volunteers who had neither allergic symptoms nor familial history and had given oral informed consent according to the regulations of the hospital ethical board. The samples were centrifuged at 120 *g* for 15 minutes at room temperature. The platelet-rich-plasma (PRP) was resuspended in wash buffer (9 mM Na_2EDTA, 26.4 mM Na_2HPO_4, 140 mM NaCl) and centrifuged at 800 *g* for 10 minutes at room temperature. Subsequently, the pellet containing the platelets was resuspended in wash buffer and centrifuged at 200 *g* for 10 minutes at room temperature. The supernatant was centrifuged at 800 *g* for 10 minutes at room temperature and the pelleted platelets were then resuspended in phosphate-buffered saline (PBS) containing 0.02% EDTA. The total yield of platelets was more than 1×10^8. We confirmed that the purity was more than 99% using both flow cytometry and microscopy.

Cell lines

THP-1 cells, human monocytic leukemia cell lines and Jurkat cells, a human T leukemia cell line, were cultured in RPMI 1640 (GIBCO RBL Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Cos-1 cells (African green monkey kidney fibroblast-like cell line) were cultured in Dulbecco's Modified Eagle's Medium (GIBCO RBL Life Technologies, Inc.) supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. These cells were obtained from American Type Culture Collection, were maintained at 37°C under humidified 5% CO_2 as stationary cultures.

Immunological reagents

Rabbit polyclonal antibodies to human CysLT1 or CysLT2 receptor (Cayman Chemical, Ann Arbor, MI, USA) and rabbit IgGs (Sigma-Aldrich, Oakville, Ontario, Canada) were used to detect the expression of CysLT1 or CysLT2 receptors in human platelets via flow cytometric analysis and Western blotting. Polyclonal antibodies against human actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were also used as an intrinsic positive control in Western blotting. To detect the binding of these antibodies we used fluorescein isothiocyanate (FITC) conjugated goat anti rabbit IgG (Sigma-Aldrich) or horseradish peroxidase(HRP)-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) as the secondary antibody for flow cytometric analysis or Western blotting, respectively. Phycoerythrin (PE) conjugated monoclonal antibody to human CD61 (BD Pharmingen, San Diego, CA, USA) was used for two-color flow cytometric analysis.

Analysis of CysLT1 and CysLT2 receptor gene expression by reverse transcription and polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from purified platelets donated by healthy individuals, peripheral blood mononuclear cells (PBMCs), THP-1 cells and Jurkat cells by the phenol-guanidium isothiocyanate method, and reverse transcribed, as previously described [6]. We were able to collect approximately 2 μg RNA from platelets of 5×10^8. CysLT1, CysLT2 receptor, CD45 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene segments were PCR amplified after reverse transcription from 1 μg of each resultant complementary DNA (cDNA) in the presence of specific sense primer (0.15 μM), antisense primer (0.15 μM), 200 μM dNTP, 0.025 U/μl Ampli Taq (Perkin Elmer, Norwalk, CT, USA), 2 mM $MgCl_2$ and PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) in a final reaction volume of 20 μl. The sequences of the specific primers were as follows:

CysLT1R (480bp): 5′-ATGACAGCCATGAGC TTTTTC-3′ (forward); 5′-CATTCTAAGGACAG AATCACA-3′ (reverse);
CysLT2R (260bp): 5′-AGCAATAACAACAGCA GGAA-3′ (forward); 5′-CCAGGTCTCCAAATAT CCAA-3′ (reverse);
CD45 (380–863bp): 5′-ATGTATTTGTGGCTT AAACTCTTG-3′ (forward); 5′-GCAGTACATGA ATTATGAGATATGG-3′ (reverse)
GAPDH (452bp): 5′-ACCACAGTCCATGCCA TCAC-3′ (forward); 5′-TCCACCACCCTGTTGC TGTA-3′ (reverse).

PCR was performed using the programmed temperature control system PC-800 (ASTEC). Each cycle included denaturation (94°C, 30s), annealing (58°C, 30s), and extension (72°C, 1 min) for 30 cycles and a final incubation (72°C, 10 min). We checked the mRNA expression of the leukocyte antigen CD45 in order to assess leukocyte contamination in the purified platelet fraction. We used RT(-) samples as negative control for checking the contamination of genomic DNA. The PCR products were electrophoresed on 2% agarose gels and, after staining with ethidium bromide, the results visualized under ultraviolet illumination.

Analysis of cell-surface or intracellular CysLT1 and CysLT2 receptor expression in human platelets by flow cytometry

Platelets were isolated as described above. Purified platelets (2×10^7) in 100 μl of 0.02% EDTA-PBS were incubated with PE-conjugated anti-CD61 monoclonal antibody at room temperature for 15 minutes and, after wash, platelets were incubated with 10 μg polyclonal antibody to human CysLT1 or

CysLT2 receptor, or with isotype-matched control antibody at room temperature for 30 min. After a subsequent wash, the platelets were incubated with 100 ng FITC-conjugated anti-rabbit IgG at room temperature for 15 min. Finally the platelets were washed again and resuspended in 0.02% EDTA-PBS and analysed for CD61 positive platelets by immunofluorescent cell sorting (Becton Dickinson, San Jose, CA, USA: FACScalibur). To determine the intracellular expression of CysLT1 or CysLT2 receptor in human platelets, the cells were pretreated with 4% paraformaldehyde in PBS supplemented with 0.1% saponin and 10 mM HEPES at room temperature for 10 min before incubation with isotype-matched control antibody or rabbit polyclonal antibody to human CysLT1 or CysLT2 receptor.

Analysis of CysLT1 and CysLT2 receptor protein expression in human platelets by Western blotting

Platelets were isolated as described above and solubilized in 2% sodium dodecyl sulfate solution containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 2 mM N-ethylmaleimide, 2 mM benzamidine and 2 mM sodium. Protein concentrations were determined using Bio-Rad protein concentration reagent. The samples were stored at −80°C and all 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, 0.5% Tween 20), the membranes were incubated with 1:200 diluted rabbit polyclonal anti-human CysLT1 receptor antibodies, 1:250 diluted rabbit polyclonal anti-human CysLT2 receptor antibodies, or 1:200 diluted rabbit anti-human actin polyclonal antibodies in TBST containing 5% non-fat dry milk for one hour at room temperature. After three washes in TBST, the membranes were incubated with 1:2000 diluted horseradish peroxidase(HRP)-conjugated goat anti-rabbit IgG (Bio-Rad) for one hour at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) and analysed by radiography. We performed these analyses on the same membrane after incubation at 70°C for 30 minutes with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 100 mM 2-mercaptoethanol) and re-blotting.

Analysis of CysLT1 receptor-mediated RANTES release from human platelets

The PRP (1×10^8/ml) from healthy individuals were washed with HEPES-Tyrode's buffer (pH 7.4, 142 mM NaCl, 6.2 mM KCl, 6.5 mM HEPES) supplemented 100 ng/ml Prostaglandin I_2 (PGI_2), and resuspended in complete HEPES-Tyrode's buffer (pH 7.4, 142 mM NaCl, 6.2 mM KCl, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 6.5 mM HEPES) supplemented 100 ng/ml PGI_2 [7, 8] and was pre-incubated on ice for 30 min to inactivate PGI_2 before incubation with cysteinyl leukotrienes. PRP was then incubated at 37°C for 60 minutes with human LTC_4, LTD_4 or LTE_4 (Sigma-Aldrich) at concentrations of 10^{-6} to 10^{-8} M with or without pretreatment of 10^{-6} to 10^{-8} M pranlukast (provided by ONO Pharmaceutical Co., Osaka, Japan) for 30 minutes at 37°C. RANTES released into the supernatant was measured by ELISA using a RANTES-specific ELISA kit (Quantikine Human RANTES Immunoassay; R&D systems, Minneapolis, MN, USA).

Statistical analysis

Statistical analysis was performed with one-way ANOVA, and a p value of less than 0.05 was taken as significant.

Results

CysLT1 and CysLT2 receptor gene expression in human platelets

We performed RT-PCR on mRNA from human platelets donated by healthy individuals. Human PBMCs and THP-1 cells were used as positive control, and Jurkat cells were used as negative control. The CysLT1 receptor, CysLT2 receptor, CD45 and GAPDH PCR products are 470 bps, 260 bps, 380–863 bps and 452 bps, respectively. The mRNA expression for the CysLT1 receptor was detected in all positive controls and human platelets from healthy individuals, but not in the Jurkat cell negative control. The level of mRNA expression in human platelets was lower than in PBMC or THP-1 cells (Figure 1A). The mRNA of CysLT2 receptor was detected in all kinds of cells, but the levels were lower compared with that of the CysLT1 receptor. We could detect CD45 mRNA in PBMC, THP-1 and Jurkat cells, but not platelets. These results show that leukocyte contamination in the purified platelet fraction did not affect amplification of mRNA from platelets. We did not use DNase before the RT-PCR reaction so we looked at RT (-) samples to check for contamination with genomic DNA and confirmed that levels of genomic DNA contamination were very low (Figure 1B).

Cell-surface and intracellular CysLT1 and CysLT2 receptor expression in human platelets

Flow cytometric analysis of human platelets from healthy individuals using polyclonal antibodies

against the human CysLT1 or CysLT2 receptor demonstrated that both the CysLT1 and CysLT2 receptor are expressed on platelet cell-surfaces. A representative result is presented in Figure 2A (11.6%, 5.8% respectively). Mean percentages of cell surface CysLT1 and CysLT2 receptor positive platelets based on six independent experiments were 10.6% (SEM 4.6) and 4.3% (SEM 1.9), respectively. We next used saponin pretreatment of the cell preparations to determine if the receptors were also expressed intracellularly. As shown in Figure 2B, CysLT1 and CysLT2 receptors were expressed in the cytoplasm with more platelets showing positive cytoplasmic expression (60.6%, 9.7% respectively) than cell-surface expression.

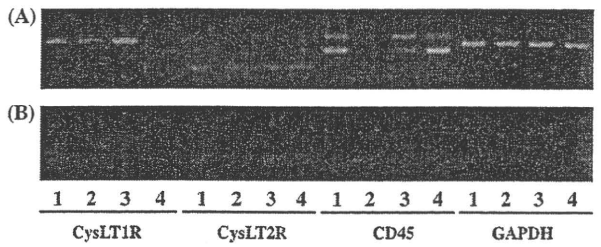

Figure 1. CysLT1 and CysLT2 receptor mRNA expression in human platelets. The CysLT1 or CysLT2 receptor mRNA expression in human platelets was analysed by RT-PCR (A). Human PBMCs or THP-1 cells were used as positive controls, and Jurkat cells were used as negative control. PCR products of the CysLT1 receptor, CysLT2 receptor, CD45 and GAPDH were amplified in the presence of specific sense and anti-sense primers. Lane 1: PBMCs, Lane 2: platelets, Lane 3: THP-1 cells, Lane 4: Jurkat cells (negative control). CD45 was used to assess leukocyte contamination in purified platelet fractions. RT(-) samples are shown as a check for genomic DNA contamination (B). Representative data are shown. Similar results were obtained in three independent experiments.

Western blot analysis of CysLT1 and CysLT2 receptor expression

Western blotting detected the CysLT1 receptor at about 42kD in isolated human platelets donated by healthy individuals [4]. Previous reports showed that the CysLT1 receptor was expressed on the cell-surface of human PBMCs and THP-1 cells, so we used these as positive controls and we were able to confirm CysLT1 receptor protein expression in the control cells [4, 9, 10]. However, we did not detect the CysLT1 receptor in negative control Jurkat cells and Cos-1 cells (Figure 3A) [11]. On the other hand, we detected the CysLT2 receptor in PBMCS, platelets, THP-1 cells and Jurkat cells, but not in Cos-1 cells by Western blotting, as shown in Figure 3B. We confirmed that the concentrations of protein in each lane were almost equal by comparison to the concentrations of actin, as shown in Figure 3C.

CysLT receptor stimulation of RANTES release

We examined whether activation of human platelets by CysLT could induce the release of RANTES. Using the CysLTs LTC_4, LTD_4 or LTE_4 at various concentrations (10^{-8}–10^{-6}M), we stimulated human platelets and evaluated RANTES release. The levels of RANTES released from platelets by stimulation of CysLTs were significantly higher than control (Figure 4). Next we investigated whether pranlukast, which is a CysLT1 receptor antagonist, inhibits RANTES release stimulated by CysLTs in human platelets. Pranlukast inhibited RANTES release from human platelets stimulated by LTC_4, LTD_4 or LTE_4, but inhibition did not reach baseline

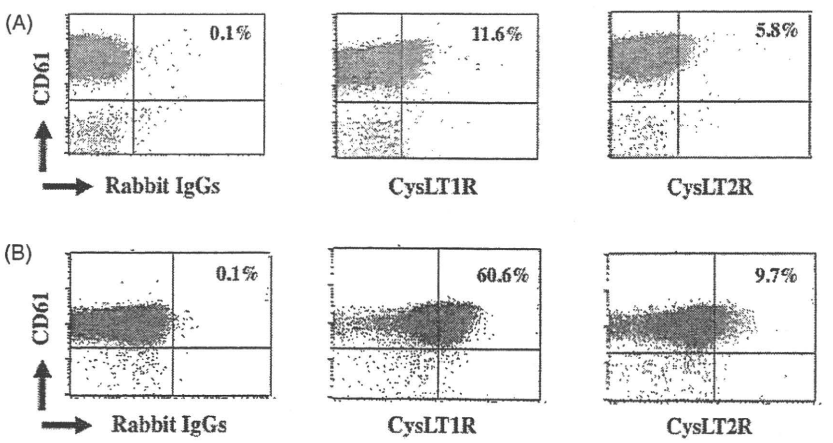

Figure 2. Expression of CysLT1 and CysLT2 receptors in human platelets analysed by two color-flow cytometry. Human platelets were incubated with rabbit polyclonal antibody against human CysLT1 or CysLT2 receptors and then stained with FITC-labeled goat anti-rabbit IgG. Rabbit IgG was used as an isotype-matched control antibody. A shows cell-surface expression, and B shows intracellular expression. Intracellular expression was analyzed after pre-treatment with 4% paraformaldehyde in PBS supplemented 0.1% saponin and 10 mM HEPES. We used PE-labeled anti-human CD61 antibody as a cell surface marker of human platelets. Representative data are shown. Similar results were obtained in six independent experiments.

levels (Figure 5). Inhibition of RANTES release stimulated by LTC_4 and LTD_4 was significant.

Discussion

Regarding the role of human platelets in allergic inflammation, some reports have shown that platelet activation may be important for pathogenesis of bronchial asthma due to platelet infiltration into bronchoalveolar lavage or the elevation of β-thromboglobulin or platelet factor 4 in plasma from asthma patients [12–18]. Additionally, in atopic dermatitis similar results were also reported [19]. These reports suggest that activated platelets may play an important role in allergic inflammation, but the role is still unclear. Furthermore it has been reported that human platelets express FcεRI, low affinity IgE receptor (FcεRII, CD23) and low affinity IgG receptor (FcγRII, CD32) and that platelets are activated via these receptors [6, 20–24]. There is also a report on CysLT receptor mRNA expression in human platelets [25]. CysLTs are important mediators in bronchial asthma and antagonists of CysLT1 receptors (such as pranlukast and montelukast) are effective in the treatment of bronchial asthma. In this study we focused on the CysLT receptors, especially the CysLT1 receptor in human platelets.

We demonstrated mRNA and protein expression of both CysLT1 and CysLT2 receptors in human platelets by RT-PCR, flow cytometry and Western blotting. We also showed that both cell-surface and intracellular expression of CysLT1 and CysLT2 receptors are present in human platelets and that levels of intracellular expression (60.6%, 9.7% respectively) in human platelets from healthy individuals are higher than that of the cell-surface expression (11.6%, 5.8% respectively). The levels of CysLT1 receptor mRNA and cell-surface expression are much higher than that of the CysLT2 receptor in human platelets from healthy individuals. The human CysLT1 receptor protein consists of 337 amino acids and has a calculated molecular weight of 38 kD, but it was detected at approximately 42kD as a monomeric form due to glycosylation or other protein modifications common in G protein-coupled receptors (GPCRs). In the Cos-1 cell negative control actin was detected, as shown in Figure 3C, which we attribute to cross-reaction between the anti-human actin antibody and monkey actin. mRNA expression levels of CysLT1 or CysLT2 receptor were especially lower than the protein level. Since platelets do not have nuclei and platelet mRNA may be derived from megakaryocytes, we

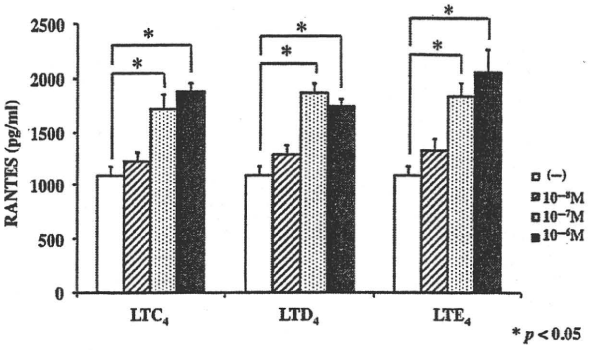

Figure 4. CysLTs induced RANTES release from human platelets. Human platelets (1×10^8/ml) were stimulated with LTC_4, LTD_4 or LTE_4 at concentrations ranging from 10^{-8}–10^{-6}M. The levels of released RANTES were measured by ELISA. Results are shown as mean ± SEM ($n = 8$).

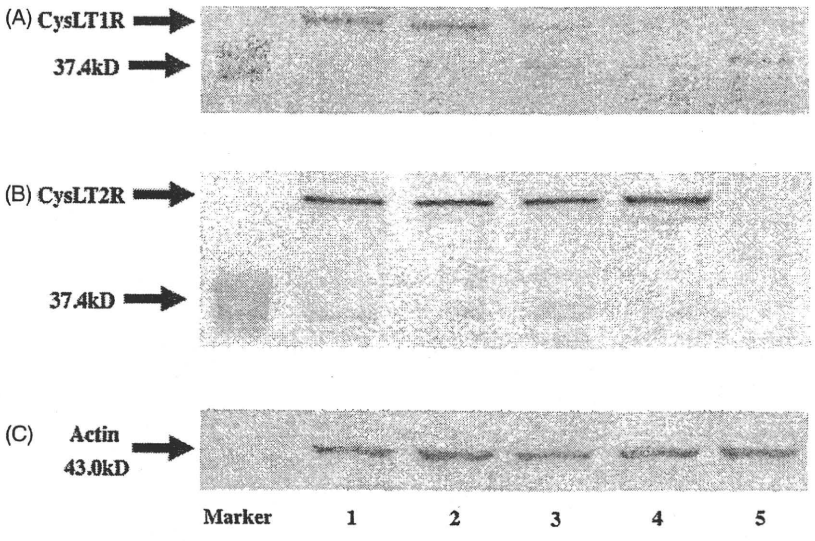

Figure 3. CysLT1 or CysLT2 receptor expression in human platelets analysed by Western blotting. Protein subjected to polyacrylamide gel electrophoresis and subsequently immunoblotted for CysLT1 receptor (A), CysLT2 receptor (B) or actin (C). Lane 1: PBMC, Lane 2: platelet, Lane 3: THP-1 cells, Lane 4: Jurkat cells, Lane 5: Cos-1 cells. Representative data are shown. Similar results were obtained in three independent experiments.

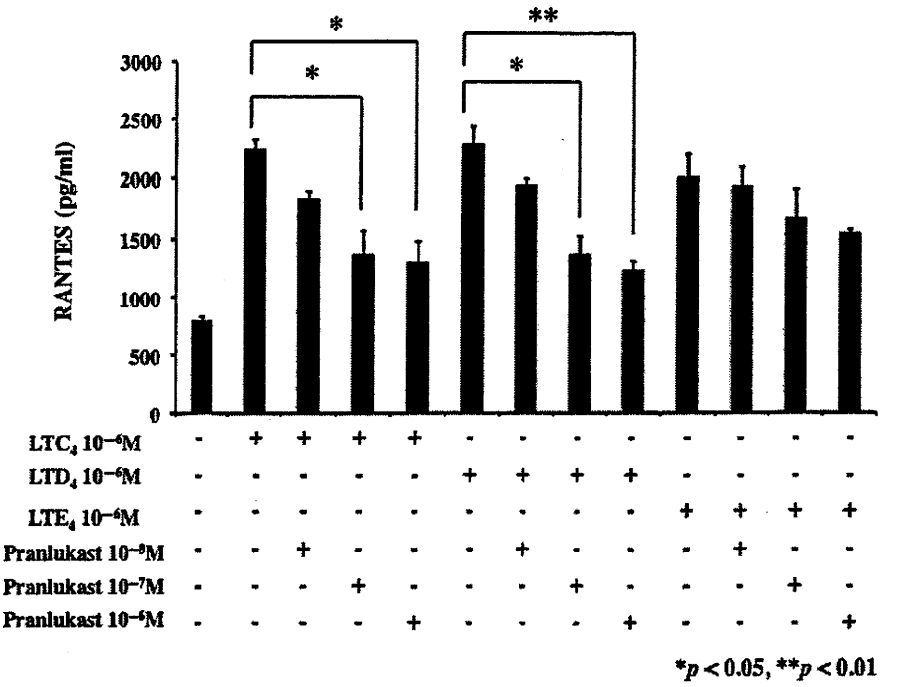

Figure 5. Pranlukast inhibits CysLT-induced RANTES release. Human platelets (1×10^8/ml) were stimulated by LTC_4, LTD_4 or LTE_4 at concentrations ranging from 10^{-8}–10^{-6}M with or without pranlukast pretreatment at 10^{-8}–10^{-6}M. The levels of released RANTES were measured by ELISA. Results are shown as mean ± SEM ($n = 5$).

speculate that there may be differences between expression levels of these mRNA and proteins. The levels of intracellular expression of both CysLT1 and CysLT2 receptors were higher than cell-surface expression. Antibodies against the CysLT1 receptor recognize the C-terminal, but we could detect cell-surface expression of CysLT1 receptor. It is our speculation that the cell membrane of platelets is fragile and may be destroyed during purification or staining. The antibodies we used against the CysLT2 receptor recognize its N-terminal. There were some observed quantitative differences in protein expression for both receptors when comparing flow cytometric analysis and Western blotting, especially for the CysLT2 receptor. The antibody against the CysLT2 receptor detected higher levels of CysLT2 expression in Western blotting compared with flow cytometric analysis. These findings were at odds with each other. Possibly the affinity for the linear structure of the CysLT2 receptor protein after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is higher than for the native structure. We conclude that cell-surface expression of both CysLT1 and CysLT2 receptors were detected by these antibodies in the total expression.

Furthermore our results showed the release of RANTES from human platelets by stimulation with the CysLTs LTC_4, LTD_4 and LTE_4. RANTES is one of the most potent and selective eosinophil chemotactic factors known to date, which, in addition induces eosinophil degranulation and preferential histamine release by basophils in allergic individuals, and its storage in platelets underscores the role of platelets in allergic inflammatory reactions [26–28]. We looked at RANTES production at 37°C for 60 minutes and compared this with the time points (10, 30, 60 minutes) in the preliminary experiments (data not shown). The level of RANTES production reached a plateau by 60 minutes of incubation. Pranlukast, which is a CysLT1 antagonist, significantly reduced the level of RANTES released from human platelets stimulated by LTC_4 or LTD_4. We checked the inhibitory effects of pranlukast not only at 10^{-6}–10^{-8}M (Figure 5), but also at 10^{-4}M and 10^{-5}M (data not shown). The inhibiton of RANTES release by pranlukast over a concentration range of 10^{-4}M to 10^{-7}M was similar, and did not result in 100% inhibition. These results suggest that RANTES may be produced via both the CysLT1 and CysLT2 receptors. It has been reported that mast cells are activated by LTE_4 through other receptors that are neither the CysLT1 receptor nor the CysLT2 receptor (such as GPR17) [29, 30]. Our results suggest that human platelets are activated by CysLTs via not only the CysLT1 receptor, but also via other receptors, such as the CysLT2 receptor. In this study, our results show that CysLT2 may also be expressed and functional in human platelets, but we could not completely determine that CysLT2 was functional, because we do not have a specific antagonist for the CysLT2 receptor.

In conclusion, we determined that both CysLT1 and CysLT2 receptors are expressed in human

platelets and demonstrated that CysLTs induced the release of significant amounts of RANTES via CysLT receptors suggesting a novel role for human platelets in CysLT-mediated allergic inflammation.

Declaration of interest

All authors have no conflict of interest.

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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 ± 49.5) than stage III patients (42.6 ± 34) ($p < 0.05$). The IFN-γ index was higher in patients older than 5 years ($n = 7$) (178.3 ± 94.6) compared to those younger ($n = 11$) (87.5 ± 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 β_1-integrin expression via E-prostanoid receptor in human monocytes/macrophages

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ABSTRACT

β_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 β_1-integrin remains unknown. In this study, we investigated the effects of PGE_2 on the expression of β_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 β_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 β_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]. β_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 β_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 β_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 β_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 (12,000g 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 β_1-integrin expression

The expression of β_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 β_1-integrin or activated β_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,

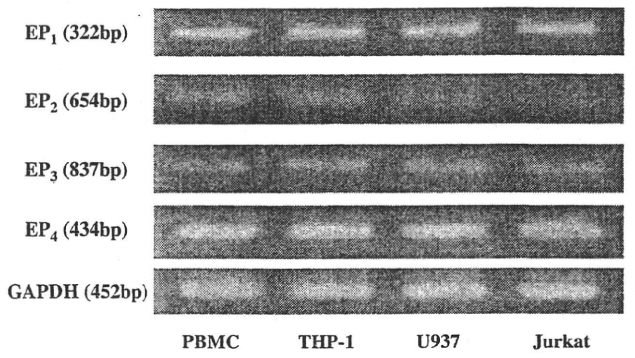

Fig. 1. The mRNA expression of EP subtypes measured by reverse transcription-PCR. mRNA expression of each EP receptor was observed in PBMCs, THP-1 cells, U937 cells, and Jurkat cells. Representative data are shown. Similar results were obtained in five independent experiments.

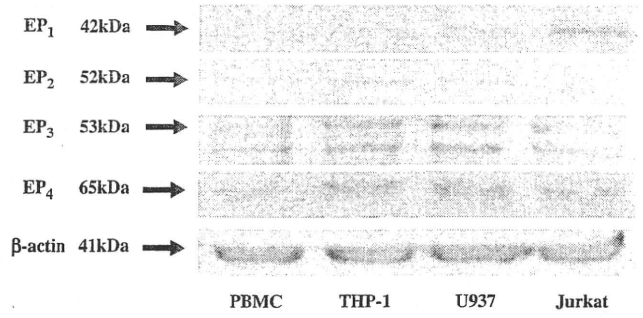

Fig. 2. Expression of EP subtypes measured by Western blot analysis in PBMCs, THP-1 cells, U937 cells, and Jurkat cells. EP_1, EP_2, EP_3, and EP_4 receptors were expressed in PBMCs and THP-1 cells. Representative data are shown. Similar results were obtained in three independent experiments.

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 β_1-integrin in $CD14^+$ human peripheral monocytes/macrophages and THP-1 cells

PGE_2 significantly reduced total or activated β_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 β_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 β_1-integrin. Fig. 4 and Table 2 present the effect of EP_{1-4} agonists, respectively, on total or activated β_1-integrin expression in THP-1 cells. EP_2, EP_3, and EP_4 agonists significantly reduced expression of total β_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 β_1-integrin, only EP_4 agonist suppressed the levels of activated β_1-integrin expression (Fig. 4 and Table 2).

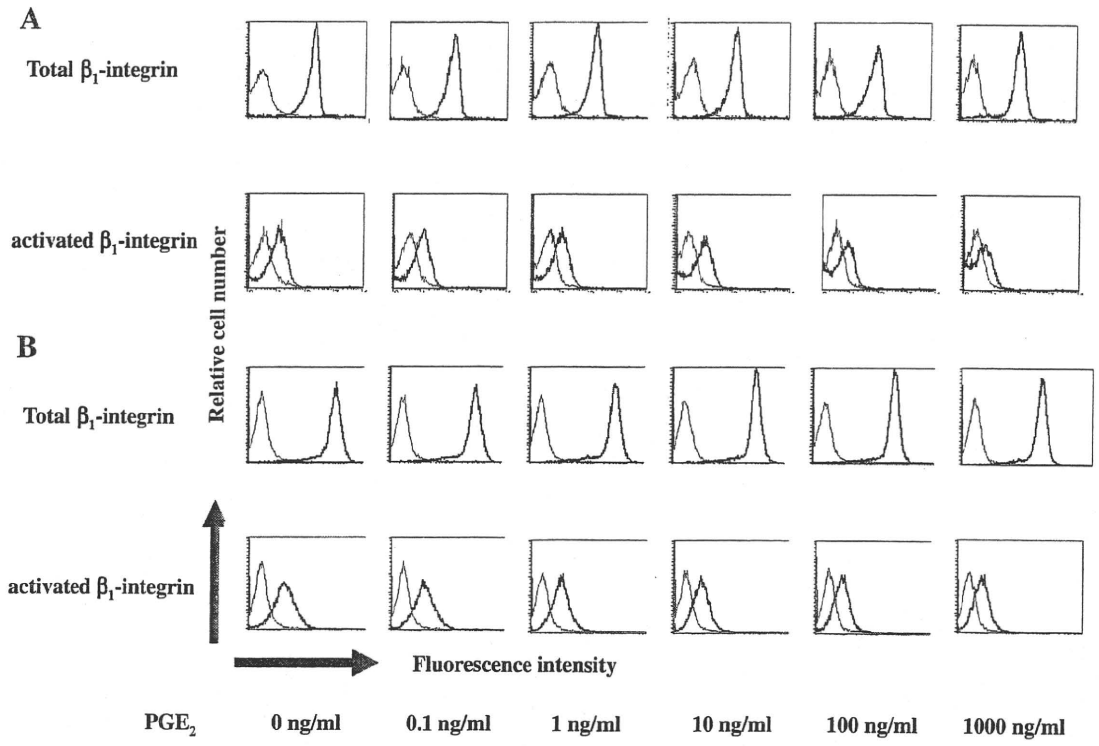

Fig. 3. Flow cytometric analysis demonstrated inhibitory effects of PGE_2 on the total and activated β_1-integrin expression in $CD14^+$ human peripheral blood monocytes/macrophages (A) and THP-1 cells (B). Cells were treated with 0, 0.1, 1, 10, 100, and 1000 ng/ml PGE_2 for 24 h. PGE_2 inhibited both total and activated β_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 β_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 β_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 β_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 β_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 β_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***

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

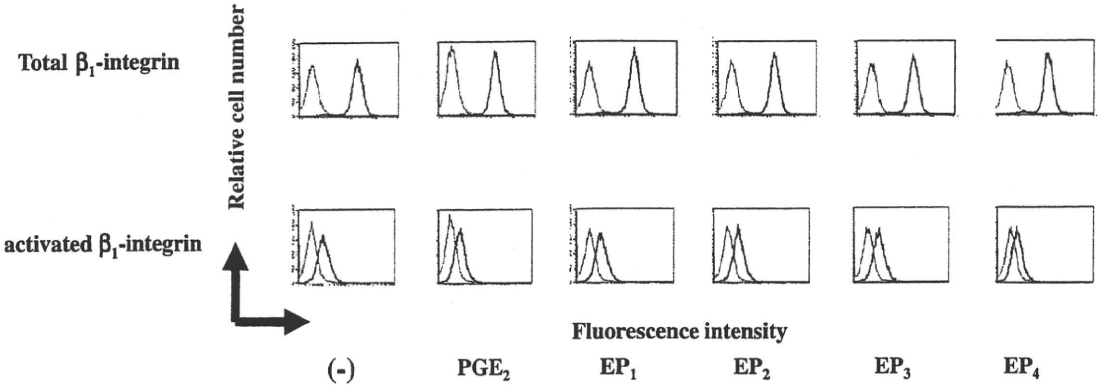

Fig. 4. Flow cytometric analysis demonstrated suppression of the total and activated β_1-integrin by EP_{1-4} agonists in THP-1 cells. Cells were stimulated with 10 μM EP_1, EP_2, EP_3, and EP_4 agonists for 24 h. EP_2, EP_3, and EP_4 suppressed total β_1-integrin. Only EP_4 suppressed activated β_1-integrin. Representative data are shown. Similar results were obtained in six independent experiments.

Table 2
Mean fluorescence intensity of both total and activated β_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 β_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 β_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**

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

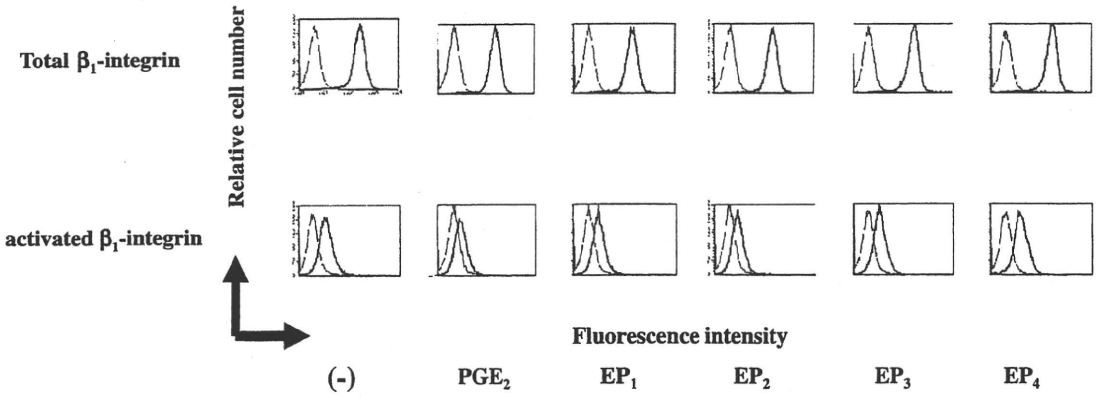

Fig. 5. Flow cytometric analysis demonstrated the effects of EP_{1-4} antagonists on total and activated β_1-integrin expression inhibited by PGE_2 in THP-1 cells. Cells were pretreated with 10 μM EP_1, EP_2, EP_3, and EP_4 antagonists for 60 min and stimulated at 10 ng/ml PGE_2 for 24 h. EP_3 and EP_4 antagonists recovered total β_1-integrin inhibited by PGE_2. Only EP_4 antagonists recovered activated β_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 β_1-integrin expression in THP-1 cells treated with EP antagonist (mean ± SD).

	(-)	PGE_2	EP_1	EP_2	EP_3	EP_4
Total β_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 β_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 β_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 β_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 β_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 β_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 β_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 β_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 β_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 β_1-integrin expression. The EP_1 agonist did not decrease the expression levels of either total or activated β_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 β_1-integrin expression induced by PGE_2. These results suggested that PGE_2 suppressed expression levels of both total and activated β_1-integrin mainly via EP_4 receptors, with some involvement in total β_1-integrin expression being mediated via EP_2 or EP_3 receptors. The EP_4 receptor plays an especially important role in activated β_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)-α production, CCL3/4 expression, and production of IL-8, macrophage inflammatory protein-1α (MIP-1α) and β, and monocyte chemoattractant protein-1 (MCP-1) [9,17–19]. PGE_2 inhibits IL-18-induced CD54 and CD86 expression and LPS-induced TNF-α 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-1α and β 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 β_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]. β_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 β_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 β_1-integrin, mainly via EP_4 receptor in human monocytes/macrophages.

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LETTER TO THE EDITOR

Elderly case of prolonged hypoglycemic coma presenting with reversible magnetic resonance imaging changes

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Dear Editor,

The aging population is leading to an increase in the prevalence of diabetes mellitus (DM).[1–4] Safer treatments for DM are required in elderly patients, because this age group suffers from a higher prevalence of geriatric syndrome and increased risk of functional dependency, frailty and hypoglycemia compared with younger patients.[1,2] In addition to DM,[1–3] recurrent severe hypoglycemia[1,5,6] is associated with cognitive impairments, and prolonged hypoglycemia can induce irreversible coma.[7–11] Thus, preventing hypoglycemia is very important, particularly for elderly patients with DM.

A 75-year-old woman developed nausea and anorexia. She had DM, dyslipidemia, atrial fibrillation, lumbar spinal canal stenosis and a history of cerebral infarction in a subcortical parietal lobe. Although she was functionally independent, she had slight cognitive and gait balance impairments. A history of stroke and peripheral diabetic neuropathy was speculated to be the causes of these impairments. She had been treated well for the DM with glimepiride and metformin hydrochloride, but had not yet started insulin therapy. Her family physician diagnosed acute enterogastritis with a cold and prescribed an antiphlogistic therapy. She had eaten very little for several days and had twice suffered disturbances in her consciousness within 3 days of the onset of the cold. Each attack revealed hypoglycemia, which was urgently restored by a glucose injection at her doctor's clinic. Although the doctor recommended hospitalization, she refused and went home. Against the doctor's recommendations, she continued to take oral blood glucose-lowering agents and went to bed. The next afternoon, she was found in a sustained coma lasting at least 18 h, and was admitted to Hyogo Brain and Heart Center at Himeji, Himeji, Japan (day 1).

On admission, her state of consciousness was Glasgow Coma Scale (GCS) 3; E1V1M1. Her blood pressure was 141/84 mmHg, her heart rate was 106 b.p.m. and her body temperature was 38.8°C. Neurological examination revealed ocular conjugate deviation to the right side. Her muscle tone was flaccid, deep tendon reflexes were absent and bilateral plantar responses were extensor. No meningeal signs were noted. Laboratory examination on admission showed a white blood cell count of $12\,000 \times 10^6$ /μl (polynuclear cells dominant) and a C-reactive protein level of 0.7 mg/dL. Her blood glucose level was 21 mg/dL, which was urgently restored to 284 mg/dL by a glucose injection. This procedure did not improve her state of consciousness. Her HbA_{1c} level was 5.9%. A urinalysis showed proteinuria and glucosuria, but was negative for bacteriuria. Cerebrospinal fluid (CSF) examination revealed pleocytosis, with a cell count of 180 cells/μL (164 polynuclear cells/μL), a protein level of 33 mg/dL and a glucose level of 177 mg/dL (day 2). This CSF analysis also proved negative for inflammatory markers (myelin basic protein and oligoclonal bands) and bacterial culture on day 5. An electroencephalogram (EEG) showed diffuse theta background activity without epileptoid discharges (day 2). We made a diagnosis of sustained hypoglycemia that resulted in hypoglycemic encephalopathy (HE) with the possibility of complicated acute encephalitis, most likely parainfectious or postinfectious acute disseminated encephalomyelitis (ADEM). She was treated with antibiotics, glycerin and methylprednisolone pulse therapy (mPSL-pulse). Her consciousness level improved a few days later (GCS 6; E4V1M1), but she remained akinetic and mute. A repeat CSF analysis on day 10 gave normal results. After 6 months, she was still in an akinetic state.

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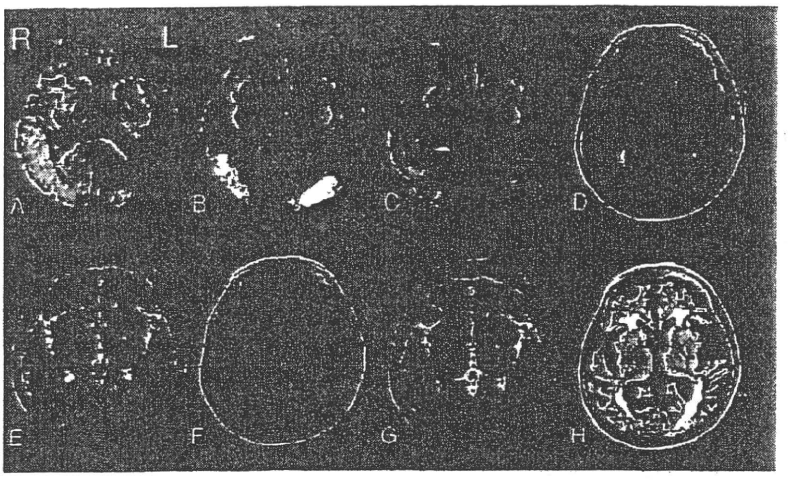

Figure 1 Temporal changes in magnetic resonance imaging findings after prolonged hypoglycemic coma. Diffusion-weighted image (DWI) on day 1 showing faint high-intensity lesions in the cortex of the bilateral occipital lobes (a). These lesions were more apparent on DWI taken on day 3, but covered a smaller area (b) and had largely disappeared by day 9 (c). Fluid-attenuated inversion recovery (FLAIR) and T2-weighted images corresponding to the DWI are also shown (d, f: FLAIR images; (e, g) T2-weighted images). (h) FLAIR images obtained 6 months later showed bilateral brain atrophy and periventricular hyperintensity.

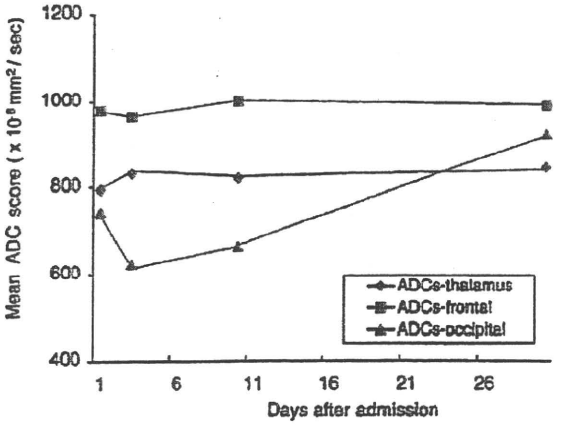

Figure 2 Time-course of mean apparent diffusion coefficient (ADC) scores after onset of hypoglycemic coma. In the early phase, the scores in the occipital lobes were lower than the scores in the thalamus and frontal lobes. After 1 month, the ADC scores in the occipital lobes had increased and were similar to those in the frontal lobes.

Brain magnetic resonance imaging (MRI) on day 1 showed faint high-intensity lesions on diffusion-weighted images (DWI), fluid-attenuated inversion recovery (FLAIR) and T2-weighted images in the cortex of the bilateral occipital lobes. These lesions had largely disappeared by day 9. MRI carried out 6 months later showed bilateral brain atrophy and periventricular hyperintensity on FLAIR images (Fig. 1). The apparent diffusion coefficient (ADC) values in the cortex of the occipital lobes showed remarkable decreases in the early phase, followed by a gradual recovery to normal levels (Fig. 2).

Because cytokine analysis can help assess the possibility of complicated acute encephalitis, we analyzed her preserved serum and CSF after we obtained informed consent from her legal representative. Examination of cytokine levels on day 1 revealed elevated interleukin (IL)-6 levels of 1371.4 pg/mL (normal <9.7 pg/ml) in her CSF, and 46.5 pg/mL (normal <19.9 pg/mL) in her serum. Repeat CSF and serum analyses on days 10 and 30 showed normal IL-6 levels. Throughout the clinical course, the serum and CSF levels of other cytokines (IL-2, IL-4, IL-10, interferon-γ and tumor necrosis factor-α) were normal.

This case reveals possible targets for improving the treatment of elderly DM patients with geriatric syndrome to avoid hypoglycemic coma. This case also showed reversible MRI changes in hypoglycemic coma[7-10,12,13] and cytokine increases in the serum and CSF.

Prevention of hypoglycemia requires monitoring of HbA_{1c} levels and self-monitoring of blood glucose, meticulous adjustment of medication and/or insulin and the education of patients, their families and care staff in coping skills for hypoglycemia and sick days.[1] This patient was elderly,[5] had not been introduced to self-monitoring of blood glucose, had impaired cognition and gait balance as part of her geriatric syndrome,[1,2] and a history of cerebral infarction,[2] became unwell and decreased intake of energy,[5] and suffered repeated episodes of hypoglycemia before the onset of coma. Decreased physiological activity in the elderly might also have affected the clinical status. Furthermore, the counter-regulatory hormone responses to hypoglycemia are worse among the elderly than younger patients, and are likely to result in an insensitivity to hypoglycemia.[5] In addition, she lived alone and might have known little about sick day management and the risk of hypoglycemic coma. All of these might have been risk factors for hypoglycemic coma in the present case.

For well-functioning DM patients free of geriatric syndrome, a HbA_{1c} level between 6.5% and 7.0% has been recommended to prevent severe hypoglycemia, diabetic complications, dementia and death.[1] In contrast, a HbA_{1c} level of 7.0% is sufficient in elderly

people,[4] and less than 8.0% is considered acceptable in patients with multiple morbidities and multiple functional impairments.[1] Thus, a HbA_{1c} level between 7.0% and 8.0% would be reasonable to prevent severe hypoglycemia in elderly DM patients with geriatric syndrome. Because our patient's HbA_{1c} level was 5.9%, a latent risk for hypoglycemia would have been present.

DWI are useful to detect acute brain injury in hypoglycemia.[7–13] Decreased ADC scores[7–9,13] are possible markers for cytotoxic edema as a result of hypoglycemic brain injury.[8,9,12] The prognosis of patients with HE who had basal ganglia involvement is poor.[7–9] Although reversible DWI changes, which indicate transitory cytotoxic edema[9] and predict good prognosis,[9,12,13] were limited in the occipital cortex in our case, the prognosis was a persistent akinetic state.

Cerebral cytotoxic edema in HE could be associated with an increase in IL-6 levels. Glucose deprivation leads to brain energy failure and membrane ionic pump failure.[8,9,13] Prolonged hypoglycemia causes hypoxia and neuronal death,[8–10] and might induce a breakdown of the blood–brain barrier. The pathological findings among HE patients have revealed superficial laminar necrosis in the cortex and severe degeneration with reactive astrocytosis and macrophage infiltration in the cerebral white matter.[11] These pathological findings support the elevated IL-6 in the CSF and serum in our case, and are also consistent with the severe white matter degeneration shown by the MRI at 6 months after coma onset. Thus, the akinetic status in our case could be more closely associated with these findings than with the reversible DWI changes.

The clinical and laboratory findings, except for hypoglycemia, were similar to the characteristics of ADEM. Additionally, the MRI findings in our case showed the involvement of the occipital lobes, which are the most frequently involved region in patients with ADEM.[14] An mPSL-pulse is an effective treatment for ADEM.[14] Although the presence of ADEM was negative in our case, it is advisable to carry out mPSL-pulse as early as possible in cases with possible ADEM, because early steroid treatment is an important factor in determining the prognosis of ADEM.[14] However, an mPSL-pulse is not a standard treatment for HE per se.

There were some differential diagnoses to consider. Although cerebral infarction, viral encephalitis or other metabolic encephalopathy might be possible, the MRI findings and serum, CSF and cytokine analysis were negative for these. Indeed, the MRI findings were similar to those of reversible posterior leukoencephalopathy syndrome,[15] but increased ADC scores as a result of vasogenic edema[15] were not confirmed in our patient. Non-convulsive status epilepticus is possible in elderly DM patients with acute confusion[16] and can be detected by timely EEG; however, the EEG findings of our patient did not support this diagnosis.

In conclusion, we report an elderly DM patient with hypoglycemic coma who had multiple risk factors for hypoglycemia. The marked correlation between the reversible MRI changes and increased IL-6 levels is noteworthy. Improving the strategies to prevent hypoglycemic coma should be an important focus of the treatment of elderly DM patients. Furthermore, a treatment strategy stratified by the patient's age, condition and the presence of geriatric syndrome might result in a more targeted and effective prevention of hypoglycemia.

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