allowing us to speculate that the *i*NKT cell defects may account for the autoimmune susceptible nature. On the contrary, transgenic overexpression of the invariant TCR of *i*NKT cells was found to protect NOD strain of mice from development of EAE. This EAE protection was associated with an inhibition of antigen-specific IFN-γ production but was independent of IL-4 (Mars et al. 2002). These results indicate an inverse correlation of *i*NKT cell numbers/functions with the susceptibility to EAE, raising a simple idea that expanding *i*NKT cells may be beneficial for treating patients with MS.

After α-GC was identified as a potent ligand for *i*NKT cells, several laboratories have examined whether in vivo injection of α-GC may modify the clinical course of EAE by stimulating *i*NKT cells. A study by Singh et al. showed that α-GC is capable of down-modulating EAE, by inducing Th2 bias of *i*NKT cells (Singh et al. 2001). Furlan et al. also showed an efficacy of α-GC in EAE, but they did not reveal a Th2 bias but rather showed an enhanced IFNγ production by the liver *i*NKT cells (Furlan et al. 2003). In an independent study by Jahng et al., injection of α-GC with aim to suppress EAE resulted in diverse outcome, which depends on the administration route, timing of injection, and dose of this glycolipid (Jahng et al. 2001). Although the reason for these discrepancies remain unclear, it is possible that source of the mice, quality of the animal facilities, or even gut flora might have influenced the results.

It was subsequently found that CD28-B7 costimulatory signals play a critical role in stimulating *i*NKT cells with α-GC. When *i*NKT cells were stimulated with α-GC in the presence of anti-B7 (CD80) antibody *in vitro*, they selectively produced Th2 cytokines (Pal et al. 2001). *In vivo* stimulation of *i*NKT cells along with blocking CD28-B7 interactions was found to suppress the onset of EAE (Pal et al. 2001). These results collectively indicated that proper stimulation of *i*NKT cells might lead to suppression of pathogenic Th1 responses. We have then explored whether a Th2 polarizing ligand could be identified among α-GC analogs. As discussed briefly in Sect. 3.1.2, we have found that an analog of α-GC, called OCH, bearing a shorter sphingosine chain could selectively induce production of IL-4 but not of IFN-γ and could modulate disease process of EAE when injected *in vivo* (Miyamoto et al. 2001). This protective effect against the development of EAE was abrogated by a simultaneous injection of anti-IL-4 antibody. Moreover, the protective effect of OCH could not be seen in IL-4 knockout mice, indicating that IL-4 produced from *i*NKT cells is involved in the disease suppression.

The molecular mechanism for the selective IL-4 production by OCH has been intensively studied in our laboratory. Owing to the truncation of sphingosine chain, OCH binds to CD1d molecule less stably compared to α-GC. We are proposing that the unstable OCH-CD1d interaction, which does not allow continuous TCR stimulation, is a key to understanding the Th2 polarizing character of OCH (Oki et al. 2004). When *i*NKT cells are stimulated by a-GC, IL-4 is produced within a few hours, which is then followed by production of a large quantity of IFN-γ (Pal et al. 2001). Of note is that de novo protein synthesis is required for the *i*NKT cell production of IFN-γ but not of IL-4 (Oki et al. 2004). Subsequent analysis has revealed that c-Rel protein is selectively induced, when *i*NKT cells are simulated by α-GC. Inhibiting c-Rel expression in *i*NKT cells has led to a selective IL-4 induction as a result of

suppressed production of IFN-γ, as seen with OCH stimulation. Taken together, it can be postulated that unstable binding of OCH with CD1d leads to disrupted TCR signaling, which does not induce expression of c-Rel and of its down-stream molecule IFN-γ. Compared with α-GC, which is capable of fully inducing c-Rel and IFN-γ, OCH would exhibit a unique Th2 polarizing effect on *i*NKT cells *in vitro* and *in vivo*. Intriguingly, in vivo injection of OCH induces defective IFN-γ production not only by NKT cells but also by NK cells (Oki et al. 2005). Mechanistic analysis has revealed that an injection of OCH induces an insufficient induction of CD40L in addition to lower primary IFN-γ production by the NKT cells, leading to a marginal IL-12 production by DCs. A combination of these differences between OCH and α-GC stimulation would account for the lower secondary IFN-γ production by NKT and NK cells by OCH. Of note, McCarthy et al. have recently confirmed that shortening of the phytosphingosine chain increased the rate of lipid dissociation from CD1d molecule and induced less sustained TCR signals (McCarthy et al. 2007). In this study, they have also demonstrated the decreased affinity of TCR to OCH bound-CD1d.

Other lipid chain truncated analogs of α-GC have been reported to display a similar skewing of cytokine profile towards Th2 but the mechanism seems to differ from that found in OCH (Goff et al. 2004; Yu et al. 2005). Taken together, altered glycolipid provides attractive means for *i*NKT cells mediated intervention of inflammatory autoimmune disease such as EAE and human MS.

4 MR1- Restricted Invariant T Cells in MS

Another novel invariant NK cell receptor-positive T cell population besides *i*NKT cells has been described in mice and humans. They are preferentially located in the gut lamina propria and are generally termed mucosal-associated invariant T (MAIT) cells (Treiner et al. 2003). Of interest, they are absent in germ-free mice, which indicates the role of gut flora for generation and maintenance of this lymphocyte. The discovery of this population is dated back to 1993, when DN T cell population expressing an invariant TCR α-chain was described along with the identification of Vα24 *i*NKT cells (Porcelli et al. 1993). It is now established that the new invariant T cells are distinct from *i*NKT cells in the expression of another conserved CDR3α sequence (Vα7.2-Jα33 in humans and Vα19-Jα33 in mice) and restricted use of Vβ2 and Vβ13 in mice and humans. Unlike *i*NKT cells selected by CD1d, they are selected by another MHC class Ib molecule, MR1, that is also highly conserved among species (Treiner et al. 2003). The mouse MAIT cells were isolated from $NK1.1^+$ T cells in the liver of CD1d deficient mice lacking "conventional" *i*NKT cells, allowing us to call the cells "Vα19-Jα33 NKT cells." As seen with "conventional" NKT cells, human MAIT cells constitutively express memory phenotype and some NK cell markers other than CD57 (Treiner et al. 2005) (Fig. 1). Several lines of evidence suggest that MR1 presents lipid ligands such as α-mannocylceramide (Shimamura et al. 2007). Although the function of MAIT cells is unclear at the moment, their cardinal features such as the semiinvariant repertoire, restriction by

monomorphic class I-like molecule and the natural memory phenotype suggest that *i*NKT cells and MAIT cells may exhibit similar and/or complementary functions.

When expression of Vα7.2 invariant TCR for human MAIT cells was investigated in MS patient samples, there was a striking difference between the MAIT and *i*NKT cell invariant TCR in their expression. Expression of the invariant TCR chain for NKT cells was clearly reduced in the peripheral blood of MS patients (Illes et al. 2000), whereas invariant TCR for MAIT cells was clearly detected in the great majority of the patients (Illes et al. 2004). Parallel analysis of CNS lesions from MS patients showed that MAIT cells would infiltrate the majority of the lesions, whereas *i*NKT cells do not (Illes et al. 2000, 2004). The differential expression of the two invariant chains in samples from MS suggests that MAIT cells and NKT cells may complement each other and MAIT cells may substitute deficiency of *i*NKT cells in MS.

The protective role of MAIT cells is further delineated by the study of mouse EAE. We found that overexpression of the invariant Vα19-Jα33 TCR in B6 mice is protective against EAE induction and progression (Croxford et al. 2006). Consistently, EAE was exacerbated in MR1 deficient mice, which lack Vα19-Jα33 invariant T cells. The protective effect was found to accompany a reduced production of inflammatory mediators as well as an increased secretion of IL-10. We have also demonstrated that IL-10 production occurred in part through interactions between B cells and Vα19 MAIT cells involving ICOS costimulatory molecule.

5 Concluding Remarks

NK cells and *i*NKT cells are groups of innate lymphocytes with multi potential qualities. Recent advances in cell biology of these cells have brought our attention to their ability in regulating autoimmune inflammatory responses. Selective induction of their regulatory properties could be an effective means for modification of autoimmune disease affecting the CNS. It is also notable that NK cells and *i*NKT cells change their phenotypes, number, and gene expression profile during disease course of MS. They could be good targets also for those who attempt to identify useful biomarkers for MS.

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Journal of the Neurological Sciences 280 (2009) 22–28

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Heterogeneity and continuum of multiple sclerosis phenotypes in Japanese according to the results of the fourth nationwide survey

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ARTICLE INFO

Article history:
Received 8 September 2008
Received in revised form 6 January 2009
Accepted 9 January 2009
Available online 7 February 2009

Keywords:
Multiple sclerosis
Japanese
Optic-spinal form
Epidemiology
Magnetic resonance imaging
Conventional form
Neuromyelitis optica

ABSTRACT

There are two distinct phenotypes of multiple sclerosis (MS) in Asians, optic-spinal MS (OSMS) and conventional MS (CMS). In 2004, we performed the fourth nationwide epidemiological survey of MS. The epidemiological features were reported elsewhere; here we report the characteristic features of patients with each MS phenotype, classified according to the clinically estimated sites of involvement and MRI findings. Among 1493 MS patients collated, 57.7% were classified as having CMS and 16.5% were classified as having OSMS. Based on MRI findings, MS patients were further subdivided into those with OSMS with or without longitudinally extensive spinal cord lesions (LESCLs) and those with CMS with or without LESCLs. Although disease duration did not differ significantly among the four groups, EDSS scores were significantly higher in patients with LESCLs than in those without LESCLs, irrespective of OSMS or CMS phenotype. Similar trends were found for the frequencies of bilateral visual loss, transverse myelitis, and marked CSF pleocytosis and neutrophilia. Increased IgG index, brain lesions fulfilling the Barkhof criteria and secondary progression were more commonly found in CMS patients than in OSMS patients, while negative brain MRIs were more commonly encountered in OSMS patients than CMS patients, irrespective of the presence of LESCLs. These findings suggest that demographic features not only vary based on CMS or OSMS phenotype, but also with the presence or absence of LESCLs, and that nonetheless, these four phenotypes constitute a continuum.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). MS is rare in Asians, but when it appears, involvement of the optic nerve and spinal cord is destructive [1]. There are two distinct subtypes of MS in Asians: the optic-spinal form (OSMS), which shows selective involvement of the optic nerve and spinal cord, and the conventional form (CMS), which affects multiple sites of the central nervous system (CNS), including the cerebrum and cerebellum [2]. The two subtypes have distinct clinical and neuroimaging features. OSMS is characterized by a higher age at onset, greater female preponderance and higher Kurtzke's Expanded Disability Status Scale (EDSS) score [3] compared with CMS [1,2]. Longitudinally extensive spinal cord lesions (LESCLs) extending over three or more vertebral segments are more commonly found in patients with OSMS than CMS patients [1]. However, reflecting the pronounced spinal cord damage seen in Asians, one-fourth of CMS patients also have such LESCLs [4,5].

In Japan, nationwide surveys of MS were conducted in 1972, 1982, 1989 and 2004 using essentially identical criteria [6–8]. In the fourth survey, we disclosed a four-fold increase in the estimated number of clinically definite MS patients (9900; crude MS prevalence, 7.7/100,000) in 2003 compared with 1972, and a shift in the peak age at onset from early 30 s in 1989 to early 20 s in 2003 [8], suggesting an

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doi:10.1016/j.jns.2009.01.008

Table 1
Clinical characteristics among each multiple sclerosis subgroups.

	OSMS		CMS	
	LESCL (+) ($n=93$)	LESCL (−) ($n=117$)	LESCL (+) ($n=121$)	LESCL (−) ($n=570$)
Sex ratio (male:female)	1:5.2[*k]	1:4.1	1:5.1[*h]	1:2.3[*h, *k]
Age at onset (years)	38.8 ± 12.8[*c, *e, *g]	33.2 ± 12.0[*d, *g]	31.1 ± 14.9[*e]	29.3 ± 11.9[*d, *e]
Age at examination (years)	49.8 ± 13.9[*c, *e, *g]	43.9 ± 13.2[*d, *g]	41.4 ± 15.6[*e]	39.7 ± 12.8[*d, *e]
Disease duration (years)	11.1 ± 8.0	10.6 ± 8.7	10.4 ± 8.8	10.4 ± 8.3
EDSS scores	5.4 ± 2.5[*a, *e]	3.2 ± 2.5[*a, *f]	4.9 ± 2.9[*b, *f]	3.2 ± 2.5[*b, *e]
Symptoms during entire course				
Bilateral visual loss	57/93 (61.3%)[*e]	51/117 (43.6%)[*d]	54/121 (44.6%)[*b]	142/563 (25.2%)[*b, *d, *e]
Transverse myelitis	58/91 (63.7%)[*a, *e]	39/113 (34.5%)[*a, *d]	57/116 (49.1%)[*b]	91/552 (16.5%)[*b, *d, *e]
Paraparesis	64/91 (70.3%)[*a, *e]	51/113 (45.1%)[*a]	67/116 (57.8%)[*b]	203/558 (36.4%)[*b, *e]
Quadriparesis	21/93 (22.6%)	18/112 (16.1%)[*f]	37/118 (31.4%)[*b, *f]	89/561 (15.9%)[*b]
Sensory impairment below a certain level	66/89 (74.2%)[*a, *e]	53/108 (49.1%)[*a, *d]	67/110 (60.9%)[*b]	130/528 (24.6%)[*b, *d, *e]
Sphincter disturbance	71/93 (76.3%)[*a, *e]	61/114 (53.5%)[*a, *f]	86/120 (71.7%)[*b, *f]	251/563 (44.6%)[*b, *e]
Severe motor disability at the time of last examination[#]	30/89 (33.7%)[*g, *e]	18/110 (16.4%)[*g, *f]	43/116 (37.1%)[*b, *f]	70/534 (13.1%)[*b, *e]
Secondary progression	7/93 (7.5%)	6/117(5.1%)[*f, *j]	22/121 (18.2%)[*j]	88/569 (15.5%)[*j]
Cerebrospinal fluid findings				
Marked pleocytosis (≥ 50 WBC/mm^3) or neutrophilia (≥ 5 neutrophils/mm^3)	16/79 (20.3%)[*a, *e]	3/96 (3.1%)[*a, *f]	17/102 (16.7%)[*b, *f]	21/511 (4.1%)[*b, *e]
Increased IgG index	12/45 (26.7%)[*e]	16/51 (31.4%)[*d]	29/59 (49.2%)	186/298 (62.4%)[*d, *e]
Brain MRI findings				
≥ 1 Gd-enhanced lesion or ≥ 9 T2 brain lesions	16/87 (18.4%)[*c, *e]	22/110 (20.0%)[*d, *f]	72/112 (64.3%)[*c, *f]	358/548 (65.3%)[*d, *e]
≥ 9 T2 brain lesions	13/87 (14.9%)[*c, *e]	21/110 (19.1%)[*d, *f]	49/112 (43.8%)[*c, *f]	281/547 (51.4%)[*d, *e]
≥ 1 Gd-enhanced lesion	5/79 (6.3%)[*c, *e]	5/99 (5.1%)[*d, *f]	43/100 (43.0%)[*c, *f]	210/481 (43.7%)[*d, *e]
≥ 1 Juxtacortical lesion	5/85 (5.9%)[*c, *e]	21/110 (19.1%)[*d, *f]	46/109 (42.2%)[*c, *f]	209/536 (39.0%)[*d, *e]
≥ 3 Periventricular lesions	21/86 (24.4%)[*c, *e]	34/111 (30.6%)[*d, *f]	69/114 (60.5%)[*c, *f]	365/546 (66.9%)[*d, *e]
≥ 1 Infratentorial lesion	11/87 (12.6%)[*c, *e]	26/107 (24.3%)[*d, *f]	69/116 (59.5%)[*c, *f]	372/559 (66.5%)[*d, *e]
Lesions fulfilling the Barkhof criteria	7/89 (7.9%)[*c, *e]	10/109 (9.2%)[*d, *f]	47/120 (39.2%)[*c, *f]	280/566 (49.5%)[*d, *e]
No cranial lesion	49/89 (55.1%)[*a, *c, *e]	39/109 (35.8%)[*a, *d, *f]	3/120 (2.5%)[*c, *f]	10/566 (1.8%)[*d, *e]
Spinal cord MRI findings				
≥ 1 T2 lesion	93/93 (100%)[*a, *e]	97/117 (82.9%)[*a, *d, *f]	121/121 (100%)[*b, *f]	354/570 (62.1%)[*b, *d, *e]
LESCL	93/93 (100%)[*a, *e]	0/117 (0%)[*a, *f]	121/121 (100%)[*b, *f]	0/570 (0%)[*b, *e]
Gd-enhanced lesion	59/75 (78.7%)[*a, *e]	39/99 (39.4%)[*a, *d, *f]	72/107 (67.3%)[*b, *f]	110/532 (20.7%)[*b, *d, *e]

#: Chair-bound or worse. CMS = conventional form of multiple sclerosis; EDSS = expanded disability status scale of Kurtzke; Gd = gadolinium; LESCLs = longitudinally extensive spinal cord lesions extending 3 or more vertebral segments; N.S. = not significant; OSMS = optic-spinal form of multiple sclerosis.
*a: $P<0.01$ (OSMS with LESCLs vs. OSMS without LESCLs), *b: $P<0.01$ (CMS with LESCLs vs. CMS without LESCLs), *c: $P<0.01$ (OSMS with LESCLs vs. CMS with LESCLs), *d: $P<0.01$ (OSMS without LESCLs vs. CMS without LESCLs), *e: $P<0.01$ (OSMS with LESCLs vs. CMS without LESCLs), *f: $P<0.01$ (OSMS without LESCLs vs. CMS with LESCLs), *g: $0.01 \leq P<0.05$ (OSMS with LESCLs vs. OSMS without LESCLs), *h: $0.01 \leq P<0.05$ (CMS with LESCLs vs. CMS without LESCLs), *i: $0.01 \leq P<0.05$ (OSMS with LESCLs vs. CMS with LESCLs), *j: $0.01 \leq P<0.05$ (OSMS without LESCLs vs. CMS without LESCLs), *k: $0.01 \leq P<0.05$ (OSMS with LESCLs vs. CMS without LESCLs), *l: $0.01 \leq P<0.05$ (OSMS without LESCLs vs. CMS with LESCLs).

increase in susceptibility to this disease among the younger generation. In this study, a successive decrease in optic-spinal involvement in clinically definite MS patients was also revealed, while the absolute numbers of CMS patients and those with MS-like brain lesions fulfilling the Barkhof criteria were found to increase rapidly with advancing year of birth. Also, the frequency of LESCLs was found to be significantly higher in OSMS patients than in CMS patients in this nationwide survey.

We recently reported that there are distinct subtypes of MS according to clinical and MRI findings using our institutional series of MS patients [9,10]. Therefore, in the present study, we aimed to clarify the characteristic features of each MS phenotype classified according to the clinically estimated sites of involvement and MRI findings unique to Asian MS patients, such as the presence or absence of LESCLs, using collated MS cases from the fourth nationwide survey of MS in Japan.

2. Methods

2.1. Survey procedures

The fourth nationwide survey of MS was conducted by the Research Committees of Neuroimmunological Diseases and of Epidemiology of Intractable Diseases, sponsored by the Ministry of Health, Labor and Welfare, Japan. The study was approved by the Kyushu University Ethics Committee. The survey was undertaken in two steps: first, a preliminary survey was undertaken to ascertain the approximate number of MS patients in Japan, and second, a survey was conducted using a questionnaire sheet for each patient. The hospitals included in the study were randomly selected from the directory of all of the registered hospitals throughout Japan. Selection was made according to a stratification based on the number of beds in each hospital; the more beds a hospital had, the higher was its probability of being selected [11]. Sampling rates were approximately 8%, 13%, 24%, 43%, 83% and 100% for the strata of general hospitals with 20 to 99 beds, 100 to 199 beds, 200 to 299 beds, 300 to 399 beds, 400 to 499 beds and 500+ beds, respectively. All university hospitals, including those in which council members of the Japanese Society of Neurology and members of the Committees of Medical Facilities for Children and the Japanese Society of Child Neurology were working, were also surveyed.

The questionnaire for the preliminary survey on MS patients who visited hospitals because of the disease in 1 year from 1 January to 31 December 2003 was mailed to 6708 departments (including 1933 neurology/internal medicine, 1227 orthopedics, 997 psychiatry, 945 pediatrics, 831 ophthalmology, 759 neurosurgery and 16 rehabilitation departments), together with the diagnostic criteria, in January 2004. In Japan, all patients with MS are requested to visit hospitals at

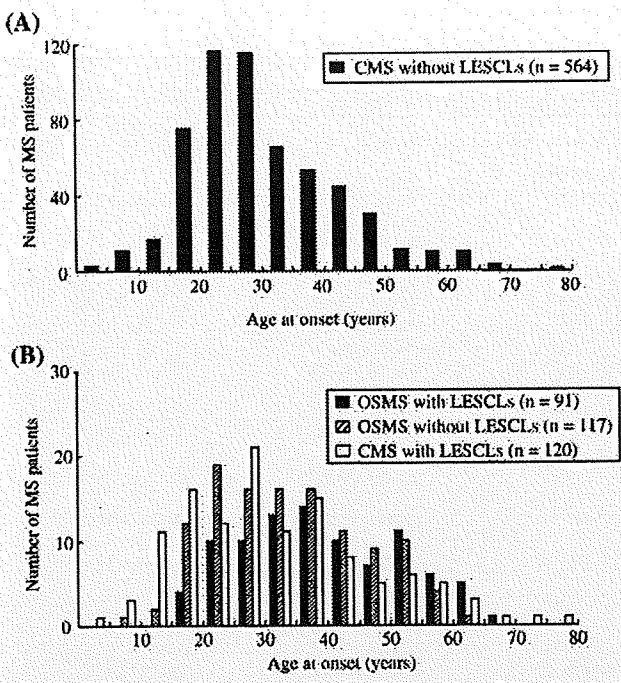

Fig. 1. Distribution of age at onset in patients with CMS without LESCLs (A), CMS without LESCLs, OSMS patients with LESCLs and OSMS patients without LESCLs (B). In (A) and (B), "anticipation" of age at onset is more pronounced in patients without LESCLs, irrespective of CMS or OSMS phenotype. The second peak around the early 50 s is not evident in CMS patients without LESCLs, but is still identifiable in the other three subtypes. CMS = conventional form of multiple sclerosis, LESCLs = longitudinally extensive spinal cord lesions extending three or more vertebral segments on MRI, n = number of patients on whom information was obtained, and OSMS = optic-spinal form of multiple sclerosis.

least once every year for registration of intractable diseases with the government in order to have their medical costs, which are not covered by health insurance, subsidized. Following the collection and collation of the first questionnaire, the second questionnaire was forwarded to those institutions reporting patients in the first survey. It requested detailed clinical information on individual patients, including age at onset and examination, sex, birthplace, present address, symptoms based on history and signs based on physical examination, laboratory findings, course, treatment and prognosis. Patients reported by more than one hospital or department were treated as duplicates.

2.2. Diagnostic criteria

The diagnostic criteria used for the present survey were based on those used for the first nationwide survey in 1972 [6], except that the limitation of age at onset was removed, as it was in the third survey [7]. The criteria required multiplicity in time and space and were essentially the same as Schumacher's criteria [12]. Briefly, the criteria used for relapsing remitting multiple sclerosis (MS) in the present survey consisted of three items for clinically definite MS: (1) symptoms and signs owing to multifocal lesions in the CNS (more than two lesions in the CNS); (2) remissions and exacerbations (multiplicity in time); and (3) other diseases, such as tumors, syphilis, cerebrovascular accident, cervical spondylosis, angiomas, subacute myelo-optico-neuropathy, neuro-Behçet, cerebellar degeneration, HTLV-I-associated myelopathy/tropical spastic paraparesis and collagen diseases, could be excluded. Clinically definite MS fulfilled all of the criteria, while a diagnosis of possible MS was made when all three criteria for clinically definite MS could not be fulfilled, but the signs were suggestive. The criteria concerning primary progressive MS (PPMS) were the same as McDonald's criteria [13].

2.3. Classification of clinical phenotype

Clinical classification of MS subtypes was based solely on the clinically estimated sites of the lesions. The second questionnaire asked answerers to report the clinically estimated sites of the lesions according to the symptomatology during the entire clinical course from among the following: optic nerve, cerebrum, cerebellum, brainstem and spinal cord. Moreover, the questionnaire also asked answerers to check for the presence of any of the signs and symptoms listed in the footnote to Table 1, during the entire clinical course. The survey center classified each case into the following clinical subtypes based on the clinically estimated lesion sites reported by each institution: OSMS involving the optic nerve and the spinal cord; optic-brainstem-spinal MS (OBSMS) involving the optic nerve, brainstem and spinal cord; brainstem-spinal MS (BSMS) involving the brainstem and the spinal cord; spinal MS (SMS) involving only the spinal cord, which was identical to recurrent myelitis without any known cause; and conventional MS (CMS), which involved multiple sites of the CNS, including the cerebrum and/or cerebellum. If there was no information about lesion sites, or the symptoms and signs during the entire course were incompatible with the lesion sites, the cases were placed into the unclassified category.

In the preliminary survey, 3749 institutions (55.9%) responded, and reported 4827 MS patients, including 849 patients with possible MS. In the second questionnaire, detailed data were collated for 1919 patients (39.3% of those in the preliminary survey), including 30 duplicate cases. The estimated number of clinically definite MS patients in 2003 was 9900 (95% CI: 9100–10,700) and the estimated crude prevalence was 7.7/100,000 (95% CI: 7.1–8.4) [8]. Based on the clinically estimated sites of lesions, 1493 patients with clinically definite MS and completed questionnaires were classified as having CMS (57.7%), OBSMS (5.8%), BSMS (4.6%), OSMS (16.5%), SMS (10.6%) or unclassified MS (4.9%). In the present study, both CMS and OSMS patients were subjected to further analyses.

2.4. MRI finding-based classification

We recently published a purely MRI findings-based classification in our institutional MS series to minimize the ambiguity of clinical finding-based classification [10]; therefore, we applied such an MRI finding-based classification to the present Japanese nationwide survey series. In the present study, MS patients were classified according to the presence or absence of LESCLs as well as the presence or absence of brain lesions fulfilling the Barkhof criteria (brain lesions fulfilling the Barkhof criteria = Barkhof brain lesions (+)). MS patients were classified into four groups based on MRI findings, Barkhof(+)LESCL(+), Barkhof(+)LESCL(−), Barkhof(−)LESCL(+), and Barkhof(−)LESCL(−), and we compared the demographic features among these groups. To conduct MRI finding-based classification and analyses, longitudinally extensive spinal cord lesions (LESCLs) were defined as those extending over three or more vertebral segments on MRIs taken during the entire clinical course. Fulfillment of the Barkhof criteria [14] was assessed by the central office according to the MRI findings described in the answer sheets. In this analysis, not only patients with OSMS and CMS, but also those with OBSMS, BSMS and SMS, were included.

2.5. Statistical analysis

Statistical analyses of numerical variables were initially performed using the Kruskal–Wallis H test. When statistical significance was found, the Mann–Whitney U test was used to determine the statistical significance of differences between subgroups. Uncorrelated P values were corrected by multiplying them by the number of comparisons

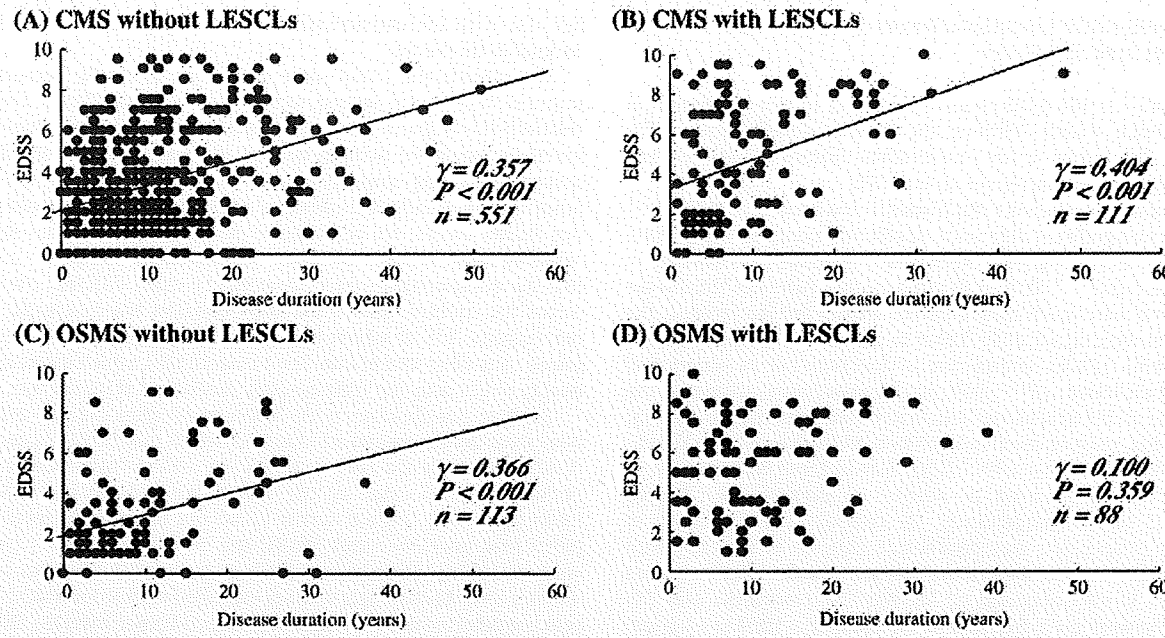

Fig. 2. Relationship between EDSS scores and disease duration in patients with each MS subtype. (A) CMS without LESCLs. (B) CMS with LESCLs. (C) OSMS without LESCLs. (D) OSMS with LESCLs. All patients except OSMS patients with LESCLs show a highly significant correlation between the two parameters. CMS = conventional form of multiple sclerosis, EDSS = Expanded Disability Status Scale of Kurtzke, LESCLs = longitudinally extensive spinal cord lesions extending three or more vertebral segments on MRI, n = number of patients on whom information was obtained, and OSMS = optic-spinal form of multiple sclerosis.

(Bonferroni–Dunn's correction) to calculate corrected P values. Differences in the ratios between two groups were tested for significance by the χ^2 test or the Fisher's exact test when the criteria for the χ^2 tests were not fulfilled.

3. Results

3.1. Demographic features of each MS subtype

Based on the MRI findings, MS patients were subdivided into those with OSMS with or without LESCLs and those with CMS with or without LESCLs (Table 1). The proportion of females was significantly greater among OSMS or CMS patients with LESCLs than among CMS patients without LESCLs ($P^{corr}<0.05$ in OSMS with LESCLs and $P^{corr}<0.05$ in CMS with LESCLs). Age at onset was significantly higher in OSMS patients with LESCLs than in other patient groups ($P^{corr}<0.05$). The peak age at onset was early 20 s among CMS or OSMS patients without LESCLs, late 20 s among CMS patients with LESCLs, and late 30 s among OSMS patients with LESCLs (Fig. 1A, B). A second peak in the early 50 s was identifiable in all groups except for CMS patients without LESCLs.

Although disease duration did not differ significantly among the four groups, EDSS scores were significantly higher in patients with LESCLs than in those without LESCLs, irrespective of OSMS or CMS phenotype ($P^{corr}<0.01$). Occurrences of bilateral visual loss, transverse myelitis, paraparesis, sensory level and sphincter disturbance were highest in OSMS patients with LESCLs among the four groups ($P^{corr}<0.01$ in all comparisons). CMS patients with LESCLs also showed significantly higher frequencies of these symptoms than CMS patients without LESCLs ($P^{corr}<0.01$ in all). Bilateral visual loss and transverse myelitis were significantly more common in OSMS patients without LESCLs than in CMS patients without LESCLs ($P^{corr}<0.01$ in both). Secondary progression was more common in CMS patients than OSMS patients, regardless of the presence or absence of LESCLs ($P^{corr}<0.05$, OSMS without LESCL vs. CMS with or without LESCL). There was a significant positive correlation between EDSS scores and disease duration in all groups ($P<0.0001$), with the exception of OSMS patients with LESCLs (Fig. 2).

3.2. Laboratory findings in each MS subtype

In the CSF, marked pleocytosis or neutrophilia was more common in patients with LESCLs than in those without LESCLs, irrespective of a diagnosis of OSMS or CMS ($P^{corr}<0.05$ in all). Increased IgG index and brain lesions fulfilling the Barkhof criteria [14] were more commonly found in CMS patients than in OSMS patients, while negative brain MRIs were more commonly encountered in OSMS patients than CMS patients, irrespective of the presence of LESCLs ($P^{corr}<0.01$ in all). Even when MS patients with a disease duration of less than 10 years were excluded, more OSMS patients showed a lack of brain lesions than CMS patients (53.8% of OSMS patients with LESCLs, 34% of OSMS patients without LESCLs, 2.1% of CMS patients with LESCLs, and 2.1% of CMS patients without LESCLs, $P^{corr}<0.01$ in all comparisons), while there were fewer OSMS patients than CMS patients with Barkhof brain lesions (5.1% of OSMS patients with LESCLs, 6.4% of OSMS patients without LESCLs, 51.1% of CMS patients with LESCLs, and 54.4% of CMS patients without LESCLs, $P^{corr}<0.01$ in all comparisons), regardless of the presence or absence of LESCLs. Gadolinium enhancement of the spinal cord lesions was significantly more common in patients with LESCLs than in those without, irrespective of clinical phenotype ($P^{corr}<0.01$ in all).

3.3. Comparison of demographic features among MS patients with contrast-enhanced spinal cord lesions

To focus on inflammatory spinal cord lesions, we compared the demographic features of MS patients with contrast-enhanced spinal cord lesions according to the clinical classification of OSMS or CMS and MRI findings of LESCL positivity. We found essentially the same tendency in this analysis as in the analysis of all the spinal cord lesions, but lost some statistical significance owing to the small sample size (Supplementary Table).

3.4. Comparison of the demographic features of MS patients according to MRI finding-based classification

We finally classified MS patients according to two hallmark MRI findings: brain lesions fulfilling the Barkhof criteria and LESCLs (Table 2). The former is the characteristic feature of Western MS, while the latter is characteristic of Asian MS.

The proportion of females was highest in the Barkhof(+)LESCL(+) group, but no significant difference was found among the four groups. The age at onset was higher in the Barkhof(−)LESCL(+) group than in any other group ($P^{corr}<0.01$ in all comparisons). Although the disease duration was not significantly different among the four groups, the EDSS scores were significantly higher in patients with LESCLs than in those without LESCLs, irrespective of the presence or absence of Barkhof brain lesions ($P^{corr}<0.01$ in all comparisons). Likewise, the frequencies of bilateral visual loss, transverse myelitis, paraparesis, quadriparesis, sensory level and sphincter disturbance were significantly higher in patients with LESCLs than in those without LESCLs, regardless of the presence or absence of Barkhof brain lesions ($P^{corr}<0.05$ in all comparisons). By contrast, the frequency of secondary progression was significantly higher in patients with Barkhof brain lesions than those without Barkhof brain lesions ($P^{corr}<0.05$ in all comparisons). Marked CSF pleocytosis and CSF neutrophilia were more frequent in the Barkhof(−)LESCL(+) group than the Barkhof (+)LESCL(−) and Barkhof(−)LESCL(−) groups ($P^{corr}<0.01$ in all comparisons), while the frequency of increased IgG index was significantly more common in the Barkhof (+)LESCL(−) group than the Barkhof(−)LESCL(+) and Barkhof(−)LESCL(−) groups ($P^{corr}<0.01$ in all comparisons).

4. Discussion

In the present study, using MS cases collated in the fourth nationwide survey in Japan, we disclose that distinct demographic features vary not only with clinical phenotype, such as OSMS and CMS, but also with the characteristic MRI findings, such as LESCLs and Barkhof brain lesions.

The present study had some limitations, primarily because the response rate in the second survey was not high. Concerning the relatively low response rate to this type of nationwide epidemiological survey in Japan, the assumption that the mean number of patients among responding hospitals is equal to that among non-responding hospitals has already been validated [15]. Therefore, we consider that our results would not be distorted seriously by the relatively low response rates. Second, the study was inevitably limited by the fact that the questionnaires were answered by many different clinicians across the country: 88% of the questionnaires were collected from neurologists, 70% of whom had previously participated in a randomized controlled trial of interferon beta-1b [16], which increases the quality of the data, but unfortunately produces a selection bias.

Subtype classification of MS based on symptomatology tends to have some ambiguity and arbitrariness, which may produce equivocal results. To minimize such a limitation, clinical classification was performed in all cases by the central office reviewing collected information. The present survey could not incorporate testing for either neuromyelitis optica (NMO)-IgG or anti aquaporin-4 (AQP4) antibody [17,18], which had not yet been discovered when this survey was initiated. As NMO-IgG, a newly identified marker for NMO [17,18], was also detected in a fraction of Japanese OSMS patients [19], OSMS is claimed to be the same disease as relapsing NMO [20]. However, recent studies from Japan have revealed that about half of OSMS patients with LESCLs are negative for anti-AQP4 antibodies [21,22], and that both NMO-IgG- and anti-AQP4 antibody-positive MS patients frequently have periventricular ovoid lesions in the brain and short spinal cord lesions in addition to LESCLs, suggesting that there is still some overlap between NMO-IgG-positive and -negative MS patients, at least among Japanese [21]. Given that these limitations exist, a nationwide survey collating a large number of Asian MS cases, including MRI findings for the first time, seems to still be relevant, especially cases of CMS, who rarely have NMO-IgG/anti-AQP4 antibody [21].

Table 2
Clinical features among each multiple sclerosis subgroups classified according to the characteristic MRI findings.

	Barkhof MRI lesion (+)		Barkhof MRI lesion (−)	
	LESCL (+)	LESCL (−)	LESCL (+)	LESCL (−)
	($n=64$)	($n=342$)	($n=213$)	($n=491$)
Sex ratio (male:female)	1:4.8	1:2.3	1:3.7	1:3.1
Age at onset (years)	29.5 ± 15.4*c	28.5 ± 11.4*d,*f	37.8 ± 13.8*b,*c, *f	32.0 ± 12.3*b,*d
Age at examination (years)	40.7 ± 15.6*c	39.1 ± 12.4*f	47.3 ± 14.2*b,*c, *f	41.2 ± 12.8*b
Disease duration (years)	11.2 ± 7.7	10.6 ± 8.2*j	9.7 ± 8.2	9.2 ± 7.7*j
EDSS scores	4.7 ± 3.0*a,*e	3.4 ± 2.5*a,*d,*f	4.9 ± 2.6*b,*f	2.7 ± 2.3*b,*d,*e
Symptoms during entire course				
Bilateral visual loss	29/64(45.3%)*a, *k	86/340 (25.3%)*a,*f	87/213 (40.8%)*b,*f	131/481 (27.2%)*b,*k
Transverse myelitis	30/63 (47.6%)*a, *e	47/335 (14.0%)*a, *d,*f	115/207 (55.6%)*b,*f	115/478 (24.1%)*b,*e, *c
Paraparesis	36/61 (59.0%)*e	146/335 (43.6%)*f	133/207 (64.3%)*b,*f	166/481 (34.5%)*b,*d,*e
Quadriparesis	23/62 (37.1%)*a, *e	60/338 (17.8%)*a	53/209 (*25.4%)*b	55/478 (11.5%)*b,*c
Sensory impairment below a certain level	36/59 (61.0%)*a, *e	86/324 (26.5%)*a, *f	127/198 (64.1%)*b,*f	158/455 (34.7%)*b, *b
Sphincter disturbance	44/64 (68.8%)*e	182/340 (53.5%)*d,*f	155/213 (72.8%)*b,*f	192/483 (39.8%)*b, *d, *e
Severe motor disability at the time of last examination#	24/58 (41.4%)*a, *e	48/326 (14.7%)*a,*f	57/203 (28.1%)*b,*f	42/455 (9.2%)*b, *c
Secondary progression	12/64 (18.8%)*k	63/341 (18.5%)*d,*l	21/213 (9.9%)*l	39/491 (7.9)*d,*k
Cerebrospinal fluid findings				
Marked pleocytosis (≥50 WBC/mm^3) or neutrophilia (≥5 neutrophils/mm^3)	5/53 (9.4%)	10/311 (3.2%)*f	35/184 (19.0%)*b,*f	20/420 (4.8%)
Increased IgG index	17/38 (44.7%)	146/220 (66.4%)*d,*f	42/109 (38.5%)*f	101/221 (45.7%)*d

#: Chair-bound or worse, EDSS = expanded disability status scale of Kurtzke; Gd = gadolimium; LESCLs = longitudinally extensive spinal cord lesions extending 3 or more vertebral segments; N.S. = not significant.

*a: $P<0.01$ (LESCLs<+>Barkhof Brain MRI lesions <+>vs. LESCLs<−>Barkhof Brain MRI lesions <+>), *b: $P<0.01$ (LESCLs<+>Barkhof Brain MRI lesions <−> vs. LESCLs<−>Barkhof Brain MRI lesions <+>),*c: $P<0.01$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<+>Barkhof Brain MRI lesions <−>), *d: $P<0.01$ (LESCLs<−>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <−>),*e: $P<0.01$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <−>),*f: $P<0.01$ (LESCLs<−>Barkhof Brain MRI lesions <+> vs. LESCLs<+>Barkhof Brain MRI lesions <−>), *g: $0.01 \le P<0.05$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <+>),*h: $0.01 \le P<0.05$ ((LESCLs<+>Barkhof Brain MRI lesions <−> vs. LESCLs<−>Barkhof Brain MRI lesions <−>), *i: $0.01 \le P<0.05$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<+> Barkhof Brain MRI lesions <−>), *j: $0.01 \le P<0.05$ (LESCLs<−>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <−>),*k: $0.01 \le P<0.05$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <−>),*l: $0.01 \le P<0.05$ (LESCLs<−>Barkhof Brain MRI lesions <+> vs. LESCLs<+>Barkhof Brain MRI lesions <−>).

Although occurrence of LESCLs was more frequent in OSMS patients than in CMS patients, LESCLs were also clearly present in a considerable fraction of Japanese CMS patients. Because not all MRI scans were performed in the relapse phase, the frequency of LESCLs in the present study could have been underestimated. There is also some ambiguity attributed to the fact that MRI films were not assessed centrally in the present study, which was a nationwide survey using questionnaire sheets and not collecting MRI films; however, importantly, the present study disclosed distinctive clinical features associated with MRI findings. LESCLs, regardless of OSMS or CMS phenotype, were related to greater female preponderance, higher EDSS scores and higher frequencies of bilateral visual loss, transverse myelitis and marked CSF pleocytosis and neutrophilia. Even when we compared the demographic features of MS patients with contrast enhancement of spinal cord lesions to focus on the inflammatory types of the lesions, we found practically the same tendency as seen for all spinal cord lesions. On the other hand, increased IgG index and secondary progression were more closely associated with the presence of brain lesions fulfilling the Barkhof criteria [14]. In addition, Barkhof brain lesions were more frequently detected in CMS patients than in OSMS patients, whereas negative brain MRIs were more commonly encountered in OSMS patients than in CMS patients, irrespective of the presence of LESCLs.

Therefore, it is reasonable to classify MS patients according to the clinically estimated sites of lesions, as previously reported, and, additionally, into four subgroups based on the presence or absence of LESCLs. Under such a classification system, OSMS patients with LESCLs represent prototypic Asian-type MS, while CMS patients without LESCLs, most of whom have Barkhof brain lesions, represent classical Western-type MS [1]; these two subgroups are supposed to exist at opposite ends of the MS spectrum. CMS patients with LESCLs shared many features with OSMS patients with LESCLs, while there were differences in age at onset, brain lesion loads, CSF IgG response and secondary progression, assigning this subtype a unique position.

Many features were also found to be common between OSMS patients without LESCLs and CMS patients without LESCLs; however, these subtypes differed in terms of age of onset, brain lesion loads, CSF IgG responses and secondary progression. Moreover, the follow-up periods of patients with intermediate phenotypes were similar to those of prototypic ones, excluding the possibility that shortness of observation periods resulted in apparently intermediate phenotypes. Although some researchers have claimed that OSMS patients without LESCLs are in fact in the early course of CMS [23], on the basis of the results of the present study and our own MS series [9,10], OSMS without LESCLs appears to be a unique subtype in Asians.

It is thus suggested that in between the two extreme ends of the MS spectrum, represented by OSMS with LESCLs and CMS without LESCLs, there exist a considerable number of patients with intermediate phenotypes, such as CMS with LESCLs and OSMS without LESCLs, showing similarities and dissimilarities to these prototypes. Solely MRI finding-based classification also yielded similar results: the Barkhof (+)LESCL(−) group represents Western-type MS and the Barkhof(−)LESCL(+) group represents Asian-type MS, while in between the two exist the Barkhof(+)LESCL(+) and Barkhof(−) LESCL(−) groups. Ikuta et al. [24] investigated a large number of Japanese and American autopsy cases with MS and found OSMS in 47% of the Japanese series, while 13% of the American cases were classified as having OSMS with frequent necrotic lesions pathologically. The results of this study suggest that even in Westerners, cases with OSMS and destructive spinal cord lesions exist with a frequency that should not be ignored.

We recently reported a decrease in peak age at onset in Japanese MS patients over the period of the four nationwide surveys [8]. The present analyses indicate that such "anticipation" of age at onset occurs in patients without LESCLs, irrespective of CMS or OSMS phenotype, but not in those with LESCLs, suggesting that changes in environmental factors associated with modernization may have differentially influenced disease susceptibility in each subtype.

In the present survey series, OSMS patients without LESCLs and CMS patients with or without LESCLs all showed a significant positive correlation between disease duration and EDSS scores, while OSMS patients with LESCLs did not. The absence of a correlation between disease duration and EDSS scores in OSMS patients with LESCLs may in part be explained by the fact that the severity of relapses determines the residual disability in anti-AQP4 antibody-positive MS/NMO patients with rare secondary progression [21], who overlap OSMS patients with LESCLs. Future nationwide surveys incorporating anti-AQP4 antibody assays and central assessment of MRI scans in Japanese will give further insight into the mechanisms underlying the phenotypic differences in MS patients.

Acknowledgments

This work was supported in part by grants from the Research Committees of Neuroimmunological Diseases and of Epidemiology of Intractable Diseases, the Ministry of Health, Labour and Welfare, Japan.

Appendix A

The chairmen of the previous nationwide survey committees were Professors Yoshigoro Kuroiwa (Department of Neurology, Kyushu University; first survey), Akihiro Igata (Third Department of Internal Medicine, Kagoshima University; second survey), and Hiroshi Nishitani (Department of Neurology, National Utano Hospital; third survey). In the fourth survey, in addition to the authors, the following were members of the Research Committee of Neuroimmunological Diseases: Drs. Susumu Chiba (Department of Neurology, School of Medicine, Sapporo Medical University), Yoshitaka Fujii (Department of Surgery II, Nagoya City University Medical School), Susumu Furukawa (Department of Pediatrics, Yamaguchi University School of Medicine), Hideo Hara (Department of Vascular Dementia Research, National Institute for Longevity Sciences, National Center of Geriatrics and Gerontology), Toshirou Hara (Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University), Kinya Hisanaga (Department of Neurology, Miyagi National Hospital), Shu-ichi Ikeda (Department of Neurology, Shinshu University School of Medicine), Shuji Izumo (Division of Molecular Pathology, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University), Ryuji Kaji (Department of Neurology, Graduate School of Medicine, Tokushima University), Takashi Kanda (Department of Neurology and Clinical Neuroscience, Yamaguchi University School of Medicine), Shosei Koh (Department of Biomedical Laboratory Sciences, School of Medicine, Shinshu University), Susumu Kusunoki (Department of Neurology, Kinki University School of Medicine), Satoshi Kuwabara (Department of Neurology, Chiba University School of Medicine), Hidenori Matsuo (Division of Clinical Research, Nagasaki Medical Center of Neurology), Hidehiro Mizusawa (Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University), Tatsufumi Nakamura (Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University), Kyoichi Nomura (Department of Neurology, Saitama Medical School), Mieko Ogino (Department of Internal Medicine III (Neurology), Kitasato University School of Medicine), Yoshiro Ohara (Department of Microbiology, Kanazawa Medical University), Mitsuhiro Osame (Department of Neurology and Geriatrics, Kagoshima University School of Medicine), Kohei Ota (Department of Health Science, Faculty of Science, Tokyo University of Science), Jun Shimizu (Department of Neurology, University of Tokyo), Akio Suzumura (Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University), Takeshi Tabira (Department of

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Appendix B. Supplementary data

Supplementary data associated with this article can befound, in the online version, at doi:10.1016/j.jns.2009.01.008.

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RESEARCH PAPER *Multiple Sclerosis* 2009; **15**: 531–541

Molecular network of the comprehensive multiple sclerosis brain-lesion proteome

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Background A recent proteomics study of multiple sclerosis (MS) lesion-specific proteome profiling clearly revealed a pivotal role of coagulation cascade proteins in chronic active demyelination. However, among thousands of proteins examined, nearly all of remaining proteins are yet to be characterized in terms of their implications in MS brain-lesion development.

Methods By the systems biology approach using four different pathway analysis tools of bioinformatics, we studied molecular networks and pathways of the proteome dataset of acute plaques, chronic active plaques (CAP), and chronic plaques (CP).

Results The database search on Kyoto Encyclopedia of Genes and Genomes (KEGG) and protein analysis through evolutionary relationships (PANTHER) indicated the relevance of extracellular matrix (ECM)–mediated focal adhesion and integrin signaling to CAP and CP proteome. KeyMolnet disclosed a central role of the complex interaction among diverse cytokine signaling pathways in brain-lesion development at all disease stages, as well as a role of integrin signaling in CAP and CP. Ingenuity pathway analysis (IPA) identified the network constructed with a wide range of ECM components, such as collagen, type I α1, type I α2, type VI α2, type VI α3, fibronectin 1, fibulin 2, laminin α1, vitronectin, and heparan sulfate proteoglycan, as one of the networks highly relevant to CAP proteome.

Conclusions Although four distinct platforms produced diverse results, they commonly suggested a role of ECM and integrin signaling in development of chronic lesions of MS. These *in silico* observations indicate that the selective blockade of the interaction between ECM and integrins in brain lesions *in situ* would be a target for therapeutic intervention in MS. *Multiple Sclerosis* 2009; **15**: 531–541. http://msj.sagepub.com

Key words: extracellular matrix; multiple sclerosis; pathway analysis; proteome; systems biology

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) presenting with relapsing-remitting and progressive clinical courses. An autoimmune process triggered by a complex interplay between genetic and environmental factors may mediate MS, although the causative agents have not yet been identified. Pathologically, MS shows remarkable heterogeneity in inflammatory demyelination, astrogliosis, and axonal degeneration [1]. Even though various drugs are lined up in clinical trials, currently, treatment options with limited efficacies, including interferon-β, glatiramer acetate, and mitoxantrone are available for ordinary clinical practice of MS [2].

The completion of the Human Genome Project in 2003 allows us to systematically characterize the comprehensive disease-associated profiles of the whole human genome [3]. The global analysis of transcriptome, proteome, protein interactome, and metabolome helps us identify disease-specific molecular signatures and biomarkers for diagnosis and prediction of prognosis, and would broaden the spectrum of molecular mechanism-based therapy for MS [4,5]. Actually, the comprehensive gene expression profiling of MS brain tissues and peripheral blood lymphocytes by DNA microarray identified a battery of genes aberrantly regulated in MS, whose role has not been previously predicted during its pathogenesis [6,7]. A recent proteomics study of MS lesion-specific proteome profiling showed

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Received 23 July 2008; accepted 12 November 2008

10.1177/1352458508101943

that overproduction of tissue factor and protein C inhibitor plays a central role in molecular events ongoing in chronic active plaques (CAP) [8]. *In vivo* administration of coagulation cascade inhibitors really reduced the clinical severity in a mouse model of experimental autoimmune encephalomyelitis (EAE), supporting the view that the blockade of the coagulation cascade would be a potential approach for the treatment of MS [8]. However, among thousands of proteins this study examined, nearly all of remaining proteins were left behind to be characterized in terms of their implications in MS brain-lesion development.

Since the global expression analysis of transcriptome and proteome usually produces high-throughput experimental data at a time, it is often difficult to find out the meaningful biological implications of the dataset. Recent advances in systems biology enable us to illustrate the cell-wide map of the complex molecular interactions by using the literature-based knowledgebase of molecular pathways [9,10]. In the scale-free molecular network, targeted disruption of limited numbers of critical components, on which the biologically important molecular connections concentrate, could disturb the whole cellular function by destabilizing the network [11]. From this point of view, the integration of comprehensive transcriptome and proteome data of disease-affected tissues with underlying molecular networks could provide the rational approach not only to characterize disease-relevant pathways but also to achieve the network-based choice of effective drug targets. By using four different pathway analysis tools of bioinformatics, this study was designed to characterize molecular networks and pathways of MS lesion-specific proteome data of Han, *et al.* [8]. Although the analysis by distinct platforms did not lead to fully identical results, they commonly suggested a role of extracellular matrix (ECM) and integrin signaling in chronic lesions of MS. These *in silico* observations indicate that ECM and integrins would be a target candidate for designing therapeutic intervention in MS.

Databases and methods

The dataset of the comprehensive MS brain-lesion proteome

In the original dataset of Han, *et al.* [8], fresh-frozen brain autopsy samples were collected from six MS patients of different clinical subtypes, acute, chronic, progressive, secondary progressive, or chronic progressive, with ages 27–54, and from two age-matched control subjects free of neurological diseases. The postmortem interval of each case ranged from 4 to 24 h. Multiple sclerosis lesions were classified into three distinct categories: acute plaques (AP), CAP (chronic active plaques), or chronic plaques (CP), based on histological evaluation of the disease activity, briefly as follows: AP showed characteristics of acute ongoing inflammation, edema, and active demyelination. CAP was characterized by chronic demyelination with active inflammation at the lesion edges, whereas CP represented chronic inactive demyelination accompanied by profound astrogliosis. Protein samples were prepared from small pieces of brain tissues isolated by laser-captured microdissection, and the tissue pieces were characterized separately by the standard histological examination. The proteins were separated on one-dimensional SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels. Then, the protein bands were dissected and digested in a gel with trypsin, and peptide fragments were processed for mass spectrometric analysis several times to obtain a saturation point. Among 2,574 proteins determined with high confidence, the application of a computational data exploration program named INTERSECT/INTERACT identified 158, 416, and 236 lesion-specific proteins that were detected exclusively in AP, CAP, and CP, respectively. In this study, we tentatively called them as the comprehensive MS brain-lesion proteome dataset.

Conversion of protein IDs into Entrez Gene IDs and KEGG IDs

We converted the protein IDs listed in the dataset described above into the corresponding the National Center for Biotechnology Information (NCBI) Entrez Gene IDs, Gene Symbols, and Kyoto Encyclopedia of Genes and Genomes (KEGG) IDs by searching them on the UniProt knowledgebase (http://www.expasy.org/sprot).

Molecular network analysis

To identify biologically relevant molecular pathways from large-scale proteome data, we have undertaken the systems biology approach. We analyzed them by using four distinct pathway analysis tools endowed with a comprehensive knowledgebase which are as follows: KEGG (http://www.kegg.jp), the protein analysis through evolutionary relationships (PANTHER) classification system (http://www.pantherdb.org), Ingenuity pathways analysis (IPA) (Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com), and KeyMolnet (Institute of Medicinal Molecular Design, Tokyo, Japan; http://www.immd.co.jp).

By importing the list of KEGG IDs, we studied molecular pathways on KEGG, a public database that systematically integrates genomic and chemical information to create the whole biological system *in silico*. KEGG contains manually curated reference pathways that cover a wide range of metabolic, genetic, environmental, and cellular processes, and human diseases [12]. Currently, KEGG contains 90,931 pathways generated from 371 reference pathways. PANTHER, a public database generated by computational algorithms that relate the evolution of protein sequence to the evolution of protein functions and biological roles, provides a structured representation of protein function in the context of biological reaction networks [13]. Currently, PANTHER includes the information on 165 regulatory and metabolic pathways, manually curated by expert biologists. PANTHER visualizes pathway maps with the format compatible with the Systems Biology Markup Language (SBML) standard. By uploading the list of Entrez Gene IDs, PANTHER identifies the genes in terms of over- or under-representation in canonical pathways, followed by statistical evaluation by multiple comparison with a Bonferroni correction.

IPA is a commercial tool built upon a knowledgebase that contains approximately 1,600,000 biological and chemical interactions and functional annotations with scientific evidence. They are collected from more than 300 selected articles, textbooks, and other data sources, manually curated by expert biologists. By uploading the list of Entrez Gene IDs, the network-generation algorithm identifies focused genes integrated in a global molecular network [14]. IPA calculates the score *P*-value, the statistical significance of association between the genes and the network by the Fisher's exact test.

KeyMolnet is a commercial database, composed of knowledge-based contents on relationships among human genes, molecules, diseases, pathways, and drugs, curated by expert biologists. They are categorized into the core contents that are collected from selected review articles with the highest reliability or the secondary contents extracted from abstracts of PubMed database and Human Reference Protein database. By importing the list of Entrez gene ID, KeyMolnet automatically provides corresponding molecules as a node on networks [15]. The "N-points to N-points" network-search algorithm identifies the molecular network constructed by the shortest route connecting the start point molecules and the end point molecules. The generated network was compared side by side with 346 human canonical pathways of the KeyMolnet library. The algorithm counting the number of overlapping molecular relations between the extracted network and the canonical pathway makes it possible to identify the canonical pathway showing the most significant contribution to the extracted network. The significance in the similarity between both is scored following the formula, where O = the number of overlapping molecular relations between the extracted network and the canonical pathway, V = the number of molecular relations located in the extracted network, C = the number of molecular relations located in the canonical pathway, T = the number of total molecular relations composed of approximately 90,000 sets, and the X = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2(\text{Score}(p))$$

$$\text{Score}(p) = \sum_{x=0}^{\text{Min}(C,V)} f(x)$$

$$f(x) = {}_C C_x \cdot {}_{T-C} C_{V-x} / {}_T C_V$$

Results

KEGG and PANTHER searches elucidated a role of ECM-mediated cell adhesion in chronic lesions of MS

First of all, we converted all protein IDs listed in the original database [8] into the corresponding NCBI Entrez Gene IDs, Gene Symbols, and KEGG IDs by searching them on the UniProt knowledgebase. After the removal of unaccepted and redundant IDs, we finally identified 155, 407, and 232 Entrez Gene IDs and KEGG IDs from the AP, CAP, and CP-specific proteome data, respectively. They are listed in Supplementary Tables 1–3*.

When the KEGG IDs of the proteome were uploaded onto the 'Search Objects in Pathway' tool of the KEGG database, the vast majority of AP, CAP, or CP-specific proteins was not mapped on any KEGG human reference pathways (Table 1). However, a battery of CAP-specific proteins were categorized as those located in the pathways linked to focal adhesion (KEGG pathway ID: hsa04510), cell communication (hsa01430), ECM-receptor interaction (hsa04512), purine metabolism (hsa00230), and other biological pathways (not shown). Likewise, a panel of CP-specific proteins was found to be involved in the pathways linked to focal adhesion, regulation of actin cytoskeleton (hsa04810), oxidative phosphorylation (hsa00190), and cell communication (Table 1). These results are derived chiefly from enhanced production and deposition of ECM and receptor components, including collagen, fibronectin, vitronection, integrin, and laminin in CAP and CP lesions. In contrast, relatively small numbers of AP-specific proteins were mapped on the

*Supplementary Tables 1–4 are available online at http://msj.sagepub.com/

Table 1 The molecular pathway relevant to multiple sclerosis (MS) brain-lesion proteome suggested by KEGG search

Stage	Rank	Functional category (KEGG Pathway ID)	Genes classified
AP	1	Unclassified	123 genes
	2	Oxidative phosphorylation (hsa00190)	NDUFS7, NDUFB9, ATP4A, ATP6V0C
	3	Regulation of actin cytoskeleton (hsa04810)	FGD1, ITGB4, SSH1, ACTA1
CAP	1	Unclassified	281 genes
	2	Focal adhesion (hsa04510)	COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, LAMA1, MYLK, SHC3, PPP1CA, PARVA, PRKCB1, MYL7, RAC3, SPP1, SRC, THBS1, VTN
	3	Cell communication (hsa01430)	NES, COL1A, COL1A2, COL5A2, COL6A2, COL6A3, KRT78, FN1, GJA1, LAMA1, KRT3, SPP1, THBS1, VTN
	4	ECM-receptor interaction (hsa04512)	COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, LAMA1, HSPG2, SPP1, THBS1, VTN
	5	Purine metabolism (hsa00230)	ADCY5, TYMP, NT5E, PDE2A, PDE3B, PDE4A, PDE4B, PRPS2, GMPS, ENTPD1
CP	1	Unclassified	166 genes
	2	Focal adhesion (hsa04510)	COL4A2, COL6A1, CRK, FYN, ITGA6, LAMB2, LAMC1, PIK3CA, ZYX
	3	Regulation of actin cytoskeleton (hsa04810)	WASF2, BAIAP2, CRK, ITGA6, PIK3CA, TIAM1, MYH14, ARHGEF7
	4	Oxidative phosphorylation (hsa00190)	NDUFB6, NDUFB8, NDUFS5, ATP5I, ATP6V1F
	5	Cell communication (hsa01430)	COL4A2, COL6A1, ITGA6, LAMB2, LAMC1

The list of KEGG IDs of MS brain-lesion proteome was uploaded onto the 'Search Objects in Pathway' tool of the KEGG database. Top 2 for AP and top 4 for CAP and CP of human reference pathways relevant to the proteome data are shown with KEGG pathway IDs and the list of genes classified.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; and CP, chronic plaques.

pathways, such as oxidative phosphorylation and regulation of actin cytoskeleton (Table 1). Thus, the KEGG search suggested that the biological process of ECM and integrin-mediated cell adhesion and communication plays a role in chronic lesions of MS.

When the Entrez Gene IDs of the proteome were imported into the 'Gene Expression Data Analysis' tool of the PANTHER database, the vast majority of AP, CAP, or CP-specific proteins were not mapped on any PANTHER canonical pathways in comparison with a reference set of NCBI human genes (Table 2). However, PANTHER identified a statistically significant relationship between a set of CAP proteins and signaling pathways of chemokines and cytokines, integrin (Figure 1), muscarinic and nicotinic acetylcholine receptors (Table 2). PANTHER suggested an involvement of integrin signaling in CP, but identified no pathways relevant to AP (Table 2). Thus, the PANTHER search indicated that integrin signaling plays a role in both CAP and CP, whereas inflammation mediated by chemokine and cytokine signaling plays a predominant role in CAP.

Table 2 The molecular pathway relevant to MS brain-lesion proteome suggested by PANTHER search

Stage	Rank	Functional category	Number of genes classified	Human reference genes	*P*-value
AP	1	Unclassified	120	22436	6.89E-02 (NS)
CAP	1	Unclassified	321	22436	1,73E-04
	2	Inflammation mediated by chemokine and cytokine signaling pathway	17	315	2,63E-03
	3	Integrin signaling pathway	14	227	3,55E-03
	4	Muscarinic acetylcholine receptor 1 and 3 signaling pathway	7	62	1,17E-02
	5	Nicotinic acetylcholine receptor signaling pathway	8	91	2,03E-02
CP	1	Unclassified	182	22436	9,75E-03
	2	Integrin signaling pathway	9	227	4,33E-02

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'Gene Expression Data Analysis' tool of the PANTHER classification system by comparing with a reference set of NCBI human genes. The canonical pathways relevant to the proteome data are shown with the number of genes classified and *P*-value evaluated by multiple comparison with a Bonferroni correction.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; CP, chronic plaques; and NS, not significant.

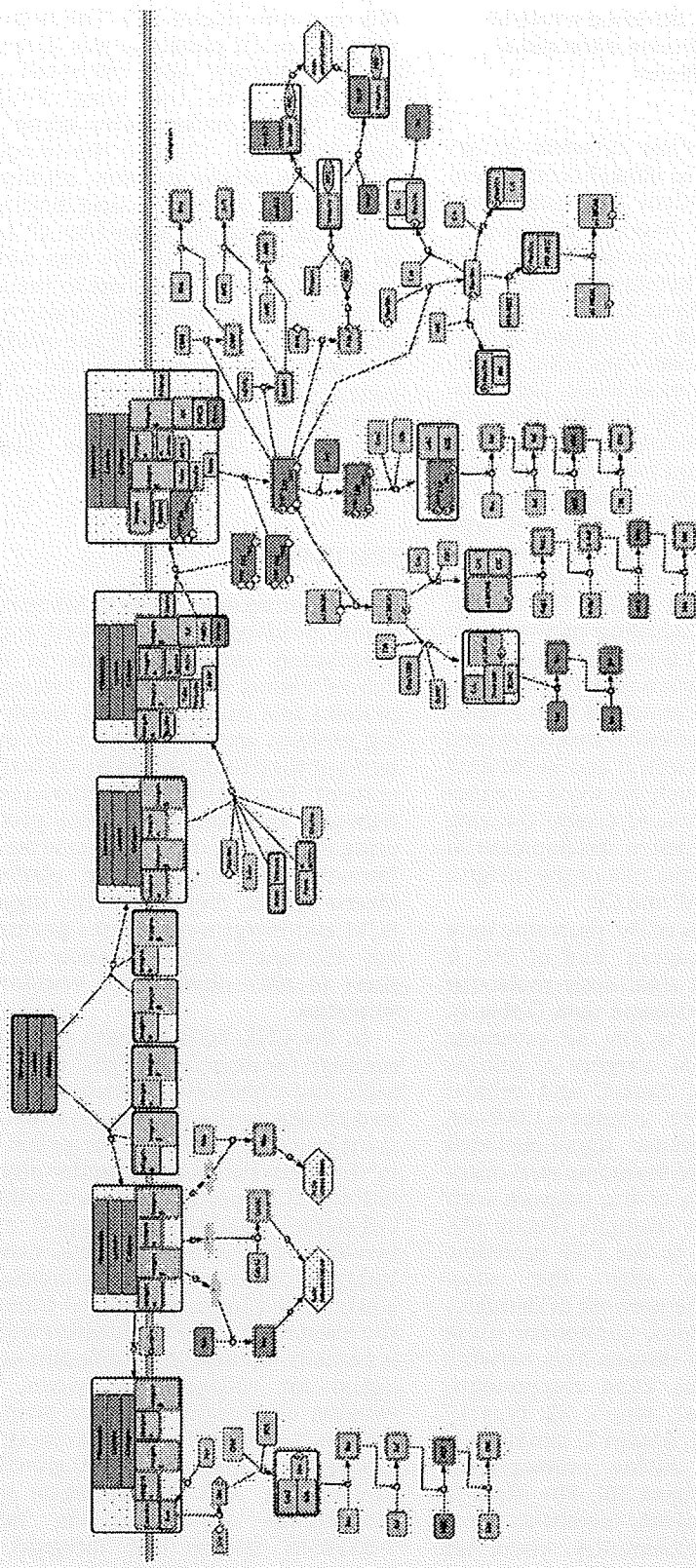

Figure 1 Integrin signaling pathway relevant to CAP proteome suggested by PANTHER. The list of Entrez Gene IDs of CAP-specific proteome was uploaded onto the 'Gene Expression Data Analysis' tool of the PANTHER classification system by comparing them with a reference set of NCBI human genes. Integrin signaling pathway was identified as one of canonical pathways statistically relevant to the CAP proteome (Table 2). The pathway is illustrated as the map compatible with the Systems Biology Markup Language (SBML) standard. The molecules colored in pink represent those included in the gene list (Supplementary Table 2). They are composed of fibronectin (Gene symbol: FN1), laminin (LAMA1), collagen (COL1A1, COL1A2, COL5A2, COL6A2, COL6A3), Rac (RAC3), MEK (MAP2K4), FAK (PTK2B), parvin (PARVA), Src (SRC), Jnk (MAP2K4), Arp2/3 (ARPC1A), and VASP (ENAH).