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ORIGINAL ARTICLE

Reduction in serum levels of substance P in patients with rheumatoid arthritis by etanercept, a tumor necrosis factor inhibitor

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Abstract We determined the effects of etanercept on the serum concentrations of neuropeptides in RA patients. In a total of 11 patients who had been injected with etanercept, the serum levels of substance P, calcitonin gene-related peptide (CGRP), and gastrin-releasing peptide (GRP) were analyzed. Average levels of serum substance P were significantly reduced from 1.53 to 0.62 ng/ml after the injection of etanercept. In the CGRP and GRP analyses, these average levels dropped from 1.57 and 0.51 ng/ml to 0.44 and 0.04 ng/ml, respectively. Etanercept appears to decrease substance P levels with an improvement in disease activities.

Keywords Calcitonin gene-related peptide (CGRP) · Etanercept · Gastrin-releasing peptide (GRP) · Rheumatoid arthritis · Substance P

Introduction

It is well known that cytokines are involved in the progression of RA. Proinflammatory cytokine TNF-α plays a central role in the pathogenesis of RA. Thus far, some anti-cytokine inhibitors [e.g., TNF (infliximab, etanercept, and adalimumab), IL-1 (anakinra), and IL-6 (MRA)] have been developed [1, 2]. Etanercept (an Fc domain of human IgG1

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and a fusion protein combined by genetic engineering and consisting of the same chains of a harmonizing recombinant human TNF-receptor p75 monomer) joined TNF together and inactivated it [3]. It has been shown in randomized double-blinded placebo control trials that etanercept treatment provides an important clinical benefit in patients who have RA with insufficient response to other DMARDs. Etanercept also has strong inhibitory effects against RA activity.

On the other hand, it has been reported that the concentrations of neuropeptides such as substance P, calcitonin gene-related peptide (CGRP), and gastrin-releasing peptide (GRP) are increased in the synovial fluids of the RA, and that they contribute to an induction of inflammation. Neurogenic inflammation is defined as edema formation, increased blood flow, and inflammatory cell involvement observed after the stimulation of sensory nerve fibers (usually C and Ad fibers) and the release of neuropeptides. Substance P is an endogenous neuropeptide that acts upon the neurokinin 1 (NK(1)) receptor, is released from sensory nerves, and is involved in neurogenic inflammation. Substance P is present in the nerve fibers of the synovium [4]. Pain-related neuropeptides such as substance P released from synovial fibroblasts have been implicated in joint destruction [5]. CGRP is a 37-amino acid neuropeptide formed from alternative splicing of the calcitonin/CGRP gene. CGRP induces vasodilatation in a variety of vessels and functions in the transmission of pain [6, 7]. It is abundant in both peripheral and central neurons [8]. In addition, it has been reported that CGRP concentrations are elevated in the synovial fluids of patients with RA [9, 10]. GRP is a peptide of 27 amino acid residues, originally isolated from the non-antral part of the porcine stomach. GRP has been known to regulate numerous functions of the gastrointestinal and central nervous systems. Although the physiological and pathophysiological roles of GRP have not been elucidated, it has been reported that it affects satiety, thermal regulation, nociception, and the activation of sympathoadrenomedullary outflow [11, 12]. Grimsholm et al. have shown that GRP is present in joint fluid in arthritis and that its levels are increased in RA [13].

In the present study, we examined whether these three neuropeptides deteriorated with the inhibition of RA activity in response to etanercept.

Materials and methods

Patients

We studied 11 patients (4 men and 7 women; mean age 57.9 ± 11.4 years, range 29–73 years) with RA who fulfilled the 1987 criteria of the American Rheumatism Association [14], and who were treated at the Department of Rheumatology and Immunology at Nagasaki University Hospital and its associated hospitals. These patients had active disease, as manifested by at least six joints that were swollen and tender, and at least one of the following: an erythrocyte sedimentation rate of 28 mm/h or greater, plasma C-reactive protein of 2.0 mg/dl or greater at the time of enrollment. Their disease durations were 1–20 years. Of these 11 patients, all were taking prednisolone (mean ± SEM 7.0 ± 2.4 mg/day; range 2–10 mg/day), 7 were

Table 1 Baseline characteristics of 11 patients with rheumatoid arthritis

Pt no.	Age (years)	Sex	Stage	Class	Duration (years)	PSL (mg/day)	MTX (mg/weeks)	Tender joints	Swollen joints	CRP (mg/dl)	DAS28-CRP
1	61	M	II	2	6	5	0	17	12	2.70	4.36
2	59	F	II	2	5	7	0	17	7	3.50	4.89
3	65	M	I	2	3	8	0	28	19	4.30	7.32
4	29	F	IV	4	9	10	8	15	8	0.48	4.08
5	53	F	IV	2	17	4	0	11	9	4.82	4.82
6	53	F	IV	2	14	2	8	2	4	0.53	3.87
7	58	F	II	1	3	7.5	0	8	20	4.74	4.40
8	62	M	II	2	8	8	0	14	11	0.80	5.62
9	56	F	III	2	20	8	8	2	3	0.38	3.08
10	68	M	I	2	1	10	8	21	20	3.43	7.39
11	73	F	IV	3	20	7.5	0	11	6	5.28	6.13
Mean	57.9				9.64	7.0		13.3	10.8	2.81	5.09

Values are the number (percentage), unless otherwise indicated

PSL prednisolone, *MTX* methotrexate, *CRP* C-reactive protein, *DAS28* disease activity score in 28 joints

taking nonsteroidal anti-inflammatory drugs (NSAIDs), 4 methotrexate (8 mg/week), 5 sulfasalazine (1.0 g/day), 1 bucillamine (200 mg/day), 1 cyclosporin (100 mg/day), 1 mizoribine (150 mg/day), and 2 tacrolimus (3 mg/day). Two patients had previously received infliximab, another TNF antagonist. The patient characteristics are summarized in Table 1. To participate in the study, each patient provided a signed consent form approved by the Institutional Review Board of Nagasaki University.

Measurement of neuropeptides

Serum concentrations of three neuropeptides—substance P, CGRP, and GRP—in the above subjects were measured by enzyme immunoassay kits according to the manufacturer's instructions (Phoenix Pharmaceuticals Inc., CA, USA). In brief, the peptides were incubated with biotin-labeled peptides. After washing, streptavidin-conjugated horseradish peroxidase (SA-HRP) was added. After washing again, tetramethyl benzidine dihydrochloride was allowed to react with bound HRP. The absorbance was read at 450 nm, and all assays were performed in duplicate. The counts bound figure was divided by the total counts and expressed as a percentage. In order to derive numerical values (ng/ml), these percentage numbers were then positioned onto the standard curve.

Statistical analysis

Data were analyzed using a parametric test for paired values of intragroup comparisons, the paired t test. A probability value of <0.05 denoted the presence of a statistically significant difference.

Results

Evaluation of serum neuropeptides at 4 weeks after etanercept therapy

We evaluated the responses of serum three neuropeptides—substance P, CGRP, and GRP—at 4 weeks after etanercept therapy. The serum concentration levels of substance P were significantly decreased from 1.53 ± 1.45 ng/ml (mean ± SEM, range 0.22–5.10 ng/ml) to 0.62 ± 0.46 ng/ml (0.089–1.59 ng/ml) ($P < 0.05$). The serum concentration levels of CGRP were decreased from 1.57 ± 1.99 ng/ml (0.08–6.80 ng/ml) to 0.44 ± 0.43 ng/ml (0.029–1.42 ng/ml) ($P = 0.053$). The serum concentration levels of GRP were decreased from 0.51 ± 1.07 ng/ml (0–3.59 ng/ml) to 0.04 ± 0.07 ng/ml (0–0.22 ng/ml) ($P = 0.091$) (Figs. 1, 2, 3).

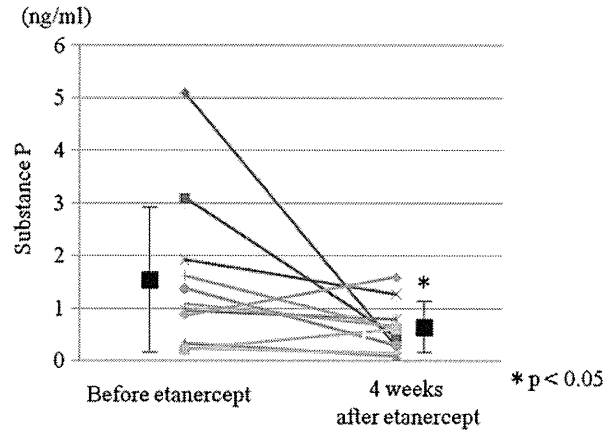

Fig. 1 Changes in serum substance P levels 4 weeks after the administration of etanercept. Changes in all patients as well as the mean change in the 11 patients are shown in the figure

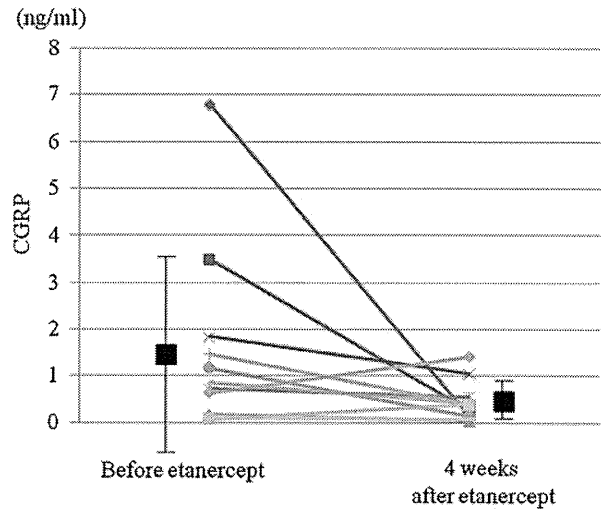

Fig. 2 Changes in serum CGRP levels 4 weeks after the administration of etanercept. Changes in all patients as well as the mean change in the 11 patients are shown in the figure

Correlation between the changes in serum concentration levels of substance P and those of DAS28-CRP

We examined the relationship between the changes in serum concentration levels of substance P and those of DAS28-CRP and found no significant correlation (Fig. 4a). Next, eleven patients were divided into two groups based on their response to etanercept as determined by serum concentration levels of substance P (Fig. 4b). Patients were placed in a "DAS-CRP good or moderate response group" ($n = 9$) or a "DAS-CRP no response group" ($n = 2$). All serum concentration levels of substance P were decreased during

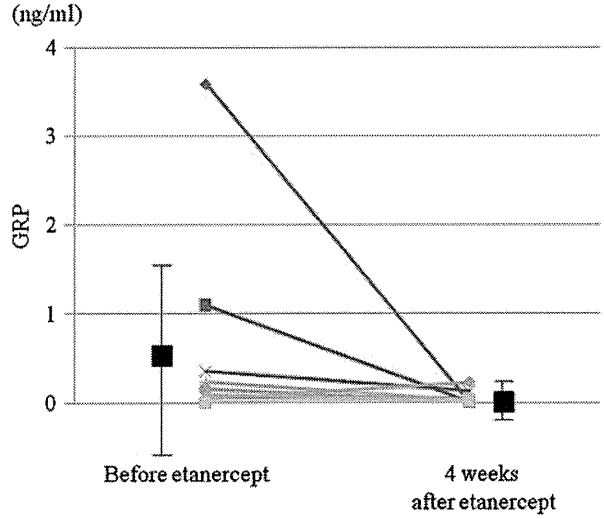

Fig. 3 Changes in serum GRP levels 4 weeks after the administration of etanercept. Changes in all patients as well as the mean change in the 11 patients are shown in the figure

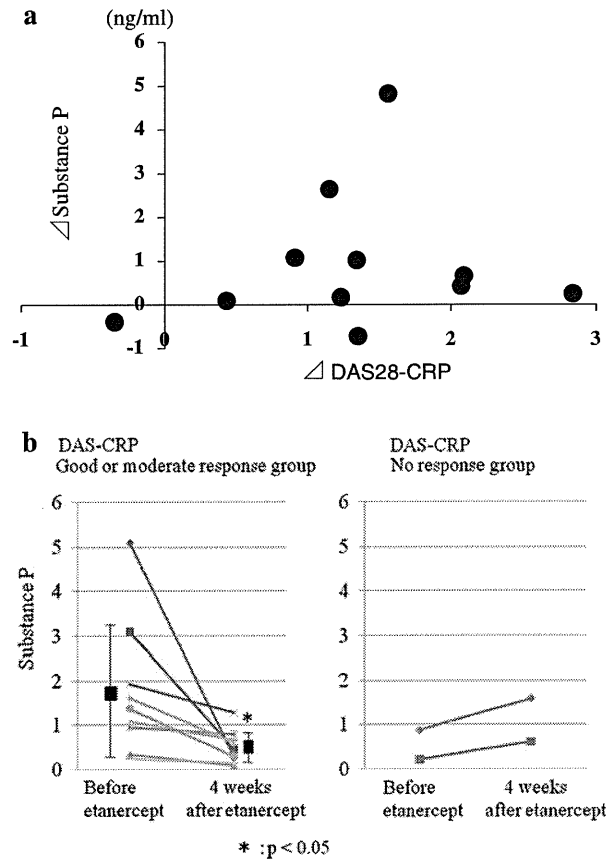

Fig. 4 Correlation between the changes in serum concentration levels of substance P and those of DAS28-CRP. Correlation between the changes in serum concentration levels of substance P and those of DAS28-CRP are shown in **a**. The 11 patients were divided into two groups depending on their response to etanercept (**b**). Patients were placed in the "good or moderate response group" ($n = 9$) or the "no response group" ($n = 2$). Changes in the serum concentration levels of substance P in the good or moderate response group are shown on the *left*, and those in the poor response group are shown on the *right*

etanercept therapy in the good or moderate response group (from 1.75 ± 1.52 to 0.51 ± 0.37 ng/ml) ($P < 0.05$). In contrast, the levels of substance P were increased during etanercept therapy in neither response group (from 0.55 ± 0.46 to 1.10 ± 0.70 ng/ml) ($P = 0.908$).

Changes in substance P and DAS-CRP parameters during etanercept therapy in 11 patients with RA

We examined the changes in substance P and DAS-CRP parameters during etanercept therapy in 11 patients with RA. The serum concentration levels of substance P were significantly decreased after etanercept therapy, except in cases 10 and 11. The DAS28-CRP scores were also decreased, except in case 11. The tender joint counts and swollen joint counts were increased only in case 11. Pain VASs were also decreased to below 60, except for case 11. All CRP levels were decreased after etanercept therapy, but the levels of CRP were still high in cases 3, 10, and 11 (Table 2).

Discussion

Our results demonstrated that etanercept suppressed serum levels of substance P and controlled disease activity in RA.

Substance P also has known proinflammatory properties that enhance the proliferation of rheumatoid synoviocytes and induce the release of prostaglandin E2 and collagenases [15]. It has been observed that the levels of substance P are increased in the synovial fluid of patients with RA [13, 16–19]. Grimsholm et al. [20] have reported that the average concentration of substance P in sera of 22 long-standing RA patients was 0.41 (0.32–0.44) ng/ml. In contrast, that in the sera of control subjects was 0.18 ng/ml. We measured the concentrations in sera of active RA patients, and the average concentration of substance P was 1.53 ± 1.45 (0.22–5.10) ng/ml. The average concentration of substance P in the active stage of RA before treatment with etanercept was significantly higher than that in the moderate stage of RA 4 weeks after treatment with etanercept. Intra-articular injection of substance P has been found to increase the severity of arthritis [21] and to lead to endothelial cell proliferation [22]. Substance P is also known to be present in innervations of the joint synovium [4, 23]. It is increasingly being reported that substance P

Table 2 Changes in substance P and DAS-CRP parameters during etanercept therapy in 11 patients with RA

Pt no.	Substance P (ng/ml)		DAS28-CRP		Tender joints		Swollen joints		Pain VAS (mm)		CRP (mg/dl)	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	5.10	0.27	4.36	2.80	17	2	12	0	77	42	2.70	0.10
2	3.10	0.45	4.89	3.74	17	11	7	5	74	51	3.50	0
3	1.93	1.27	7.32	5.23	28	16	19	4	90	27	4.30	3.00
4	1.62	0.60	4.08	2.74	15	0	8	0	32	9	0.48	0.01
5	1.37	0.29	4.82	3.91	11	14	9	2	58	41	4.82	0.02
6	1.08	0.66	3.87	1.80	2	1	4	0	64	5	0.53	0.08
7	0.96	0.79	4.40	3.17	8	1	20	10	14	2	4.74	0.68
8	0.34	0.09	5.62	2.78	14	2	11	1	79	46	0.80	0.20
9	0.25	0.15	3.08	2.65	2	0	3	0	15	6	0.38	0.09
10	0.87	1.59	7.39	6.04	21	14	20	13	93	65	3.43	1.83
11	0.22	0.61	6.13	6.48	11	16	6	8	85	85	5.28	3.56
Mean	1.53	0.62	5.09	3.76	13.3	7.0	10.8	3.9	61.9	34.5	2.82	0.87

can be produced by non-neuronal cells [24, 25] in the synovial tissue [26].

It is known that other neuropeptides such as CGRP and GRP are related to inflammation. CGRP has also been found in synovial fluid from patients with RA [19, 27]. It has been reported that the average concentration of CGRP in the sera of long-standing RA patients was found to be 0.82 ng/ml [20]. In contrast, it was 0.31 ng/ml in the sera of control subjects. CGRP enhanced IL-6 production induced by IL-1β or TNF-α in fibroblasts [28]. Nevertheless, CGRP inhibits IL-2 production by murine T lymphocytes [29], macrophage activation induced by IFN gamma, and the functional capacity of macrophages acting as antigen-presentating cells [30]. The effects of CGRP on inflammation are controversial.

Increased levels of GRP have also been found in joint fluid of RA [13]. Grimsholm et al. [20] have determined the levels of GRP in blood from patients with RA [1.70 ng/ml (1.60–2.00 ng/ml) vs. 0.34 ng/ml (control)] and have shown that these levels correlate with the levels of proinflammatory cytokines. They have also noted the occurrence of GRP-containing fine nerve fibers in association with the inflammatory infiltrates in arthritic mice [31]. GRP are also reported to be expressed in chondrocytes [32].

The correlation between cytokines and neuropeptides in RA has been studied for several years. Grimsholm et al. investigated possible interrelations between five neuropeptides [substance P, CGRP, GRP, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY)] and the three cytokines TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) in the synovial fluid of patients with RA [20, 33, 34]. They found correlations between substance P and GRP and the cytokines. The concentrations of substance P were grouped together with those of TNF-α, and substance P and GRP were also grouped together with IL-6. These results suggest that substance P and GRP are involved together with cytokines in the neuroimmunomodulation that occurs in arthritic joints. It has been suggested that regulatory neuroimmune pathways in the joints are important mechanisms modulating the expression of arthritis [35]. Conversely, it has been reported that substance P and CGRP can stimulate the production of IL-1, IL-6, and TNF-α from various immune cells [33] [36–38]. Therefore, substance P and CGRP cooperate with TNF-α and enhance their own production in RA patients. These results suggest that proinflammatory cytokines and neuropeptides are involved in a vicious cycle to enhance the activity of RA.

In this study we postulated that one TNF inhibitor, etanercept, stopped the vicious cycle of proinflammatory cytokines and neuropeptides, leading to an inactive state of RA. Our results demonstrated that etanercept inhibited the activities of RA as well as the release of substance P. It was suggested that etanercept might block the release of substance P through the inhibition of the COX/prostaglandin pathway, the desensitization of nociceptors, or the blockade of the transport of substance P to serum from synovial fluid. We therefore suggest that the inhibition of TNF-α by etanercept stops the vicious cycle between cytokines and neuropeptides, leading to control over the activity of RA.

Conclusion

We observed for the first time that etanercept, an anti-cytokine blocker, inhibited the increased concentrations of substance P in patients with RA. These results indicated

that the levels of serum substance P were correlated with RA activity in RA patients. In a future study, we would like to investigate the correlation between neuropeptides and pain in RA patients.

Conflict of interest None.

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Original article

A familial case of LEOPARD syndrome associated with a high-functioning autism spectrum disorder

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Abstract

A connection between LEOPARD syndrome (a rare autosomal dominant disorder) and autism spectrum disorders (ASDs) may exist. Of four related individuals (father and three sons) with LEOPARD syndrome, all patients exhibited clinical symptoms consistent with ASDs. Findings included aggressive behavior and impairment of social interaction, communication, and range of interests. The coexistence of LEOPARD syndrome and ASDs in the related individuals may be an incidental familial event or indicative that ASDs is associated with LEOPARD syndrome. There have been no other independent reports of the association of LEOPARD syndrome and ASDs. Molecular and biochemical mechanisms that may suggest a connection between LEOPARD syndrome and ASDs are discussed.
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Keywords: LEOPARD syndrome, Noonan syndrome; Autism spectrum disorders (ASDs); RAS/MAPK signal transduction pathway

1. Introduction

LEOPARD syndrome (OMIM#151100) is a rare autosomal dominant disorder characterized by **L**entigines, **E**lectrocardiogram abnormalities, **O**cular hypertelorism, **P**ulmonic valvular stenosis, **A**bnormalities of genitalia, **R**etardation of growth, and **D**eafness. This syndrome is caused by germline missense mutations in the *PTPN11* gene that encodes Src homology 2 domain-containing tyrosine phosphatase 2 (Shp2): non-receptor protein-tyrosine phosphatase comprising two N-terminal SH2 domains, a catalytic domain, and a C terminus with tyrosylphosphorylation sites and a proline-rich stretch. The mutations induce catalytically impaired Shp2 by a "dominant negative effect" [1–2].

In the more common Noonan syndrome, approximately 50% of patients have *PTPN11* mutations scattered over the entire Shp2, including the catalytic domain. The mutations resulting in the Noonan phenotype are the "gain-of-function" mutations, and they exhibit substantially increased catalytic ability. Although LEOPARD syndrome and Noonan syndrome are caused by *PTPN11* mutations resulting in opposite effects, they share many common clinical features, including physical dysmorphic findings and intellectual disability [1].

The term "autism spectrum disorders (ASDs)" was first used by Lorna Wing [3] and then widely used as a category comprised of autistic disorder, Asperger's

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disorder, and other related conditions [4]. These conditions are very common neurobehavioral disorders that are characterized by impairments in three behavioral domains, including social interaction, language/communication/imaginative play, and a range of interests and activities [3–5].

At least ten genes have been reported to be associated with ASDs [6]. Except for Rett syndrome, the other pervasive developmental disorder (PDD) subtypes including autistic disorder, Asperger's disorder, disintegrative disorder, and PDD Not Otherwise Specified (PDDNOS) are not tightly linked to any particular gene mutations. Several common genetic syndromes are known to be associated with ASDs. Autism is frequent in patients with tuberous sclerosis (TSC) [7], with neurofibromatosis type 1 [8,9] and with Fragile X syndrome [10]. Studies of psychological profiles of adults with Noonan syndrome did not suggest a specific behavioral phenotype, but difficulties with social competence and emotional perceptions were noted [11]. A case of Noonan syndrome who was also diagnosed with autism was reported [12]. The present study of neuropsychiatric evaluation in a familial case of LEOPARD syndrome indicates all patients satisfied the criteria of ASDs. An association of LEOPARD syndrome and ASDs has not been reported previously. The familial case presented in this report may suggest such an association.

2. Patients and methods

After obtaining written informed consent, fifteen coding exons in *PTPN11* were sequenced in each patient following the methods described somewhere else [13].

Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV-TR) [5] and The high-functioning Autism Spectrum Screening Questionnaire (ASSQ) [14] were used in neuropsychiatric evaluation of the subjects.

Patient 1 is a 20-year-old male who was born as the second child to a non-consanguineous Japanese couple. His early developmental milestones were reportedly unremarkable. He was clinically diagnosed with LEOPARD syndrome at age 7 years based on findings that included lentigines, multiple café-au-lait spots, electrocardiogram (ECG) abnormalities, ventricular septal defect, ocular hypertelorism, short stature, and unilateral renal hypoplasia. *PTPN11* mutation analysis revealed a heterozygous mutation of 1403C > T (T468 M). The patient was diagnosed as having Asperger's disorder based on ASSQ and DSM-IV-TR, at age 12 years. His intelligence quotient (IQ) by the Wechsler Intelligence Scale for Children-third edition (WISC-III) was 85 (verbal: 77, performance 98). His ASSQ score by mother's rating was 41. He met the DSM-IV-TR diagnostic criteria of Asperger's disorder with all subcategories in the category of Qualitative impairment in social interaction (Category 1), three subcategories (1,2, and 4) in the category of Restricted repetitive and stereotyped patterns of behavior, interests and activities (Category 2), and the rest of the four categories (Table 1).

Patient 2 is a 15-year-old younger brother of Patient 1. His early infantile developmental milestones were unremarkable. He was diagnosed with growth retardation at age 2½ years. At age 12 years his clinical findings of a few café-au-lait spots, ocular hypertelorism, and undescended testes led us to obtain *PTPN11* mutation analysis, which showed the same heterozygous mutation of 1403C > T. At age 9 years, a diagnosis of Asperger's disorder was made based on ASSQ and DSM-IV-TR. His full-scale IQ by WISC-III at age 9 years was 99 (verbal 104, performance 92). His ASSQ score by parental rating was 32 at age 15 years. He also met the Asperger's disorder diagnostic criteria with all subcategories of Category 1, three of Category 2 (1, 2, and 4), and the rest of the categories (Table 1).

Patient 3 is the 22-year-old eldest brother of Patients 1 and 2. His developmental milestones were normal, although his ritualistic behavior and difficulties in relating to peers were noted in his childhood. He had a surgical repair of bilateral undescended testes and inguinal hernia. He was diagnosed with Wolff-Parkinson-White syndrome at age nine years. He has ocular hypertelorism and short stature. The same *PTPN11* heterozygous mutation found in the two younger siblings was identified in this patient. He attends college, and was diagnosed as having PDDNOS, because he also had impaired development of reciprocal social interaction associated with communication skills, repetitive routine, and ritualistic behavior. His ASSQ score was 7 at age 22 years (Table 1).

Patient 4 is a 55-year-old male who is the father of the siblings. He has prominent lentigines, bilateral mild hearing loss, cardiac anomalies, ECG abnormalities, short stature, and apparent ocular hypertelorism. His early developmental milestones are not well known. He has been noted to have obsession with a specific topic, repetitive routine and rituals, and clumsy movements. At age 50 years, his social skills and aggressive behavior were noted to be deteriorating, and consequently he was suspected of having Asperger's disorder based on DSM-IV-TR. He met the diagnostic criteria of Asperger's disorder with Category 1 (1 and 3), Category 2 (1 and 2), and the rest of the four categories. His ASSQ score was 20 at age 55 years by his wife's evaluation. He has the same heterozygous *PTPN11* mutation (Table 1).

3. Discussion

The presented familial case of LEOPARD syndrome included individuals (patients 1, 2, and 4) diagnosed with or suspected of having Asperger's disorder, and

Table 1
Summary of clinical findings and *PTPN11* mutation.

	Pt. 1 Male	Pt. 2 Male	Pt. 3 Male	Pt. 4Male
Age	20 y	15 y	22 y	55 y
Physical findings				
Skin: café-au-lait spots	multiple	a few	a few	a few
Lentigines	+++	+++	–	+++
Cardiac defects	VSD	No	No	No
EKG abnormalities	+	No	WPW	No
Ocular hypertelorism	+	+	+	+
Pulmonary stenosis	No	No	No	No
Abnormal genitalia	No	Und. Testes*	Und. Testes*	No
Renal anomalies	R-hypoplasia	No	No	No
Retardation of growth	Yes	+	+	No
Deafness	No	No	No	Yes
Miscellaneous:				
Rocker bottom feet	Yes	Yes	Yes	No
Macrocephaly	Yes	Yes	Yes	No
PTPN11 mutation	T468 M	T468 M	T468 M	T468 M
Neuropsychological				
Diagnosis	AD**	AD**	PDDNOS***	AD**
ASSQ score(1) (age)	41 (12 y)	32 (15 y)	7 (22 y)	20 (50 y)
WISC-III(2) (age)	85 (12 y)	99 (9 y)	n/a	n/a
-Verbal/performance	77/98	104/92	n/a	n/a

* Und. Testes, undescended testes.
** AD, Asperger's disorder.
*** PDDNOS, Pervasive developmental disorder not otherwise specified.
(1) ASSQ score, Autism Spectrum Screening Questionnaire Score. The cutoff score of 3 predicts 91% of the true positive rate of Autistic spectrum disorders.
(2) WISC-III, Wechsler Intelligence Scale for Children-third edition.

patient 3 was diagnosed as having PDDNOS, which may lead to the diagnosis of ASD. ASDs were first introduced by Lorna Wing, who suggested that Asperger's disorder is a type of ASD and described in detail its various manifestations in speech, nonverbal communication, social interaction, motor coordination, motor clumsiness, and idiosyncratic interests [3]. Patient 3 did not have enough clinical symptoms to meet the diagnostic criteria for Asperger's disorder; however, he had some symptoms suggestive of ASD in his childhood that led to a diagnosis of PDDNOS.

The ASSQ is a 27-item checklist for completion by lay informants when assessing characteristic symptoms of Asperger's disorder and high-functioning autism in children and adolescents with normal intelligence or mild mental retardation. The ASSQ allows for rating on a 3-point scale (0, 1, or 2; 0 indicating normality, 1 some abnormality, and 2 definite abnormality). The range of possible scores is 0–54. The mean ASSQ parent scores in the Asperger's disorder validation sample were 25.1 (SD, 7.3) [14]. The cutoff score of 13 is 91% of the true positive rate of ASDs. The ASSQ score was established as a screening tool primarily for children between 6 and 17 years of age by parents and/or teachers. The delayed evaluation of patient 3 may account for the difference in diagnosis between this patient and his siblings.

ASDs are known to be associated with particular genetic disorders such as fragile X syndrome [10,15, 16], tuberous sclerosis (TSC) [7], and neurofibromatosis type 1 [8,9]. Fifty percent of children with TSC have behavioral problems in the form of ASDs. Gene mutations in either *TSC1* or *TSC2* influence neural precursors, resulting in abnormal cell differentiation and dysregulated control of cell size. These cells migrate to the cortex to generate an abnormal collection of inappropriately positioned neurons, causing widespread cortical disorganization and structural abnormalities [7]. Mutations in *PTPN11* causing LEOPARD syndrome induce catalytically impaired Shp2. In situ hybridization detected Shp2 expression in the neural ectoderm and nervous system in mouse embryos, suggesting an involvement of Shp2 in neural development. Shp2 is a critical signaling molecule in the coordinated regulation of progenitor cell proliferation and neuronal/astroglial cell differentiation. The studies with mutant mouse strains with Shp2 selectively deleted in neural precursor cells showed a dramatic phenotype of growth retardation, early postnatal lethality, and multiple defects in proliferation and cell fate specification in neural stem/progenitor cells [17]. The product of the TSC2 gene tuberin is known to up-regulate the B-RAF/MEK/MAPK signal transduction pathway. B-RAF is required for neuronal differentiation, suggesting another possible link between B-RAF signaling and the clinical manifestations of TSC including ASDs [18]. Disturbed neuronal cell differentiation and development due to mutations in

the TSC genes and the *PTPN11* gene are likely to contribute to the development of ASDs in patients with these syndromes.

NF-I is well known to be associated with ASDs. The prevalence of autism in patients with NF-I was reported to be 4% [9]. The well-known function of the NF-I protein is to act as a RAS-GTPase-activating protein known to be involved in the regulation of the RAS-mitogen-activated protein kinase (MAPK) pathway. Mutations in the NF-I gene are thought to result in activation of the RAS/MAPK signal transduction pathway [2]. Clinical overlap between LEOPARD syndrome and NF-I is also well known [19].

Approximately 50% of patients with Noonan syndrome are due to missense *PTPN11* mutations [20]. *PTPN11* encodes SHP2, a protein tyrosine phosphatase, that is involved in the activation of the RAS/MAPK cascade [2]. Noonan syndrome is caused by "gain of function" *PTPN11* mutations [1,2], and the SHP2 mutants due to the *PTPN11* mutations causing Noonan syndrome cause prolonged activation of the RAS/MAPK pathway [2]. Schubbert et al. [21] reported that germline KRAS mutations cause Noonan syndrome through the hyperactive RAS/MAPK pathway.

Herault et al. [22] reported a positive association of the HRAS gene and autism. The psychological profiles of adults and children with Noonan syndrome have been studied, and deficiencies in social and emotional recognition and expression have been identified in adults, while low verbal IQ, clumsiness, and impairment of developmental coordination have been reported in children [23].

To date, there have been no reports to suggest an association of LEOPARD syndrome and ASDs. Our observations in this familial case may suggest at least some degree of association between LEOPARD syndrome and ASD phenotypes possibly through the RAS/MAPK signal transduction pathway. Further studies with more patients with LEOPARD syndrome are needed to establish the association and to investigate the genetic contributing factors causing ASDs, leading to the prevention and earlier detection of ASDs and better management of patients with these disorders.

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Case report

A long-term survival case of arginase deficiency with severe multicystic white matter and compound mutations

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Abstract

Neuropathology and neuroimaging of long-term survival cases of arginase deficiency are rarely reported. The magnetic resonance imaging (MRI) of our case showed severe multicystic white matter lesions with cortical atrophy, which were more severe compared with previous reports. In this patient, low-protein diet successfully reduced hyperammonemia, but hyperargininemia persisted. These severe neurological and MRI findings may be explained by a compound heterozygote, inheriting both of severe mutant alleles from her parents.
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Keywords: Argininemia; Long-term survival; MRI; Multicystic encephalomalacia

1. Introduction

Argininemia is an autosomal recessive urea cycle disorder resulting from deficiency of arginase. The clinical features of this rare disease differ from other urea cycle enzyme deficiencies. In most patients with argininemia, hyperammonemia tends to be moderate, and they are typically seen in late infancy to the second year of life with progressive spastic paraplegia, retardation in motor and mental development, seizures, and poor growth [1].

Neuropathological and neuroimaging findings of long-term survival cases of argininemia are rarely reported. The brain of patients with urea cycle disorders is swollen with flat gyri, narrow sulci, and reduced ventricular size in acute cases. The protoplasmic astrocytes are swollen (Alzheimer type II glia) probably due to accumulation of intracellular glutamine and water. In chronic cases, the brain may be atrophic [2].

We report the neuroimaging findings in a long-term survival patient with argininemia, which showed severe morphological brain changes and molecular defect.

2. Case report

This woman of 32 years of age is the first child of healthy non-consanguineous Japanese parents. She was born by normal delivery at 42 weeks of gestation. Her birth weight was 3300 g, and head circumference was 34.5 cm. She was well until the 18th day of life, when she began to vomit after feeding. At the age of 23 days, she developed fever with clonic convulsions. Since then, convulsive episodes continued even during afebrile

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periods in spite of treatments with various anticonvulsants. At 4 months of age, her mother noticed that the baby appeared "drowsy" after feeding. Her extremities were hypertonic with opisthotonic posture. Her neurological development was markedly delayed during infancy. At the 8th month of age, the head circumference was 39.5 cm (−2 SD). Computed tomography (CT) at 12 months revealed dilatation of the lateral ventricles and subdural fluid collection [3].

At the age of 4 years, she was admitted to the hospital because of pneumonia, where she was found to have hyperammonemia (960 μg/dl) and hyperargininemia (341–1049 μmol). Argininemia was diagnosed by the elevated plasma arginine concentration and virtually nil activity of arginase in red blood cells (16 and 19 μmol/h/g hemoglobin, control; 3716 ± 2236).

In the amino acid analysis, arginine level was 1.91 mg/dl (normal range; 0.15–0.55 mg/dl) in the cerebro spinal fluid (CSF), 19 mg/g Cr (normal range; ~10 mg/g Cr) in urine. Glutamine level was 16.4 mg/dl (normal range; 3.45–16.61 mg/dl) in serum and 10.1 mg/dl (normal range; 3.22–11.64 mg/dl) in CSF. Excretion of orotic acid in urine was elevated.

In the examination of arginase activity of red blood cells, her father and mother showed low activities of arginase (1103 and 1440 μmol/h/g hemoglobin, respectively) and delayed patterns in the arginine loading test, suggesting that both of parents were obligatory carriers. Genomic DNAs from the patient and from her parents were amplified by the polymerase chain reaction method. The patient showed a compound heterozygote, inheriting an allele with the four-base deletion from the father and the other allele with the one-base deletion from the mother. There was a four-base deletion at protein-coding region 262–265 or 263–266 in exon 3 that would lead to a reading-frame shift after amino acid residue 87 and make a new stop codon at residue 132. The other was a one-base deletion at 77 or 78 in exon 2 that would lead to a reading-frame shift after residue 26 and make a stop codon at residue 31.

3. Course and treatment

Argininemia was treated with low-protein diet, sodium benzoate supplement, and short-term administration of an arginine-free essential amino acid mixture. Natural protein intake was 0.5–1.0 g/kg daily. This dietary treatment was successful in ameliorating hyperammonemia, but high plasma arginine levels (432–757 μmol) persisted. The electroencephalogram (EEG) revealed sharp waves in the temporal and frontal lobes. CT showed dilatation of both lateral ventricles predominant in the anterior horn and the third ventricle. The frontal lobes showed relatively low density. At 32 years of age, she showed spastic quadriplegia, scissoring, increased deep tendon reflexes, sardonic laugh seizures, and periodic vomiting. She could not speak or walk. Severe developmental delay was noted in all areas of motor and mental functions. Plasma ammonium levels were within normal limits after dietary treatment, however, plasma arginine levels changed into the normal range and remained at that level at 32 years of age.

4. Magnetic resonance imaging findings

Magnetic resonance imaging (MRI) at the age of 30 and 32 years showed marked cerebral atrophy with multiple cystic cavities predominantly seen in the cerebral white matter, marked dilatation of lateral and third ventricles (Figs. 1 and 2), sparing of the brain stem and cerebellum (Fig. 3). In addition, significant atrophy and increased signal on T2-weighted image were detected at the bilateral basal ganglia and thalami (Fig. 1). Bilateral hippocampal atrophy was also noted (Fig. 2). These findings are consistent to multicystic encephalomalacia (MCE), but diffuse cystic changes in the white matter are characteristic.

5. Discussion

So far several articles have been published on the neuroimaging findings of argininemia, in which cerebral and/or cerebellar atrophy [4–6], ischemic changes and edema [4], abnormal myelination [5], and no significant abnormality [6] were reported. Our case is the first argininemia case to develop MCE. MCE is the final common result of a variety of insults to the immature brain including perinatal anoxia, infection, trauma, and toxic agents. MCE is characterized by (i) multiple cystic cavities of irregular size and shape, separated by glial septations, distributed throughout the bilateral cerebral hemispheres, and (ii) relative sparing of the brainstem, cerebellum, and spinal cord. The glial septations result from well developed glial reactivity, suggesting that cysts with septation are formed either during the late gestational period or after birth [7].

In argininemia, there are phenotypic variations and differences in clinical responses to dietary treatment between patients. Some patients show a response to controlled diets with a marked improvement in neurological symptoms, whereas our case showed very severe clinical symptoms which were resistant to treatment. Researches which addressed molecular biology and pathology of this disease suggest that differences in clinical responses to the dietary treatment are based on molecular heterogeneity in mutant arginase alleles. In our patient, the degree of metabolic defect induced by these mutations was considered to be "severe", as was that associated with the two deletions (77delA and 262-5delAAGA) [8].

The pathogenesis of the neurological disorder in argininemia is not clear [1,4]. There is "glutamine hypothesis". In intermediate hyperammonemic cases the serum

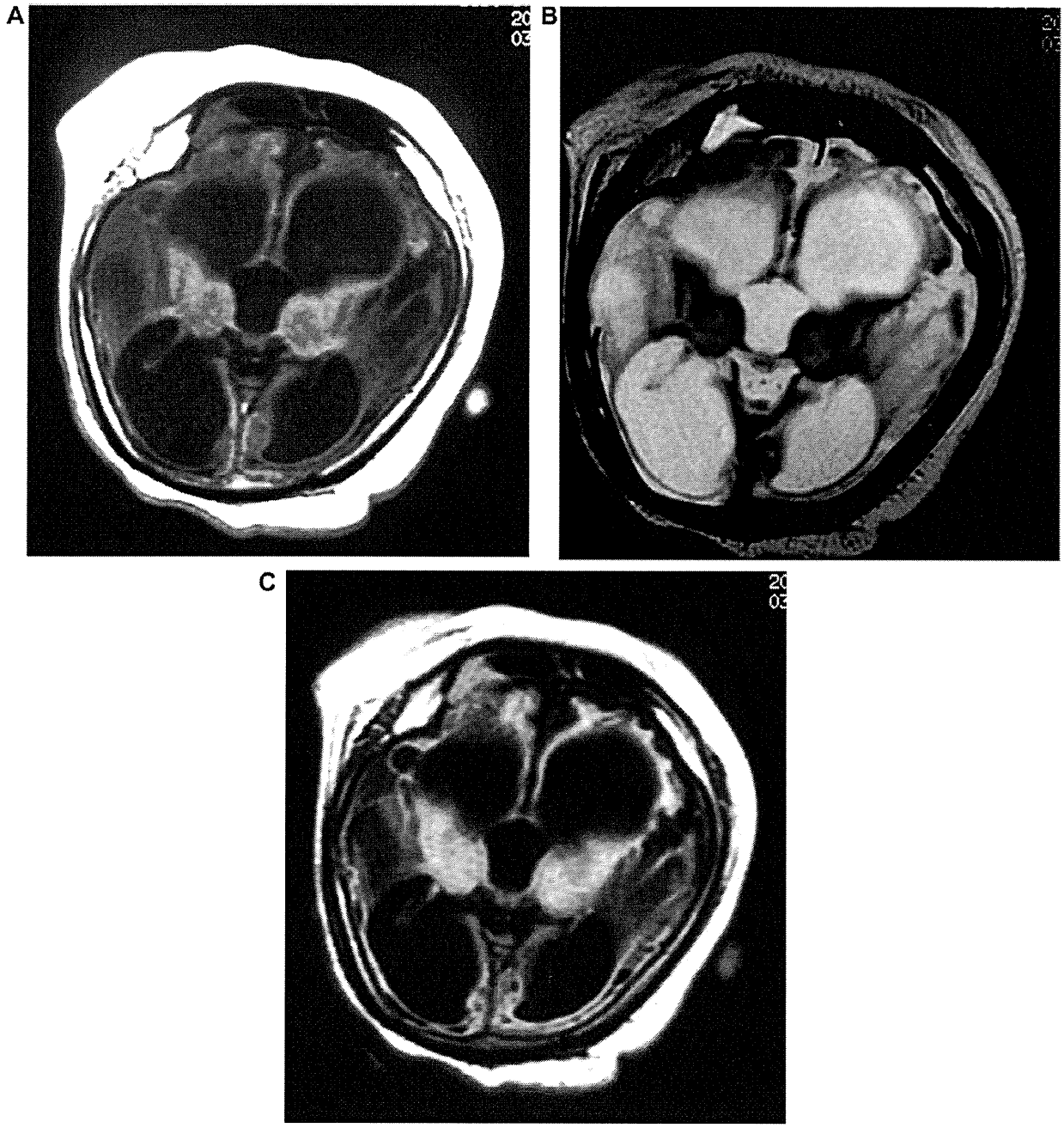

Fig. 1. MRI at 32 years of age. T1-weighted (A) and T2-weighted (B) and fluid attenuated inversion recovery (FLAIR) (C) axial images show marked dilatation of bilateral ventricles and marked cerebral atrophy with cystic formation predominant in the white matter. Significant atrophy and T2 hyperintensity are noted on the basal ganglia and thalami.

and CSF glutamine levels are increased. To eliminate cerebral ammonia, glutamine enters to the astrocyte and may alter the affect an osmotic regulation. However, our patient showed normal CSF and serum glutamine levels, which is inconsistent with the "glutamine hypothesis". In our case of argininemia the concentrations of plasma ammonium were within normal levels after protein diet, however neurological manifestations were progressive. This means that hyperammonemia is not the sole factor related to neurological damage in this patient. Elevated concentrations of plasma arginine or its five metabolites, which are argininic acid, guanidinoacetic acid, β-guanidinopropionic acid, β-guanidinobutyric acid, and *N*-α-acetylarginine seem to be directly linked to the neurological symptoms seen in these patients [9]. These guanidine compounds may lead to

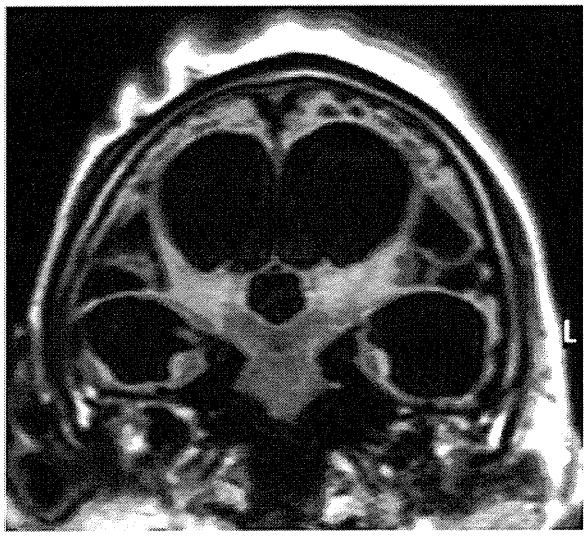

Fig. 2. MRI at 30 years of age. Coronal FLAIR image shows cortical thinning and ulegyria.

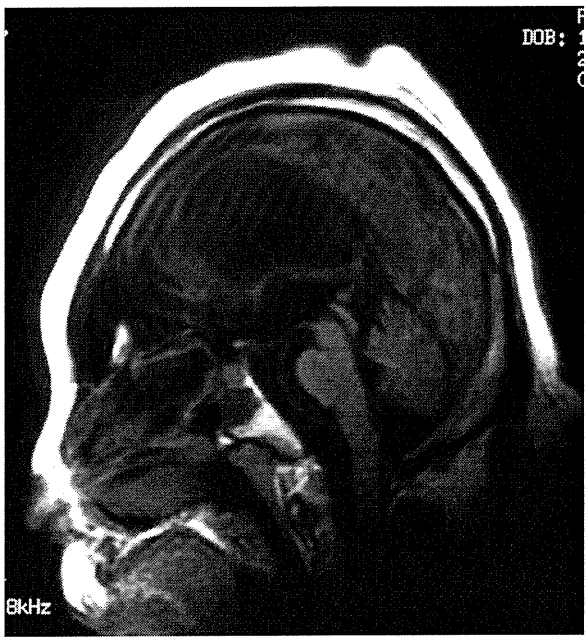

Fig. 3. MRI at 32 years of age. Sagittal T1-weighted image shows, the brain stem and cerebellum are spared.

increased neurotoxicity, inhibit the activity of transketolase and may result in demyelination. The accumulation of guanidine compounds may affect GABAergic neurotransmission, resulting in epileptogenic properties. These compounds may induce seizures by decreasing the fluidity of the plasma membrane.

On the other hand arginine may act as a precursor to glutamate or GABA and may cause damage on an excitotoxic basis. Argininemia is also a precursor of nitric oxide, and elevated levels of arginine may lead to increased synthesis of nitric oxide. The pathophysiology of arginase deficiency may be related to cerebrovascular disturbances, via occasional hyperammonemia, hyperargininemia, and potentially high plasma and tissue levels of monosubstituted guanidine compounds. Thus, the overproduction of nitric oxide may play an important role in the pathogenesis of leukoencephalopathy [10].

In summary, the MRI of our case showed severe multicystic encephalomalacia predominant in the cerebral white matter. These lesions were the most severe of the reported patients with arginase deficiency.

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Identification of CD93 expression on hematopoietic stem cells in human neonatal umbilical cord blood cells

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Abstract

Human CD93 is a heavily *O*-glycosylated type I transmembrane protein consisting of unique C-type lectin domains (CTLDs) containing glycoprotein. CD93 is mainly expressed on myeloid cells (monocytes and granulocytes) and endothelial cells. However, the expression patterns of CD93 on various other kinds of cells are not well understood. In this study, we found that CD93 was recognized by a CD93 monoclonal antibody (mAb) (mNI-11) that was established in our laboratories and was expressed on a broad hematopoietic stem cell population ($CD34^+$ cells) from human neonatal umbilical cord blood cells (UCBCs), as shown using a two-color flow cytometric analysis. In addition, the CD93 recognized by mNI-11 was also expressed on a narrow hematopoietic stem cell population ($CD34^+CD45^{dim+}$ cells) in which the non-specific reactivity of CD34 mAb from human neonatal UCBCs was excluded using a three-color flow cytometric analysis. Taken together, these results provide the first evidence concerning the identification of CD93 expression on hematopoietic stem cells. These cell populations ($CD34^+CD93^+$ and $CD34^+CD45^{dim+}CD93^+$ cells) in human neonatal UCBCs are thought to have an important role in cell biology, transplantation, and immature/mature immune responses.

Key words : CD93, flow cytometric analysis, hematopoietic stem cells, umbilical cord blood cells (UCBCs).
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Introduction

Human CD93 has a molecular weight of about 100 - kDa. CD93 belongs to the Group XIV family of transmembrane glycoproteins. This molecule is a heavily *O*-glycosylated type I transmembrane protein consisting of C-type lectin-like domains (CTLDs) containing glycoprotein, followed by a series of epidermal growth factor (EGF)-like repeats.[1] Although CD93 was previously identified as a receptor for complement component 1, subcomponent q phagocytosis (C1qRp) that interacts with defense collagens such as C1q, mannose-binding lectin (MBL), and pulmonary surfactant protein A (PS-A) and is involved in the C1q-mediated enhancement of phagocytosis for various antigens,[2, 3] several recent studies have reported that CD93 is not a C1q receptor involved in the C1q-mediated enhancement of phagocytosis against various antigens and the clearance of apoptotic cells.[4, 5] However, the detailed immunological functions of CD93 remain uncertain.

On the other hand, CD93 is mainly expressed on myeloid cells (monocytes and granulocytes) and endothelial cells.[5] CD93 is strongly upregulated after the exposure of human monocyte-like cell line (U937) to a

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protein kinase C (PKC) activator, phorbol myristate acetate (PMA), and this upregulation is controlled by a PKC delta isoenzyme inhibitor, Rottlelin.[6] However, the pattern and regulation of CD93 expression on various kinds of cells other than those with a myeloid cell lineage, especially hematopoietic stem cells possessing multipotent activities in immune responses, are not well understood.

In this study, we focused on CD93 expression in broad and narrow human hematopoietic stem cell populations ($CD34^+$ and $CD34^+CD45^{dim+}$ cells) and investigated the expression patterns of CD93 on these cell populations in human neonatal umbilical cord blood cells (UCBCs) using a CD93 monoclonal antibody (mAb) (mNI-11) established in our laboratories and flow cytometry to define the expression pattern on immature multipotent hematopoietic stem cells.

Materials and Methods

Donors and preparation of peripheral blood mononuclear cells from normal adults and umbilical cord blood cells from neonates

We prepared human peripheral blood mononuclear cells (PBMCs) from four normal adults and human neonatal umbilical cord blood cells (UCBCs) from the umbilical veins of 17 neonates according to a previously described methodology.[7] Informed consent was obtained from all the donors/parents.

Antibodies

A phycoerythrin (PE)-conjugated CD93 monoclonal antibody (mAb) (mNI-11) (mouse IgG1) [8] was established in our laboratories. Fluorescein isothiocyanate (FITC)-conjugated CD34 mAb (My10) (mouse IgG1), allophycocyanin (APC)-conjugated CD34 mAb (My10) (mouse IgG1), and peridinin chlorophyll protein (PerCP)-conjugated CD45 mAb (2D1) (mouse IgG1) were purchased from BD Biosciences Co. (USA). Isotype-matched normal mouse IgG1 was purchased from MBL Co. (Nagoya, Japan).

Flow cytometry

The cells were washed in cold phosphate-buffered saline (PBS) containing 0.1% NaN_3 (subsequently referred to as the washing buffer) and were then incubated in PBS containing 25% normal goat serum, 1 mg/mL of normal human IgG, and 0.1% NaN_3 for 10 min on ice to block the Fc receptor of IgG. The cells were then incubated with an optimal concentration of PE-conjugated CD93 (mAb) (mNI-11) and FITC-conjugated CD34 mAb (My10) for 40 min at room temperature. In some experiments, the cells were then incubated with an optimal concentration of APC-conjugated CD34 mAb (My10), PerCP-conjugated CD45 mAb (2D1), and PE-conjugated CD93 (mAb) (mNI-11) for 40 min at room temperature. Negative controls were provided by incubation with isotype-matched normal mouse IgG1 under the same conditions. Following a final wash with the washing buffer and resuspension in PBS containing 2% fetal calf serum (FCS) and 0.1% NaN_3, the cellular debris and aggregates were excluded. The percentages of positively stained cells were analyzed using two-color and three-color flow cytometry using a FACScan (Becton Dickinson Co.). The analysis was repeated in triplicate per sample.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD). The statistical analysis was performed using the Student *t*-test. Differences with a P value of less than 0.05 were considered significant.

Results and Discussion

First of all, we investigated the percentage of broad hematopoietic stem cells ($CD34^+$ cells) among normal adult PBMCs and neonatal UCBCs using an FITC-conjugated CD34 mAb (My10) and flow cytometry with side scattering; the gated $CD34^+$ cells were counted. The results are summarized in Fig. 1. As shown in Fig. 1, the percentages of gated $CD34^+$ cells among normal adult PBMCs and neonatal UCBCs were 0.026 ± 0.015% and 0.365 ± 0.178%, respectively. The percentage of $CD34^+$ cells among the neonatal UCBCs was significantly ($P<0.01$) larger than that among the normal adult PBMCs. Figure 2 shows typical histograms for the $CD34^+$ cells among normal adult PBMCs (panel A) and neonatal UCBCs (panel B). The results indicated that $CD34^+$ cells do not exist among normal adult PBMCs. Thus, only neonatal UCBCs were used for all subsequent

experiments in this study.

Next, we investigated the expression of CD93 on the gated $CD34^+$ cells from neonatal UCBCs using a PE-conjugated CD93 mAb (mNI-11), an FITC-conjugated CD34 mAb (My10), and two-color flow cytometry. The results are summarized in Table 1. As shown in Table 1, the percentage of CD93 expression on the $CD34^+$ cells ($CD34^+CD93^+$ cells) was 89.1%, while the percentage of $CD34^+CD93^-$ cells was 10.9%. Figure 3 shows typical histograms for the CD93 expression on $CD34^+$ cells.

Recently, a narrow hematopoietic stem cell population, in which the non-specific reactivity of CD34 mAb was excluded, was identified as $CD34^+CD45^{dim+}$ cells.[9,10] We also investigated the percentage of $CD34^+CD45^{dim+}$ cells among neonatal UCBCs using an APC-conjugated CD34 mAb (My10), a PerCP-conjugated CD45 mAb (2D1), and two-color flow cytometry; the gated $CD34^+CD45^{dim+}$ cells were counted. The results are summarized in Table 2. As shown in Table 2, the percentage of gated $CD34^+CD45^{dim+}$ cells among the neonatal UCBCs was 0.24 ± 0.069%. Next, we investigated the expression of CD93 on gated $CD34^+CD45^{dim+}$ cells using a PE-conjugated CD93 mAb (mNI-11) and three-color flow cytometry. The results are summarized in Table 2. As shown in Table 2, the percentage of $CD34^+CD45^{dim+}CD93^+$ cells was 74.9%, while the percentage of $CD34^+CD45^{dim+}CD93^-$ cells was 25.1%. Figure 4 shows typical histograms for CD93 expression on $CD34^+CD45^{dim+}$ cells.

Human CD93 has a molecular weight of about 90 – 100 kDa and is a heavily *O*-glycosylated type I transmembrane protein consisting of unique C-type lectin domains[11] that exhibits a strong homology (67 – 87% identity) with rat and mouse CD93 (C1qRp), also known as the AA4.1 antigen.[11,12] The selective expression of CD93 myeloid cell lineages (granulocytes and monocytes) and endothelial cells has been reported.[5] In addition, the regulation of CD93 expression has been investigated in a variety cells, particularly granulocytes, and the inflammatory peptide FMLP has been shown to upregulate the expression of this molecule rapidly.[13] Furthermore, CD93 is strongly upregulated after the exposure of a human monocyte-like cell line (U937) to a PKC activator, PMA, and this upregulation is controlled by a PKC delta isoenzyme.[6]

On the other hand, mouse CD93, known as the AA4.1 antigen, was found to be expressed in primitive hematopoietic stem cells ($CD34^+$ cells),[14] suggesting that this molecule may be involved in some important biological functions of immune-related cells. These findings led to the speculation regarding the possibility that human CD93 may also be expressed on hematopoietic stem cells similar to mouse CD93 (AA4.1 antigen). From this point of view, we investigated the expression of CD93 on human hematopoietic stem cells in neonatal UCBCs. As shown in this study, CD93 defined by CD93 mAb (mNI-11) was clearly expressed in both broad and narrow hematopoietic stem cell populations ($CD34^+$ and $CD34^+CD45^{dim+}$cells, respectively) from neonatal UCBCs. Together, these findings suggest that human CD93 on hematopoietic stem cells may be associated with immune biological activities, such as cell proliferation and differentiation/maturation.

Human immature multipotent hematopoietic progenitor cells (hematopoietic stem cells) ($CD34^+$ and $CD34^+CD45^{dim+}$ cells) reportedly have a much higher frequency among the UCBCs of neonates than among normal adult PBMCs.[15] In this regard, human neonatal UCBCs have been used as a source of hematopoietic stem cells for transplantation to enable hematopoietic reconstitution and reduce the occurrence of acute graft-versus-host-disease (GvHD) in recipients after myeloablative therapy during allogenic transplantation.[16,17] Thus, the identification of these new hematopoietic stem cell populations, $CD34^+CD93^+$ and $CD34^+CD45^{dim+}CD93^+$cells, among neonatal UCBCs in this study may provide important information with potential application to the fields of basic and clinical immunology. Recently, we found that CD93 is also expressed on human naive T-lymphocytes ($CD4^+CD45RA^+$ cells) among neonatal UCBCs using a CD93 mAb (mNI-11) probe and flow cytometry and western blot analyses.[18] These results indicate that multiple forms of CD93 exist on various cells, particularly on immature immune-related cells, and suggest that CD93 may be closely associated with the development and regulation of immature cells in neonatal immune responses, such as cell proliferation, differentiation, maturation.

In conclusion, we have found that CD93 is expressed on broad and narrow hematopoietic stem cell

populations ($CD34^+$ and $CD34^+CD45^{dim+}$) and that these cell populations ($CD34^+CD93^+$ and $CD34^+CD45^{dim+}CD93^+$cells) exist as novel hematopoietic stem cell populations in neonatal UCBCs. Further analyses are needed to demonstrate the detailed properties of these cell populations at the cellular and molecular (messenger RNA expression of CD93) levels in both basic and clinical fields.

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Table1. Percentages of CD93 expression on $CD34^+$ cells in neonatal UCBCs

Donor	$CD34^+$ (%)	$CD34^+CD93^+$ (%)	$CD34^+CD93^-$ (%)
1	0.38	87.3	12.7
2	0.30	60.8	39.2
3	0.23	91.4	8.6
4	0.29	91.8	8.2
5	0.30	92.0	8.0
6	0.34	94.2	5.8
7	0.83	88.3	11.7
8	0.25	96.8	3.2
9	0.40	95.9	4.1
10	0.51	91.5	8.5
11	0.18	89.0	11.0

The neonatal UCBCs (n=11) were incubated with an FITC-conjugated CD34 mAb (My10) and a PE-conjugated CD93 mAb (mNI-11) for 40 min at room temperature. Negative controls were incubated with isotype-matched normal mouse IgG1. The percentages of positively stained cells were determined using two-color flow cytometry. The analysis was repeated in triplicate per sample

Table 2. Percentages of CD93 expression on $CD34^+CD45^{dim+}$ cells in neonatal UCBCs

Donor	$CD34^+CD45^{dim+}$ (%)	$CD34^+CD45^{dim+}CD93^+$ (%)	$CD34^+CD45^{dim+}CD93^-$ (%)
1	0.23	84.9	15.1
2	0.12	59.9	40.1
3	0.33	82.9	17.1
4	0.27	54.7	45.3
5	0.26	86.9	13.1
6	0.23	80.6	19.4

The neonatal UCBCs (n=6) were incubated with an APC-conjugated CD34 mAb (My10), a PerCP-conjugated CD45 mAb (2D1), and a PE-conjugated CD93 mAb (mNI-11) for 40 min at room temperature. Negative controls were incubated with isotype-matched normal mouse IgG1. The percentages of positively stained cells were determined using three-color flow cytometry. The analysis was repeated in triplicate per sample.