2$. In this study, the inhibitory effects of $\beta_1$-integrin expression in THP-1 cells occurred mainly via $EP_4$ receptor and partially via $EP_2$ and $EP_3$ receptors. Our results are consistent with these previous reports.

It has also been reported that activated $\alpha_4\beta_1$-integrin on the surface of endothelial cells binds fibronectin, which binds to $\alpha_4\beta_1$-integrin on the surface of monocytes and enables firm adhesion of monocytes to endothelial cells [19]. $\beta_1$-Integrins play important roles in the cell–cell adhesion of inflammatory reactions. Previously, it was believed that $PGE_2$ accelerates inflammatory reactions, but it is now known that $PGE_2$ has anti-inflammatory effects that are mediated via different EP receptors. Our results suggest that $PGE_2$ may suppress the migration of human monocytes/macrophages by inhibiting $\beta_1$-integrin expression.

In conclusion, human monocytes/macrophages constitutively express $EP_1$, $EP_2$, $EP_3$, and $EP_4$ receptors. $PGE_2$ suppresses both total and activated $\beta_1$-integrin, mainly via $EP_4$ receptor in human monocytes/macrophages.

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Short communication

# Posterior reversible encephalopathy syndrome following measles vaccination

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ABSTRACT

Due to recent domestic measles outbreaks in Japan, the Japanese government has mandated measles vaccination at ages 14 and 17 since April 2008. Since then, the number of people receiving measles vaccination has increased in Japan. Measles vaccination may cause serious neurological complications including encephalopathy, although the incidence is very low. We report here an adult case of posterior reversible encephalopathy syndrome (PRES) and myeloradiculoneuropathy following measles vaccination. Brain MRI demonstrated typical findings of PRES, high intensity signals in the occipital lobes on FLAIR imaging, isointensity signals on diffusion weighted imaging, with an increase in the apparent diffusion coefficient (ADC). Vasoconstriction mainly in the posterior cerebral arteries was detected by MRA. Physicians should keep in mind the possible occurrence of PRES and myeloradiculoneuropathy following measles vaccination.



## 1. Introduction

Measles is one of the best known neurotropic viruses. Postinfectious encephalopathy occurs in approximately 0.1% of patients with natural measles infection and shows a mortality rate of 10% to 20%. The majority of survivors have residual permanent CNS impairment. Measles vaccination also causes serious neurological complication including encephalopathy, though the incidence is very low at approximately 0.0001% [1]. Domestic measles outbreaks in Japan have occurred in 1998, 2001, 2006, and 2007 [2]. The Japanese government has mandated measles vaccination at the ages of 14 and 17 since April 2008. Since then, the number of people receiving measles vaccine has increased in Japan.

Posterior reversible encephalopathy syndrome (PRES) is a disorder showing typical radiologic findings of bilateral gray and white matter abnormalities in the posterior regions of the cerebral hemispheres [3–8]. Typical clinical symptoms include headache, convulsion, loss of consciousness and visual disturbance. We report here an adult case of PRES and myeloradiculoneuropathy following measles vaccination.

## 2. Case report

A healthy 19-year-old university student received measles vaccination. After 8 h, she felt itching on the bilateral thighs, which gradually changed to pain. Over the next 3 days, pain with itching gradually expanded to include her back and four extremities, and she developed difficulty in handling chopsticks. Four days after the vaccination, the pain became severe, and spread over her whole body. Three of her school mates who underwent measles vaccination from the same lot also experienced similar itching of the thighs, however those cases were mild and subsided quickly.

Her blood pressure (BP) was 145/99 mmHg, which was higher than usual (90/60 mmHg). Pulse was regular at 78/min, and body temperature was 36.8 °C. Neurologically, the consciousness level was alert and she showed normal mentality. There were no meningeal irritation signs or symptoms, including headache. She did not show any abnormalities of the cranial nerves, including visual field and acuity. She showed mild to moderate weakness of the upper and lower extremities. Deep tendon reflexes were decreased in her four limbs with bilateral positive Chaddock signs. She showed disturbance of touch and pain sensations below the C6 level. However, vibratory sensations were normal. There was a decreased secretion of sweat below the T1 level. She also complained of difficulty in urination.

Laboratory studies of CSF showed elevated protein concentration (51 mg/dl) and IgG index (0.74; normal level <0.65) with a normal cell count. Serological tests for collagen diseases and anti-ganglioside antibodies were all negative.

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Nerve conduction study demonstrated elongation of the distal latency of the F wave in the median nerve. Delayed motor conduction velocity in the tibial nerve and low amplitude of the sensory nerve action potential in the sural nerve were detected.

She presented with dysesthesia on the left side of her face and oral cavity on the 10th day after onset. To rule out the brainstem lesion, brain MRI was performed on the 12th day after onset. FLAIR imaging of brain MRI (GE-Signa, 3 T, repetition time/echo time 10002/121.3) demonstrated high intensity signals in the white matter of the bilateral occipital lobes (Fig. 1a). On diffusion weighted imaging, this lesion showed an iso-intense signal (Fig. 1b), with an increase in the apparent diffusion coefficient (ADC) indicating vasogenic edema rather than cytotoxic edema (Fig. 1c). MRS demonstrated a normal pattern, including an absence of lactate elevation. These findings were compatible with PRES. MRA showed narrowing of the posterior cerebral artery, indicating arterial vasoconstriction (Fig. 1d). Continuous arterial spin labeling (CASL) perfusion images were obtained as demonstrated by Kimura et al. [9]. The method was performed using arterial spin labeling with electromagnetically labeled arterial water used as a freely diffusible intrinsic tracer to demonstrate cerebral blood flow [9]. CASL imaging detected hypo-perfused regions in the bilateral occipital lobes with left side dominance (Fig. 1e arrow).

The patient received steroid pulse therapy (1 g/day) for 3 days beginning 11 days after onset. The neurological symptoms gradually subsided, and BP also returned to the usual levels (90/56 mmHg) within 3 days after steroid administration. MRI and MRA findings returned to normal after steroid therapy (Fig. 2a and b).

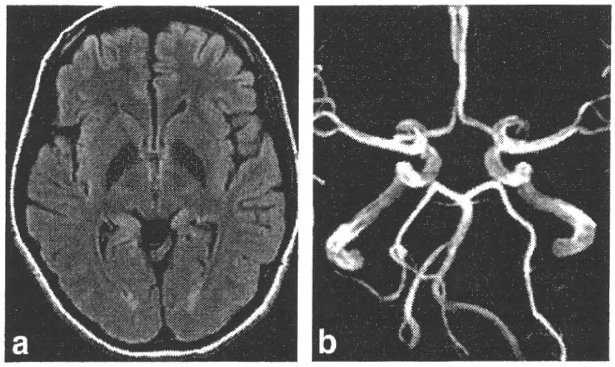


**Fig. 2.** After methylprednisolone pulse therapy, FLAIR imaging on MRI demonstrated that high intensity signals in the occipital lobes were improved (a). MRA demonstrated that vasoconstriction of posterior cerebral arteries was also improved (b).

## 3. Discussion

Clinical, electrophysiological and MRI findings suggested PRES and myeloradiculoneuropathy. Guillain–Barré syndrome (GBS) following measles [10], rubella [11], influenza [12], and poliovirus vaccination have been reported. Along with the GBS-like symptoms, this patient showed neurological signs suggesting spinal cord involvement including sensory disturbances below the C6 level, and bilateral positive Chaddock signs. Mihara et al. reported post infectious myeloradiculoneuropathy mimicking GBS with cranial nerve involvement associated with human herpes virus 7 infection [13]. Actually, an autopsied case of inflammatory polyradiculoneuropathy with spinal cord involvement following hepatitis B vaccination [14] has been reported. The possibility of acute disseminated encephalomyelitis (ADEM) was also suspected in this patient, but MRI findings in this patient were typical for PRES, and the interval between measles vaccination and onset was only 8 h. In cases of ADEM, it usually takes 2 days to 4 weeks after vaccination [15]. In fact, there are two similar reports of PRES occurring acutely during influenza A infection [16,17].

PRES has been induced under many conditions, e.g. transplantation [18], infection, including influenza A [16,17], sepsis, shock, toxemia, pregnancy, autoimmune disease [8], and chemotherapy. As one of the triggers of PRES in this patient, rapid change in BP due to autonomic dysfunction of myeloradiculoneuropathy induced by

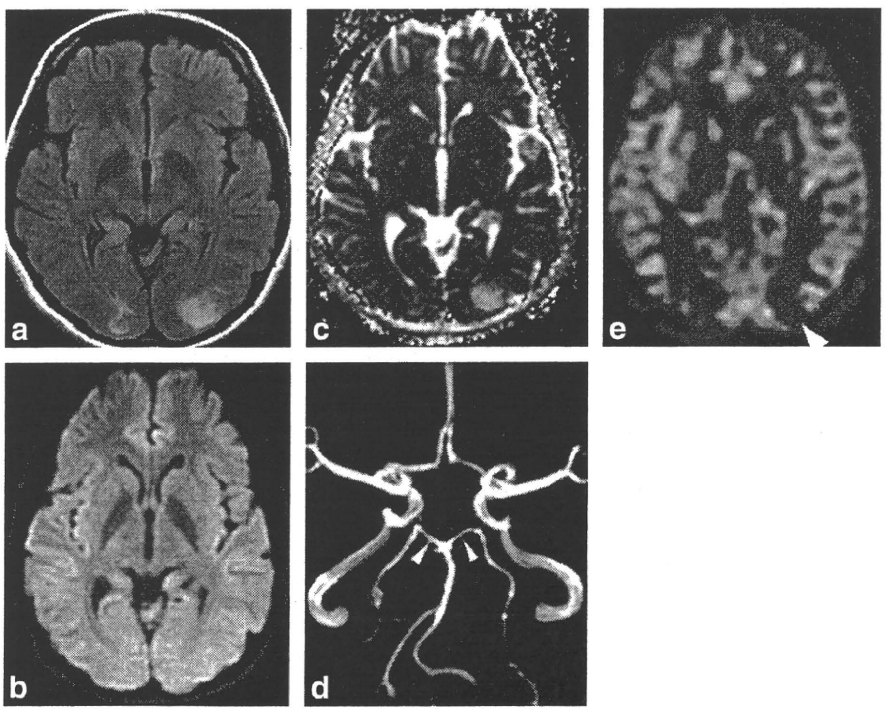


**Fig. 1.** FLAIR imaging (GE-Signa 3.0 T, repetition time/echo time: 10002/121.3) on MRI detected high intensity signals in the occipital lobes with left side dominance (a). Diffusion weighted imaging (repetition time/echo time: 6000/64.6) showed an iso-intense signal (b). However, increase in the apparent diffusion coefficient (c) was observed, indicating vasogenic, rather than cytogenic edema. These findings were compatible with posterior reversible encephalopathy syndrome. MRA showed vasoconstriction of the posterior cerebral arteries (d: arrow). Continuous arterial spin labeling (CASL) perfusion images detected hypo-perfused regions in the bilateral occipital lobes with left side dominance (e: arrow).

measles vaccination was suspected. Van Diest et al. reported a patient having both disturbances, GBS and PRES. The authors speculated that acute arterial hypertension, probably caused by autonomic dysfunction in the context of GBS, may have been the cause of PRES [7]. The authors also indicated that one other possibility was the influence of cytokines produced in the context of GBS, on permeability of the BBB.

Although the hemodynamic mechanisms underlying PRES are not yet clear, three theories have been proposed to date. 1) The current more widely accepted theory suggests that severe hypertension leads to failed autoregulation, subsequently causing hyperperfusion with endothelial injury/vasogenic edema. 2) The earlier, original theory suggests that hypertension or rapid BP change leads to cerebral autoregulatory vasoconstriction, ischemia and subsequent brain edema [3–5]. 3) More recently, it has been suggested that the immune system triggering with T-cell activation, endothelial activation, and T-cell trafficking, accompanying vasculopathy with vasoconstriction leads to sustained hypoperfusion and PRES edema [18]. Vascular endothelial growth factor (VEGF) expression was observed in reversible encephalopathy after cardiac transplantation [18]. In this case, reversible vasoconstriction mainly in the posterior cerebral arteries was detected by MRA, and hypoperfusion in the bilateral occipital lobes in the setting of BP elevation was detected by perfusion imaging using CASL. Based on these findings, the latter two theories are more likely, at least in this patient.

Aydin et al. reported a 9-year-old boy with PRES and Adie's pupil association that occurred after measles vaccination [6]. This patient presented with diminished deep tendon reflexes, with bilateral pyramidal signs, like our patient, but there was no description indicating any change in BP in that patient [6].

To our knowledge, this is the first report describing an adult case of PRES and myeloradiculoneuropathy following measles vaccination. Cases demonstrating neurological complications including PRES and myeloradiculoneuropathy following measles vaccination should be accumulated and carefully examined.

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Short communication

# Autoantibodies to glutamate receptor GluRε2 in a patient with limbic encephalitis associated with relapsing polychondritis

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ABSTRACT

Limbic encephalitis is a rare central nervous system (CNS) manifestation of relapsing polychondritis (RP). Vasculitis is assumed to be the cause of CNS involvement in RP. Several studies, however, have described CNS involvement in RP with no evidence of vasculitis but with a more nonspecific inflammatory picture. We report a patient with limbic encephalitis associated with RP who presented with anti-glutamate receptor (GluR) ε2 (NR2B) autoantibodies in his cerebrospinal fluid and sera. Brain MRI showed a high signal intensity lesion in the medial temporal lobe and progressive atrophy without multifocal abnormality on fluid-attenuated inversion recovery scanning. Our patient's results raise the interesting possibility that anti-GluRε2 (NR2B) antibodies function in the development of limbic encephalitis in certain patients with RP.


## 1. Introduction

Relapsing polychondritis (RP) is a disorder of unknown etiology manifesting as episodic and progressive inflammation of cartilaginous structures throughout the body that include the ears, joints, nose, eyes, respiratory tract, cardiovascular system and skin [1]. The etiology and pathogenesis of RP are unknown, but autoantibodies to type II collagen restricted to cartilage have been detected in the sera of 30–50% of patients studied [2]. Rarely, the central nervous system (CNS) is involved in RP with clinical manifestations of headache [3–5], meningitis [5], encephalitis [3,6–8], cerebral infarction [9], hemiplegia [3], ataxia [10,11], seizures [3,5,7], confusion [3,8,10–12], psychosis [3,5,10,12] or dementia [4,6–9,11]. We report a patient with limbic encephalitis associated with RP who presented with anti-glutamate receptor (GluR) ε2 (NR2B) antibodies.

## 2. Case report

A 62-year-old man was admitted to our hospital because of generalized tonic–clonic convulsion followed by recurrent focal seizures. He had a previous history of bilateral ear swelling, erythema and tenderness which emerged at age 58, lasted 6 months and was followed for about 1 year by nasal deformity and conjunctivitis. At age 60, he experienced fever and headache. The diagnosis was aseptic meningitis, which subsided spontaneously in a month. At age 61, he again experienced fever and headache accompanied by delirium. Aseptic meningitis again was diagnosed and subsided in a month. Results of a cerebrospinal fluid (CSF) study and medication details given at these events are not available. No specific immunosuppressant therapy had been performed for his systemic symptoms. During exacerbation of his symptoms, finger deformity emerged bilaterally. Three months before admission, his family had noticed such psychiatric and behavioral abnormalities as visual and auditory hallucinations, agitation, disinhibition, cognitive decline and seizure emergence.

On admission, a neurological examination showed disturbed consciousness and recurrent clonic convulsion in the left face and upper and lower limbs. Both ears were swollen (Fig. 1A), and his nose and fingers (Fig. 1B) deformed. Deep tendon reflexes were reduced in his lower limbs. Laboratory blood tests revealed an inflammatory reaction that included increased C-reactive protein (CRP) of 19.1 mg/dl accompanied by a high body temperature of 38.0 °C. His white blood cell count was 7400/mm$^3$. He also had increased HbA1C, indicative of diabetes mellitus. Serum rheumatoid factor and antinuclear antibodies were negative. His CSF was sterile with an increased cell count of 39/mm$^3$ (59% lymphocytes) and an increased protein level of 68 mg/dl. A polymerase chain reaction DNA for herpes simplex virus was negative. A fluid-attenuated inversion recovery MRI scan of the brain at that time showed a slightly high signal intensity lesion in the medial temporal lobe that included the hippocampus and amygdala (Fig. 1C), and mild chronic ischemic change in the putamen. Diffusion-weighted MR images were normal. An electroencephalogram showed slowing of background activities to 4–5 Hz. Limbic

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