pial peripheral white matter lesions might be created by the prominently high expression of AQP4 in not only central gray matter,[26] but also white matter[25] within spinal cord tissues in contrast with brain. This finding might be caused by direct invasion of lymphocytes and macrophages derived from meningeal infiltration in NMO, and broader spectrum of immunogenic specificities for not only strict and limited target antigens such as AQP4, but also other antigens such as myelin.[29-32]

All these findings could be why NMO displays homogeneity of pathogenic effector immune mechanisms through the long-term course, while MS should be recognized as a heterogeneous 2-stage disease that could switch from inflammatory to degenerative phase.

In NMO, good prognosis after immunotherapy for patients with limited NMO or definite NMO in the early stage was suggested to be produced as follows. First, in terms of the results of accumulated lesions at the same spinal cord level, cases of definite NMO could display greater axonal injury and incrementally increased disability compared with limited NMO (figure 2B and table e-2). Second, we supposed that the most crucial points were large inflammatory responses on the basis of the same immunologic pathogenesis in definite NMO. Definite NMO at the nadir of relapses showed significantly higher amounts of IL-1β and IL-6 in CSF, compared with limited NMO (MY) and MS (figure 1). These cytokines are reportedly important inflammatory cytokines influencing not only antigen-specific immune responses and inflammatory reactions, but also differentiation/induction of the T_H-17 lineage.[33] Definite NMO might have more inflammatory responses with more IL-17–producing cells, such as T_H-17 cells, and could more easily disrupt and pass through the blood-brain barrier by higher anti-AQP4 antibody titers compared with limited NMO. Larger inflammatory responses might produce larger lesions with more axonal damages and cavity formations in definite NMO compared with limited NMO.

Even though we analyzed limited and definite NMO using a uniform shorter period of <5 years (table e-3 and figure e-3), all findings including immunologic and other characteristic features for limited and definite NMO were consistent with data for the entire disease courses (figures 1, 2A, and 2B and tables e-1 and e-2). We therefore concluded that limited and definite NMO as described above exhibit distinct characteristic features, irrespective of disease duration.

In our series, IV corticosteroid therapy was commonly used as the initial treatment for acute attacks of NMO. Maintenance immunosuppressive therapy for reducing relapses of NMO was performed using a combination of oral prednisone and immunosuppressive agents, including azathioprine and tacrolimus. Recent reports have described long-term treatment with oral agents such as azathioprine[34] or mycophenolate mofetil[35] for patients with relatively mild disease, and rituximab[36] for those with treatment-refractory disease. We agree with the previously proposed concept[8] that patients with LEM and seropositivity for NMO-IgG should be treated for a minimum of 5 years, because the risk of "conversion" to NMO seems highest in the first 5 years based on a prior clinic-based series of NMO,[2] consistent with our data indicating that conversion from limited NMO with myelitis to definite NMO happened at a mean of 2.9 ± 1.5 years from onset (table e-1). Early detection and start of immunotherapy at limited NMO would be key to the long term-prognosis of patients with NMO.

We have documented 2 basic characteristic phenotypes in the NMO spectrum: a limited form of NMO as an early type and a definite form of NMO as a complete type. Based on not only high specificity of NMO-IgG but also characteristic homogeneity of pathogenic effector immune mechanism, we emphasized that NMO should be recognized as a distinct pathologic entity with a fundamentally different etiology from MS with heterogeneous 2-stage disease.

ACKNOWLEDGMENT

The authors thank Y. Watanabe and M. Kaneko for technical assistance with assays for anti-AQP4 antibodies.

Received March 24, 2009. Accepted in final form July 17, 2009.

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Research Paper

Multiple Sclerosis

Multiple Sclerosis
15(12) 1450–1458

DOI: 10.1177/1352458509350305
msj.sagepub.com
SAGE

Relationship between Barkhof criteria and the clinical features of multiple sclerosis in northern Japan

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Abstract

We previously reported that the prevalence of multiple sclerosis (MS) in the Tokachi Province of Hokkaido increased from 8.6 to 13.1 per 100,000 individuals between 2001 and 2006. Here, we study the frequency of MS patients who fulfill the Barkhof criteria and identified their common features. All 47 subjects in our previous study, who fulfilled Poser's criteria, were included in this study. Of these, 33 satisfied the Barkhof criteria. In 2006, 9.2 per 100,000 MS patients fulfilled the Barkhof criteria; the percentage of patients who fulfilled these criteria was significantly higher among patients born after 1960 than among those born before 1960 (84.3% and 40.0%, respectively). The proportion of patients with conventional MS (C-MS) who fulfilled the Barkhof criteria was higher than that of patients with opticospinal MS (OS-MS) who fulfilled these criteria (93.9% and 71.4%, respectively). Longitudinally extensive spinal cord lesions (LESCLs) were not associated with the brain lesions defined in the Barkhof criteria (Barkhof brain lesions). In Tokachi Province, the increased percentage of MS patients who fulfill the Barkhof criteria was associated with increased C-MS incidence and an increase in the proportion of C-MS patients with Barkhof brain lesions among people born after 1960.

Keywords

multiple sclerosis, prevalence, incidence, magnetic resonance imaging, spinal cord, epidemiology

Date received: 9th February 2009; accepted: 2nd September 2009

Introduction

The 2001 McDonald diagnostic criteria for multiple sclerosis (MS) outline the findings of brain magnetic resonance imaging (MRI) that indicate dissemination in space (DIS) and dissemination in time (DIT). In patients with a clinically isolated syndrome (CIS), these two phenomena are diagnostic of clinically definite MS (CDMS). In these criteria, typical brain abnormalities seen on MRI scans are primarily used to diagnose MS.[1,2] The diagnostic criteria for DIS are based on those recommended by Barkhof et al.[3] and Tintoré et al.[4] These criteria are highly accurate for the diagnosis of MS in patients with a CIS in western countries,[3–9] and according to these criteria, at least one of the main clinical features of MS must be present for the diagnosis of DIS. However, few long-term follow-up examinations have been conducted to determine the proportion of MS cases in which the Barkhof criteria are fulfilled.[10]

In Japan, MS is generally classified into two clinical forms, namely, a conventional form (C-MS) and an opticospinal form (OS-MS).[11,12] The latter describes cases in which clinically identified lesions are restricted to the optic nerve and the spinal cord and is considered to be the 'Asian type,' while the former refers to cases with symptoms involving the brain and is considered to be analogous to MS in western countries.[11,12] Some patients with OS-MS have minor brainstem symptoms (e.g. nystagmus and transient double vision),[12] and some of these patients might have an early form of C-MS. Furthermore, MRI criteria for the differentiation between these two forms of brain lesions have not yet been established.[13] It has been reported that C-MS in the Japanese population is significantly associated with the HLA-DRB1*1501 allele,[12,14,15] as is the case in western countries,[16] while OS-MS is associated with

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the HLA-DPB1*0501 allele.[17,18] OS-MS might also occur due to lack of the HLA-DPB1*0301 allele.[19] Although the clinical diagnosis of OS-MS is controversial, it has been proposed that OS-MS be considered an independent entity since it shows characteristic clinical signs and is associated with the HLA-DPB1*0501 allele significantly more frequently than C-MS.

We reported the prevalence of MS in Tokachi Province in March 2001[20] and March 2006.[21] The incidence of MS increased from 8.6 to 13.1 per 100,000 individuals over these five years. The mean annual incidence of MS also increased from 0.15 (1975–1989) to 0.68 (1990–2004) per 100,000 individuals, and this increase was mainly because of the increase in the incidence of C-MS after 1990.[20,21] Another study conducted in Japan also reported a marked increase in the proportion of C-MS to OS-MS in patients born after 1960 (4.7:1).[22] Recent studies conducted in western countries report that the annual incidence of MS is 3.0–9.2 per 100,000 individuals,[23,24,25,26] which is much higher than the incidence in Japan. The proportion of OS-MS in western patients with MS is much lower than that in the Japanese patients, and the proportion of C-MS is much higher in western countries than in Japan.[27] The increasing incidence of MS and the progressive predominance of the C-MS phenotype in Japan may reflect the westernization of MS in Japan. However, C-MS in Japan has several characteristics that are different from those observed in western countries; Japanese patients probably have a lower incidence of cerebellar hemispheric lesions[28] and a higher incidence of longitudinally extensive spinal cord lesions (LESCLs) (14.3–31.5%).[10,29,30]

With the recent discovery of the serum anti-aquaporin 4 (AQP4) antibody,[31] neuromyelitis optica (NMO) was introduced as a distinct disease entity.[32] In Japan, NMO is differentiated from OS-MS by testing for the serum anti-AQP4 antibody.[30,33] Although NMO can be associated with brain lesions,[34] the differences between NMO in the Asian and western populations remain largely unknown, and these differences warrant further research.

We estimated the extent of westernization of MS in patients in Tokachi Province on the basis of the incidence of MS and the proportion of the different types of MS. We used the Barkhof criteria to measure the above two parameters and discuss the factors influencing the change in the extent of westernization of MS. We also determined the proportion of Japanese patients who satisfied the Barkhof criteria and possessed the LESCLs characteristic of Japanese MS. In addition, we examined the influence of the serum anti-AQP4 antibody on the incidence of brain lesions as defined in the Barkhof criteria (hereafter referred to as Barkhof brain lesions).

Materials and methods

Case ascertainment

This study was conducted in the Tokachi Province of Hokkaido, the northernmost island of Japan. The population of this province was 358,439 at the end of March 2006. The subjects of this study were those included in our previously published second epidemiological study conducted from March to May 2006.[21] This study was approved by the Ethical Committee of Obihiro Kosei General Hospital. Information regarding the clinical features of the subjects was collected from the processing sheets based on medical records, which were used in our previous study. We recorded information of the cases in other institutions with a unified form. The criteria of Poser et al.[35] were used for the diagnosis of MS in patients in whom the clinical course of disease was the relapse and remitting (RR) type or the secondary progressive (SP) type. The primary progressive (PP) type was diagnosed using the criteria of Polman et al.[2] On the basis of the distribution of lesions, cases were categorized into two types, namely, OS-MS and C-MS. OS-MS was defined as MS in which the clinically identified lesions were restricted to the optic nerve and spinal cord, and C-MS was defined as MS with clinical signs involving the cerebrum, brainstem, or cerebellum.[11,12] Clinical disability was estimated using Kurtzke's Expanded Disability Status Scale (EDSS).[36]

MRI analysis

MRI scans obtained at 1.5, 1.0, or 0.5 Tesla for each subject were used in this study. A set of scans for each subject contained T2-weighted images (T2) or T2-weighted fast-fluid attenuated-inversion recovery images (FLAIR) with axial slices through the brain. The slice thickness was 5–10 mm. Gadolinium enhancement and sagittal slices of FLAIR images were used whenever possible.

The MRI scans were evaluated by two of the authors (M Nakamura and H Houzen). Hyperintense brain lesions larger than 3 mm in cross-sectional diameter were considered as significant lesions, according to the 2001 McDonald diagnostic criteria for MS.[1] These lesions were classified as infratentorial or supratentorial. The latter were divided into juxtacortical (contiguous with the cortex), periventricular (contiguous with the ventricle), or deep white matter not in contact with the cortex or the ventricle. Barkhof et al.[3] have defined four criteria for the diagnosis of MS lesions by using MRI: (1) the presence of at least one gadolinium-enhancing lesion or nine T2 hyperintense lesions; (2) the presence of at least one infratentorial lesion; (3) the presence of at least one juxtacortical

lesion, and (4) the presence of at least three periventricular lesions. If the demyelinating lesions fulfilled three of the four abovementioned criteria, the Barkhof criteria were considered to have been fulfilled.[3,4] We also noted the presence of ovoid lesions of the corpus callosum. Ovoid lesions were defined as oval-shaped, hyperintense lesions oriented perpendicular to the corpus callosum in sagittal FLAIR images. We considered the spinal cord lesions only for the diagnosis of MS, not as alternatives for the brain lesions for the fulfillment of the Barkhof criteria. LESCLs were defined as the spinal cord lesions extending over three contiguous vertebral segments. Long spinal cord lesions that consisted of several short spinal cord lesions involving discrete vertebrae were excluded.

Anti-AQP4 antibody measurement

The serum level of anti-AQP4 antibody in MS patients was measured using an indirect immunofluorescence method reported previously.[37] This method involved the use of HEK-293 cells transfected with an AQP4 expression vector containing a full-length complementary DNA (cDNA) of human AQP4. The serum anti-AQP4 antibody level was measured by one of the authors (K Tanaka).

Subgroup analysis

In Japan, the ratio of the incidence of OS-MS to that of C-MS among individuals born in the 1960s showed a very steep increase (1:4.67). In contrast, that among individuals born before the 1960s showed a slight increment (1:0.5 to 1:1.7).[22] No significant changes in this ratio were seen among the cohort of people born after the 1960s, although a mild increase in this ratio was noted.[22,38] Our second epidemiological survey in Tokachi Province also showed an exceedingly large difference in the C-MS to OS-MS ratio between patients born in the 1950s and those born in the 1960s.[21] Thus, the clinical aspects of Japanese patients with MS have changed to a large extent in the population born after the 1960s. We classified our patients into two subgroups: those born before and those born after 1960, and compared the following clinical parameters between these two subgroups: gender ratio, age at onset, phenotypic ratio, presence of Barkhof brain lesions, LESCLs, and serum anti-AQP4 antibody.

Statistical analysis

The gender ratio, type of clinical course (RR, SP, or PP), clinically apparent lesion distribution (C-MS and OS-MS), presence or absence of LESCLs, and presence or absence of Barkhof brain lesions were assessed in all patients. The statistical significance of the differences between the above parameters in the two groups (e.g. the number of patients fulfilling or not fulfilling the Barkhof criteria, or the number of patients born before or after 1960) was estimated using Fisher's exact probability test. Age, disease duration, and EDSS scores were calculated as mean ± SD. The statistical significance of the differences in these parameters between the two groups was estimated using the Mann–Whitney *U*-test.

Results

In our second study on the prevalence of MS, we obtained information from 47 subjects (35 women and 12 men) with MS at the end of March 2006; 44 of these were diagnosed with CDMS and three with laboratory-supported MS (LSMS).[21] The mean age of the subjects was 41.0 ± 13.1 years, and the mean disease duration was 12.6 ± 10.0 years. RR was the most common clinical course (70.2%), followed by SP (25.5%); PP was observed in only two patients (4.3%).[21] Most patients had C-MS (87.2%); OS-MS was observed in only six patients (12.8%). The mean EDSS score was 3.1 ± 3.1 (range, 0–9.5) (Table 1).

For the analysis of the brain lesions, MRI scans obtained from April 2005 to January 2008 were used for 32 of the 47 subjects, and 28 of these 32 subjects underwent MRI after our second epidemiological survey in March 2006. In 15 subjects, the latest scans were obtained before April 2005. At the time of the collection of MRI scans, treatment for the prevention of relapse had been administered to 14 subjects, and 24 subjects had not been given any such treatment. We could not obtain any information about the treatment regimen for the other nine subjects. Of the 14 subjects

Table 1. Clinical features of the patients included in this study

Female/Male	35/12
Mean age (years) ± SD (range)	41.0 ± 13.1 (15–80)
Mean age at onset (years) ± SD (range)	28.4 ± 11.7 (7–60)
Mean disease duration (years) ± SD (range)	12.6 ± 10.0 (2–48)
RRMS/SPMS/PPMS	33 (70.2%)/12 (25.5%)/2 (4.3%)
C-MS/OS-MS	41 (87.2%)/6 (12.8%)
Mean EDSS ± SD (range)	3.1 ± 3.1 (0–9.5)

RRMS, relapsing-remitting type multiple sclerosis; SPMS, secondary progressive type multiple sclerosis; PPMS, primary progressive type multiple sclerosis; C-MS, conventional multiple sclerosis; OS-MS, opticospinal multiple sclerosis; EDSS, Kurtzke's expanded disability scale.

who received treatment, interferon β (IFNβ)-1b was administered to 11, three of whom were also administered a low dose of prednisolone ((PSL); 5–15 mg/day). Two subjects were administered only PSL. One subject was treated with PSL and mitoxantrone. Of the 24 subjects not receiving any treatment for the prevention of relapses, three had been administered IFNβ-1b, but by the time the MRI scans used in this study were obtained, the drug had been withdrawn owing to adverse effects. Of these 24 subjects, two had been treated with PSL and one with azathioprine. To detect spinal cord lesions, we used the spinal MRI scans that had been taken in all the 47 subjects before March 2006.

The Barkhof criteria were fulfilled in 33 patients (70.2%), and the prevalence of Barkhof brain lesions was 9.2 per 100,000 individuals in Tokachi Province. The mean age of the patients fulfilling the Barkhof criteria was significantly lower than that of the patients not fulfilling the Barkhof criteria (37.0 ± 10.1 years and 49.5 ± 15.2 years, respectively; $P = 0.01$) (Table 2). The mean age at onset was also significantly lower in the patients with Barkhof brain lesions than those without Barkhof brain lesions (24.5 ± 7.7 and 36.9 ± 14.7, respectively; $P = 0.003$) (Table 2). The incidence of C-MS tended to be higher among the patients fulfilling the Barkhof criteria than among those not fulfilling the Barkhof criteria (93.9% and 71.4%, respectively) (Table 2). The gender ratio, mean disease duration, proportion of each clinical course, and mean EDSS score did not significantly differ between the patients fulfilling the Barkhof criteria and those not fulfilling these criteria (Table 2).

We analysed the distribution of brain lesions in 45 cases, according to the each item of Barkhof criteria (Table 3). Although we had information as to whether each case fulfilled the Barkhof criteria in all 47 cases, the analysis of each item of the Barkhof criteria was flawed in two cases. Thus, we excluded these two cases from this analysis. Most of the cases with Barkhof brain lesions fulfilled the following items of the Barkhof criteria: 'at least nine T2 hyperintense lesions,'

Table 2. Comparison of clinical features between cases fulfilling and not fulfilling Barkhof criteria

	Barkhof (+) ($n = 33$)	Barkhof (−) ($n = 14$)
Female/Male	26 (78.8%)/7 (21.2%)	9 (64.3%)/5 (35.7%)
Mean age (years) ± SD (range)*	37.0 ± 10.1 (15–59)	49.5 ± 15.2 (23–80)
Mean onset age (years) ± SD (range)**	24.5 ± 7.7 (12–48)	36.9 ± 14.7 (7–60)
Mean disease duration ± SD (range)	12.5 ± 9.3 (2–40)	12.6 ± 11.1 (2–48)
RRMS/SPMS/PPMS	23 (69.7%)/8 (24.2%)/2 (6.1%)	10 (71.4%)/4 (28.6%)/0 (0.0%)
C-MS/OS-MS	31 (93.9%)/2 (6.1%)	10 (71.4%)/4 (28.6%)
Mean EDSS ± SD (range)	2.9 ± 3.0 (0–9.5)	3.6 ± 3.2 (0–9)

Barkhof (+), cases fulfilling Barkhof criteria; Barkhof (−), cases not fulfilling Barkhof criteria. Other abbreviations are as defined in Table 1.
*$P = 0.01$, by Mann–Whitney U-test, for difference between Barkhof (+) cases and Barkhof (−) cases.
**$P = 0.003$, by Mann–Whitney U-test, for difference between Barkhof (+) cases and Barkhof (−) cases.

Table 3. Lesion distribution in brain MRI of our cases

Lesions	Barkhof (+) ($n = 32$) [C-MS ($n = 30$):OS-MS ($n = 2$)]	Barkhof (−) ($n = 13$) [C-MS ($n = 10$):OS-MS ($n = 3$)]
Nine T2 hyperintense lesions	32/32 (100%) [30/30 (100%):2/2 (100%)]	4/13 (30.8%) [3/10 (30.0%):1/3 (33.3%)]
One gadolinium-enhancing lesion[a]	8/27 (29.6%) [8/25 (32.0%):0/2 (0%)]	0/9 (0%) [0/6 (0%):0/3 (0%)]
One infratentorial lesion	16/32 (50.0%) [16/30 (53.3%):0/2 (0%)]	3/13 (23.1%) [2/10 (20.0%):1/3 (33.3%)]
One juxtacortical lesion	32/32 (100%) [30/30 (100%):2/2 (100%)]	8/13 (61.5%) [6/10 (60.0%):2/3 (66.7%)]
Three periventricular lesions	31/32 (96.9%) [29/30 (96.7%):2/2 (100%)]	2/13 (15.4%) [2/10 (20.0%):0/3 (0%)]
Ovoid lesions[b]	22/28 (78.6%) [21/26* (80.8%):1/2 (50.0%)]	2/12 (16.7%) [2/9* (22.2%):0/3 (0.0%)]
Cerebellar hemispheric lesions	6/32 (18.8%) [6/30 (20.0%):0/2 (0.0%)]	2/13 (15.4%) [1/10 (10.0%):1/3 (33.3%)]

The objects were limited to 45 of 47 cases for this analysis (see Materials and methods).
[a]The gadolinium enhancing was conducted for 36 of 45 cases at analysis.
[b]The ovoid lesions of corpus callosum were examined in 40 of 45 cases.
*$p = 0.003$, by Fisher's exact probability test, for difference between C-MS with Barkhof brain lesions and C-MS without Barkhof brain lesions in the rate of the cases with ovoid lesions of the corpus callosum.

'at least one juxtacortical lesion,' and 'at least three periventricular lesions.' Infratentorial lesions and gadolinium-enhancing lesions were detected in a relatively small proportion of the study population with or without Barkhof brain lesions. However, in the case of C-MS, patients with Barkhof brain lesions showed the above two types of lesions more frequently than those without Barkhof brain lesions. Sagittal slices of FLAIR images of brain were conducted in order to detect ovoid lesions of the corpus callosum in 40 cases. These ovoid lesions were more frequent in the case of C-MS than in the case of OS-MS, although the difference was not statistically significant (65.7% and 20%, respectively). The incidence of ovoid lesions was significantly higher among the C-MS patients who had Barkhof brain lesions than among those who did not (80.8% and 22.2%, respectively; $P=0.003$). Five C-MS patients with Barkhof brain lesions did not have ovoid lesions of the corpus callosum, and two C-MS patients without Barkhof brain lesions had these ovoid lesions. Of five OS-MS patients, only one had ovoid lesions, and this patient fulfilled the Barkhof criteria. The other four patients without ovoid lesions did not fulfill the Barkhof criteria. A few of the study patients had cerebellar hemispheric lesions (8 of 45; 17.8%) (C-MS: 7 of 40, 17.5%; OS-MS: 1 of 5, 20.0%).

We determined the proportion of patients with Barkhof brain lesions and categorized our subjects into two subclasses on the basis of their birth year (born before or after 1960). The proportion of patients fulfilling the Barkhof criteria was significantly higher among the subjects born after 1960 than among the subjects born before 1960 (27 of 32 patients, 84.3% and 6 of 15 patients, 40.0%, respectively; $P=0.003$) (Figure 1). Barkhof brain lesions were more common among patients with C-MS. The proportion of C-MS patients who fulfilled the Barkhof criteria was significantly higher among subjects born after 1960 than among those born before 1960 (25 of 29 patients, 86.2% and 6 of 12 patients, 50.0%, respectively; $P=0.02$) (Figure 1). Barkhof brain lesions were not present in the three OS-MS patients who were born before 1960, while two of the three OS-MS patients born after 1960 fulfilled the Barkhof criteria (Figure 1). In the case

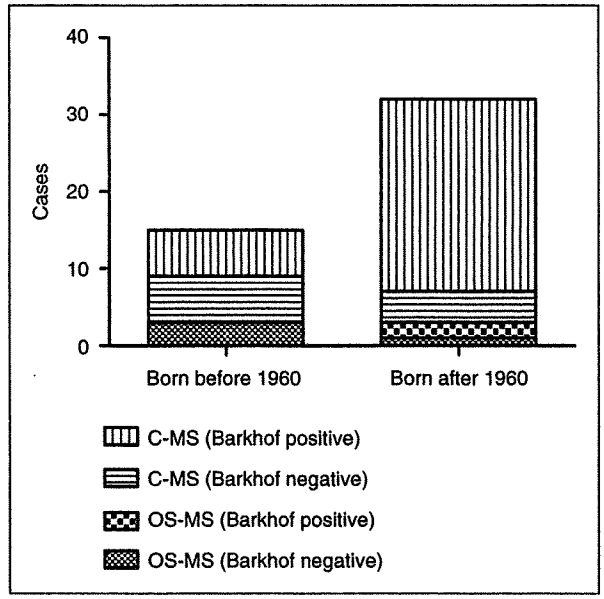

Figure 1. A total of 47 patients with MS were categorized into two subclasses according to birth year (before or after 1960). Next, the patients in each subclass were further divided into those who had C-MS and fulfilled the Barkhof criteria, those who had C-MS but did not fulfill the Barkhof criteria, those who had OS-MS and fulfilled the Barkhof criteria, and those who had OS-MS but did not fulfill the Barkhof criteria. The proportion of patients fulfilling the Barkhof criteria was significantly higher among subjects born after 1960 than among those born before 1960 (27 of 32 patients, 84.4% and 6 of 15 patients, 40.0%, respectively; $P=0.003$). Of the 32 patients (90.6%) born after 1960, C-MS was present in 29, and 25 of these 29 patients (86.2%) fulfilled the Barkhof criteria. In contrast, C-MS was present in 12 of the 15 patients (80.0%) born before 1960, and 6 of these 12 patients (50.0%) fulfilled the Barkhof criteria. The proportion of C-MS patients who fulfilled the Barkhof criteria was significantly higher among the subjects born after 1960 than among those born before 1960 (25 of 29 patients and 6 of 12 patients, respectively; $P=0.02$). The increase in the number of patients with Barkhof brain lesions may be attributable in part to the increase in the number of C-MS patients and mainly to the increase in the proportion of C-MS patients born after 1960 who fulfill the Barkhof criteria.

of C-MS, the mean age at onset was significantly greater in the subjects born before 1960 than in those born after 1960 (37.6 ± 14.2 years and 23.3 ± 6.5 years, respectively; $P = 0.004$ by the Mann–Whitney U-test), while not detected in OS-MS.

LESCLs were detected in nine patients, of which, seven had C-MS and two had OS-MS. These patients accounted for 17.1% (7 of 41) C-MS patients and 33.3% (2 of 6) OS-MS patients. We studied the relationship between the presence of LESCLs and the fulfillment of the Barkhof criteria among patients with C-MS and OS-MS. Most C-MS patients had Barkhof brain lesions but not LESCLs (61.0%), while very few patients without Barkhof brain lesions had LESCLs (2.4%) (Table 4). The proportion of C-MS patients who fulfilled the Barkhof criteria did not vary with the presence or absence of LESCLs (6 of 7 patients, 85.7% and 25 of 34 patients, 73.5%, respectively) (Table 4). Thus, Barkhof brain lesions can be present even in C-MS patients with LESCLs. In the case of OS-MS, LESCLs were not present in many subjects (2 of 6 subjects; 33.3%), and most patients had neither Barkhof brain lesions nor LESCLs (50.0%) (Table 5).

The serum anti-AQP4 antibody was examined in 29 patients with C-MS and four patients with OS-MS; further, of nine patients with LESCLs (seven C-MS and two OS-MS patients), this antibody was examined in seven (five C-MS and two OS-MS patients). Of the above 33 patients, only one (3.0%; female) was positive for the serum anti-AQP4 antibody. She was diagnosed with C-MS, she had LESCLs, and fulfilled the Barkhof criteria. None of the OS-MS patients were positive for the serum anti-AQP4 antibody.

Table 4. Relationship between LESCLs and Barkhof criteria in C-MS

	Barkhof	
C-MS ($n=41$)	(+)	(−)
LESCLs		
(+)	6 (14.6%)	1 (2.4%)
(−)	25 (61.0%)	9 (22.0%)

Table 5. Relationship between LESCLs and Barkhof criteria in OS-MS

	Barkhof	
OS-MS ($n=6$)	(+)	(−)
LESCLs		
(+)	1 (16.7%)	1 (16.7%)
(−)	1 (16.7%)	3 (50.0%)

Discussion

In our previous two studies, we showed that the prevalence of MS in Tokachi Province was the highest in East Asia and has been increasing in the past five years.[20,21] In the current study, we focused on the brain MRI data of the subjects included in our previous study.[21] The main findings of this study are as follows:

(i) a relatively high proportion (70.2%) of the subjects in Tokachi Province fulfilled the Barkhof criteria in March 2006;
(ii) Barkhof brain lesions and ovoid lesions of the corpus callosum were considerably more frequent among C-MS patients than among OS-MS patients;
(iii) cerebellar hemispheric lesions were rare in both C-MS and OS-MS patients;
(iv) the incidence of Barkhof brain lesions was higher among the C-MS patients born after 1960 than among those born before 1960;
(v) the incidence of Barkhof brain lesions among C-MS patients was independent of the presence of LESCLs;
(vi) the most common lesion pattern among C-MS patients was the presence of Barkhof brain lesions, but not LESCLs;
(vii) the presence of the serum anti-AQP4 antibody was rare in Tokachi Province.

The brain and spinal cord MRI data used in this study were not obtained under uniform conditions, for example, there were different magnetic strengths (0.5, 1.0, and 1.5 Tesla) during MRI. This drawback might have led to the underestimation of the number of brain lesions on MRI scans. We might have also overlooked some ovoid lesions since we could not examine sagittal FLAIR images in all patients. Furthermore, the small number of patients in our study, particularly those with OS-MS, might limit the sensitivity of clinical differences. Therefore, a more extensive study is needed in the future.

The Barkhof criteria were proposed to predict the conversion of CISs to CDMS,[3,4] and in Japan, we found only one article (from Kyushu, in the southern part of Japan) describing the proportion of patients with CDMS who were found to fulfill the Barkhof criteria during a long-term follow-up.[10] In their study of 136 subjects with C-MS, OS-MS, or the PP type of MS,[10] 52.9% fulfilled the Barkhof criteria. This percentage is much lower than that in our study (70.2%). They also reported that the Barkhof criteria are fulfilled in a considerably greater proportion of C-MS patients (73.1%) than OS-MS patients (24.6%);[10] this trend is similar to that observed in our study. The most significant difference between our study and the reported

study[10] is the C-MS:OS-MS ratio among the study subjects (6.83 and 1.18, respectively), and this difference might account for the difference in the percentage of patients fulfilling the Barkhof criteria. In Japan, the prevalence of OS-MS might not differ greatly between the southern and northern islands.[39] In contrast to the north–south gradient of total MS prevalence in Japan, there is an inverse south–north gradient in the proportion of OS-MS. This gradient is probably an artifact of the north–south gradient in the prevalence of C-MS.[39] Furthermore, the fourth nationwide survey of MS in Japan (2003) also reported that the C-MS:OS-MS ratio and the percentage of MS cases with Barkhof brain lesions were significantly higher in the northern part than in the southern part of Japan and that these ratios are increasing all over Japan.[38]

A study conducted in a western country reported that 67.3% of patients with CDMS fulfilled the Barkhof criteria, as assessed using MRI scans obtained within six months of diagnosis.[9] We could not find any other clear description of the proportion of CDMS patients with Barkhof brain lesions in western countries, although some studies have reported that the proportion of patients with a CIS and Barkhof brain lesions at the onset who converted to CDMS for short periods (1–3 years) was 49–71%.[5–7] In these studies, many patients with CDMS were presumed to fulfill the Barkhof criteria at the time of the survey, despite the subjects being in an early phase of the disease. Considering that our survey was conducted in a longer follow-up period, the proportion of patients with CDMS and Barkhof brain lesions in our study might be lower than that in western countries. However, we found a close correlation between the fulfillment of the Barkhof MRI criteria and the presence of clinical features indicative of C-MS, suggesting that they are applicable in the diagnosis of this form of MS in Japanese populations, as they are in the diagnosis of MS in western countries. On the other hand, in the case of OS-MS, the above association was weaker; therefore, these MRI criteria may be less sensitive when used for the diagnosis of this type of MS.

Most of the C-MS patients had cerebral lesions characteristic of western MS, while the OS-MS patients tended to not have these lesions. We consider that this reflects the difference in the genetic backgrounds of the patients with C-MS and OS-MS, and that these two types of MS could be genetically classified into different entities.[12,14,15,17–19] However, the fact that some OS-MS patients exhibit cerebral lesions typical of western MS indicates the diversity in the clinical presentations of OS-MS.[10,13] Furthermore, like the C-MS patients in Japan, some OS-MS patients respond to IFNβ-1b, which is administered for the prevention of relapses.[40] These observations suggest the existence of a subgroup of OS-MS patients who share the genetic background of C-MS patients; thus, these OS-MS patients exhibit clinical characteristics that are similar to those of C-MS due to some environmental factors. We consider that OS-MS and C-MS might form a continuous clinical spectrum, as reported in another article,[10] and that Barkhof brain lesions might be present in a subgroup of OS-MS patients.

Cerebellar hemispheric lesions were rarely observed in this study (17.8% in 45 patients), regardless of the clinical phenotypes. A study conducted on 66 Japanese patients with MS from Tohoku Province reported that patients with cerebellar hemispheric lesions accounted for only 6.4% of the study population.[28] The low incidence of cerebellar hemispheric lesions is presumably a feature of MS in Japanese people.

In this study, the proportion of patients fulfilling the Barkhof criteria was significantly greater among those born after 1960 than among those born before 1960. This increase may be attributed in part to the increase in the proportion of patients with C-MS among the total number of patients with MS among individuals born after 1960. An increase in the incidence of C-MS has also been observed in Kyushu,[22] and recently, this trend was reported to occur throughout Japan, as determined by the fourth nationwide survey.[38] Moreover, patients with C-MS born after 1960 fulfilled Barkhof criteria more frequently than those with C-MS born before 1960 in this study. We consider this phenomenon is more strongly associated with the increase of MS patients with Barkhof brain lesions among those born after 1960. We also found that in the case of C-MS, the age at onset was higher among subjects born before 1960 than among those born after 1960. This trend was not observed among the OS-MS patients. The lower age at onset might be associated with the higher rate of fulfillment of the Barkhof criteria among C-MS patients born after 1960. These changes in the number and nature of Japanese patients with MS could be a result of the westernization of the Japanese environment along with the rapid economic growth that began in the 1960s.[22]

In western countries, MS patients typically exhibit multiple spinal cord lesions that involve less than two contiguous vertebral segments.[41,42] One study reported that only three of 68 (4.4%) patients with CDMS and clinically probable MS, which were independently diagnosed before the introduction of the Poser criteria, had spinal cord lesions involving >3 contiguous vertebral segments.[41] Another study reported the high incidence of long spinal cord lesions in western countries (14 of 91 MS patients; 15.3%).[43] However, the above study included a large number of patients with the PP type of MS (31 of 91 cases), and most patients with long spinal cord lesions had the PP type of MS (10 of 14

patients), which is not considered to be typical MS. Another study found that within six months of diagnosis, 13 of 104 patients with CDMS or LSMS (12.5%) developed diffuse spinal cord lesions, with a mean length of 11.2 ± 5.7 vertebral segments.[9] The incidence of LESCLs among the C-MS patients (17.1%) in our study is higher than that observed in western countries. In our study, most of the C-MS patients with LESCLs fulfilled the Barkhof criteria. In Japan, no significant difference has been reported in the length of the spinal cord lesions between C-MS and OS-MS patients.[12] Furthermore, some recent studies conducted in Japan have shown that many C-MS patients exhibit LESCLs (14.3–31.5%)[10,29,30] and that many of these patients fulfilled the Barkhof criteria (85.0–88.2%).[10,30] The trend that some C-MS patients have both LESCLs and Barkhof brain lesions could be considered to be one of the characteristic features of Japanese C-MS patients.

We detected the serum anti-AQP4 antibody in only one patient. This patient had C-MS with LESCLs; none of the OS-MS patients tested positive for the serum anti-AQP4 antibody. The percentage of patients with serum anti-AQP4 antibodies has been reported to be 14.2% (16 of 113 consecutive patients with CDMS) in Kyushu Province,[30] and the percentage of patients with serum NMO-immunoglobulin G (IgG), which is similar to the serum anti-AQP4 antibody, has been reported to be 40% (14 of 35 patients with CDMS) in Tohoku Province.[33] The prevalence of the serum anti-AQP4 antibody in Tokachi Province (3.0%) was lower than the prevalence rates reported in the abovementioned two studies. The population in this study is based on an epidemiological study in Tokachi Province, and we suppose our data could reflect the relatively precise prevalence of patients with the serum anti-AQP4 antibody in northern Japan. A more extensive study is required to clarify the relationship between the Barkhof criteria and the anti-AQP4 antibody.

Acknowledgements

We thank the following colleagues for enrolling patients in our study: Dr. Kamaya, Department of Neurology, Obihiro Nishi Hospital; Dr. Maeda, Department of Medicine, Kurosawa Hospital; and Dr. Yoshikawa, Tokachi Neurosurgical Hospital.

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Journal of Neuroimmunology 219 (2010) 105–108

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

High prevalence of autoantibodies against phosphoglycerate mutase 1 in patients with autoimmune central nervous system diseases

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ARTICLE INFO

Article history:
Received 11 July 2009
Received in revised form 7 November 2009
Accepted 19 November 2009

Keywords:
Autoantibody
Multiple sclerosis
Neuromyelitis optica
Phosphoglycerate mutase 1
Proteome

ABSTRACT

We identified the autoantibody against phosphoglycerate mutase 1 (PGAM1), which is a glycolytic enzyme, in sera from multiple sclerosis (MS) patients by proteomics-based analysis. We further searched this autoantibody in sera from patients with other neurological diseases. The prevalence of the anti-PGAM1 antibody is much higher in patients with MS and neuromyelitis optica (NMO) than in those with other neurological diseases and in healthy controls. It was reported that the anti-PGAM1 antibody is frequently detected in patients with autoimmune hepatitis (AIH). Results of our study suggest that the anti-PGAM1 antibody is not only a marker of AIH but also a nonspecific marker of central nervous system autoimmune diseases.

1. Introduction

Over the past few years, compelling data on the roles of B cells as sensors, coordinators and regulators of the immune response have strengthened the view that B cells and autoantibodies are fundamental factors for activating T cells and/or mediating tissue injury in several autoimmune-mediated diseases of the central nervous system (CNS) (Dalakas, 2008; Hasler and Zouali, 2006). In this study, we identified the autoantibody against phosphoglycerate mutase 1 (PGAM1) in sera from multiple sclerosis (MS) patients by proteomics-based analysis. Phosphoglycerate mutase is a glycolytic enzyme that catalyzes the interconversion of 3- and 2-phosphoglycerate with 2, 3-bisphosphoglycerate as the primer of the reaction (Fothergill-Gilmore and Watson, 1989). In mammalian tissues, there are two types of phosphoglycerate mutase: type M (also known as PGAM2) in muscles and type B (also known as PGAM1) in other tissues (Omenn and Cheung, 1974; Zhang et al., 2001). However, there are few reports on the anti-PGAM1 antibody (Lu et al., 2008; Zephir et al., 2006) and the specificity of this autoantibody is not clearly understood. To evaluate the specificity of this autoantibody, we assessed the prevalence of this autoantibody in sera from patients with various neurological diseases and healthy controls.

2. Patients and methods

2.1. Patients

Serum samples were obtained from patients with MS [$n=21$; male:female = 9:12; age range, 31–75; mean age, 49], neuromyelitis optica (NMO) [$n=13$; male:female = 2:11; age range, 26–79; mean age, 49], multiple cerebral infarctions (MCI) [$n=20$; male:female = 9:11; age range, 53–83; mean age, 71], infectious meningoencephalitis (IME) [$n=19$; male:female = 14:5; age range, 15–71; mean age, 45], and Parkinson's disease (PD) [$n=21$; male:female = 11:10; age range, 50–85; mean age, 68], and from healthy controls [$n=17$; male:female = 7:10; age range, 25–74; mean age, 44]. All the MS patients were diagnosed with clinically definite MS according to the criteria of Poser et al. (1983). All the NMO patients satisfied the 2006 revision to the Wingerchuk diagnostic criteria (Wingerchuk et al., 2006).

2.2. Preparation of tissue proteins

Under ether anesthesia, adult Wistar rats were sacrificed. Their cerebrums were immediately removed and frozen in dry-ice powder. The frozen brain tissue was homogenized in lysis buffer (7 M urea, 2 M thiourea, 0.4% CHAPS, 0.1% DTT, 0.5% Triton X-100, and 0.2% SDS) and centrifuged at 100,000 × g for 40 min. The obtained supernatant was used in all experiments.

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doi:10.1016/j.jneuroim.2009.11.014

2.3. Screening for autoantibodies against protein sample in sera from MS patients

We examined autoantibodies against a prepared protein sample in sera from five MS patients and five healthy controls by one-dimensional electrophoresis (1DE) and immunoblotting. The extracted proteins were applied at 20 μg/well to 4–20% polyacrylamide gel for western blotting. The proteins were separated by SDS-PAGE and separated proteins were blotted onto polyvinylidine difluoride (PVDF) membranes at 0.8 mA/cm^2 for 1 h using a semidry blotting apparatus (Trans-Blot SD semidry transfer cell, Bio-Rad Laboratories). Subsequently, the membranes were incubated in blocking solution overnight in a cold room, and then reacted with the sera from MS patients and healthy controls (diluted at 1:1500) for 1 h at room temperature, followed by washing. Then the membranes were incubated with HRP-conjugated anti-human Ig (A + G + M) antibodies (Zymed) (diluted at 1:2000) for 1 h at room temperature and reacted with the ECL-Plus Western blotting detection system (GE Healthcare).

2.4. Two-dimensional electrophoresis (2DE) and immunoblotting

A sample was loaded onto an immobilized and rehydrated dry strip (pH 3–10, nonlinear 18 cm long, GE Healthcare). Up to 100 μg of extracted proteins was applied to the dry strip for western blotting. Isoelectric focusing was carried out at 20 °C for 85,000 Vh at a maximum of 8000 V using a horizontal electrophoresis system (Coolphorestar IPG-IEF Type-PX, Anatech). This IPG strip was transferred to 12.5% polyacrylamide gel. The second-dimension run was carried out vertically using an electrophoresis apparatus (Coolphorestar SDS-PAGE Dual-200 K, Anatech) at 30 mA/gel. After the electrophoresis, the SDS-PAGE gels were stained with SyproRuby (Bio-Rad Laboratories) or used for protein transfer onto PVDF membranes (Toda and Kimura, 1997). Separated proteins were electrophoretically transferred to a PVDF membrane at a constant voltage of 32 V for 3 h using a buffer transfer tank with cool equipment (Toda et al., 2000). Subsequently, this membrane was incubated in a blocking solution (5% skim milk in 1× TBST and 1× TBS containing 0.1% Tween 20) overnight in a cold room, and then reacted with serum from a patient diluted (1:1500) with 1% skim milk in 1× TBST for 1 h at room temperature. The PVDF membrane was washed five times with 1× TBST and reacted with peroxidase-conjugated goat anti-human Ig (A + G + M) antibodies (Zymed) diluted (1:2000) with 1% skim milk in 1× TBST for 1 h at room temperature. After six washes, the membrane was incubated with the WB detection reagent (ECL-Plus, GE Healthcare) for 5 min and then scanned using a variable-mode imager (Typhoon 9400, GE Healthcare). The antibody-reactive protein spots were matched with the protein spots stained with SyproRuby (Bio-Rad Laboratories) using image analysis software (Adobe Photoshop 6.0).

2.5. In-gel digestion and mass spectrometry

Proteins were detected by staining with SyproRuby (Bio-Rad Laboratories). For mass spectrometric identification, the target protein spot on the SyproRuby-stained 2D electrophoresis gel was excised using FluoroPhoreStar 3000 (Anatech). In-gel digestion was performed in according with a standard protocol (Toda and Kimura, 1997) with minor modifications. Briefly, gel pieces were dehydrated and the dried gel pieces were rehydrated in 5 μl of 100 mM ammonium bicarbonate containing 10 μg/ml trypsin (Promega) for 3 h at 37 °C. After digestion, tryptic peptides were extracted twice with 50 μl of 66% acetonitrile in 0.1% trifluoroacetic acid (TFA) in a sonicator. The extracted peptides were dried, redissolved in 0.1% TFA, and injected onto a MonoCap 0.1 mm × 250 mm monolithic C18 column (Kyoto Monotech) with Prominence Nano (Shimadzu). The column eluent was spotted every 15 s onto a μFocus MALDI plate (Shimadzu GLC) with α-cyano-4-hydroxycinnamic acid (Sigma Aldrich) as a matrix using AccuSpot (Shimadzu). Buffer A consisted of 5% acetonitrile and 0.1% (v/v) TFA and buffer B consisted of 90% acetonitrile and 0.1% (v/v) TFA. The separation gradient was 5–60% buffer B over 30 min at a flow rate of 1 μl/min. The digests were

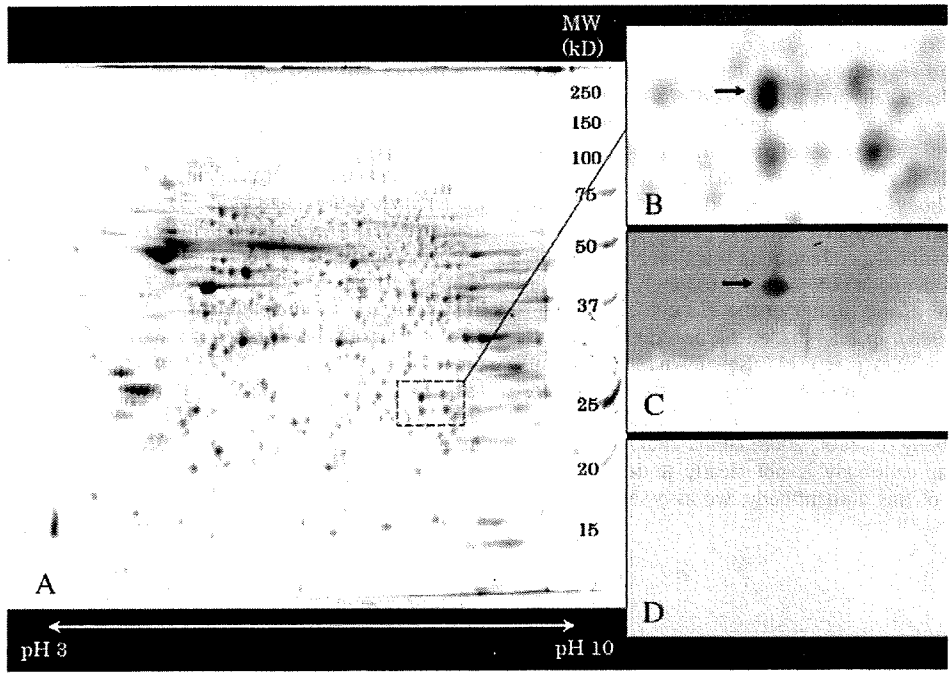

Fig. 1. Autoantibodies visualized by two-dimensional electrophoresis (2DE) and immunoblotting in sera from patients with multiple sclerosis (MS) and from healthy controls. (A, B) Total protein extracts of homogenized rat brain tissue were separated by 2DE, followed by SyproRuby staining. Arrow indicates the protein spot matching the immunoreactive spot detected by western blotting of MS patients' sera. Subsequently, this spot was identified as phosphoglycerate mutase 1 (PGAM1) by MALDI TOF-MS; (C) 1:1500-diluted MS patient's sera; arrow indicates the protein spot (PGAM1) recognized in sera from MS patients; and (D) 1:1500-diluted sera from healthy controls.

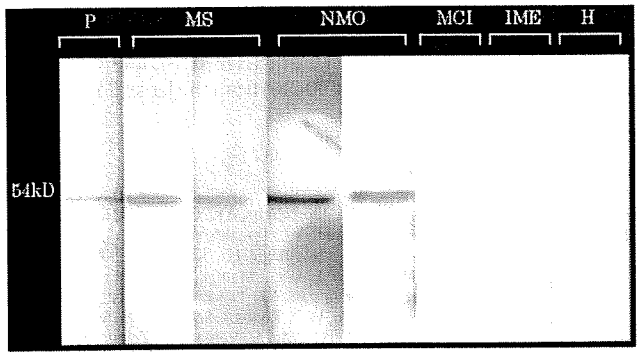

Fig. 2. Immunoblotting of human phosphoglycerate mutase 1 (PGAM1) full-length recombinant protein with GST. The recombinant protein (0.28 μg) was applied to each well. P, 1:300-diluted anti-PGAM1 monoclonal antibody (Abnova); MS, 1:1500-diluted sera from patients with multiple sclerosis; NMO, 1:1500-diluted sera from patients with neuromyelitis optica; MCI, 1:1500-diluted sera from patients with multiple cerebral infarctions; IME, 1:1500-diluted sera from patients with infectious meningoencephalitis; H, 1:1500-diluted sera from healthy controls.

analyzed on a MALDI TOF/TOF instrument, AXIMA Performance (Shimadzu). By utilizing information on the *x*–*y* positions of spotted samples on AccuSpot, autoexperiments using AXIMA Performance were performed to analyze the samples on the plates. Every autoexperiment and protein identification were performed using an integrated software, Kompact Ver.2.8. Protein identification was carried out using the MS/MS ion search database, Mascot (http://www.matrixscience.com/; Matrix Science Ltd.).

2.6. Immunoreactivity of sera from patients with various neurological diseases against human PGAM1 full-length recombinant protein

We examined the anti-PGAM1 antibodies in sera from 21 MS, 13 NMO, 21 PD, 20 MCI, to 19 IME patients, and 17 healthy controls by 1DE immunoblotting using the commercially available human PGAM1 full-length recombinant protein with GST (Abnova). Immunoblotting was carried out as described in Section 2.3. The screening dilution of sera from all patients and healthy controls was 1:1500.

2.7. Statistical analysis

We used the chi-square test with Yates' continuity correction to assess the difference in the prevalence of the anti-PGAM1 antibody between groups. Differences were considered significant at $P<0.05$.

3. Results

3.1. Screening and identification of target antigen of MS patients' autoantibodies (Fig. 1)

We examined the target antigen that reacted selectively with MS patients' sera. We detected by 1DE immunoblotting an approximately 30 kDa band corresponding to a protein that reacted with antibodies in sera from two out of five MS patients, but not with sera from five healthy controls. The same sample was subjected to 2DE, and one spot (observed MW/p*I*: 26,000/6.9) with a similar molecular weight reacted with the sera from these two MS patients but not with the sera from the healthy controls. We analyzed this spot by MALDI TOF-MS. This protein spot was identified as PGAM1 (accession number, P25113; score/coverage identification (%), 660/40; number of matched peptides, 11; theoretical MW/p*I*, 28,948/6.67).

3.2. Immunoreactivity of sera from patients with various neurological diseases against human PGAM1 full-length recombinant protein (Figs. 2 and 3)

To investigate whether the anti-PGAM 1 antibody is specific for MS, we examined this autoantibody in sera from patients with various neurological diseases (21 MS patients, 13 NMO patients, 21 PD patients, 20 MCI patients, and 19 IME patients) and 17 healthy controls by 1DE immunoblotting using the human PGAM1 full-length recombinant protein with GST (Figs. 2 and 3). As a result, the positivity rates were 81% (17 of 21) in MS patients, 77% (10 of 13) in NMO patients, 24% (5 of 21) in PD patients, 30% (6 of 20) in MCI patients, 32% (6 of 19) in IME patients, and 24% (4 of 17) in healthy controls. Statistically, the prevalence of the anti-PGAM1 antibody was significantly higher in patients with MS than in patients with PD ($P<0.001$), MCI ($P<0.003$), and IME ($P<0.005$), and in healthy controls ($P<0.002$). The prevalence of the anti-PGAM1 antibody was also significantly higher in patients with NMO than in patients with PD ($P<0.008$), MCI ($P<0.03$), and IME ($P<0.04$), and in healthy controls ($P<0.02$). These findings indicate that the anti-PGAM1 antibody has a stronger correlation with MS and NMO than with PD, MCI, IME, and being healthy.

4. Discussion

We identified PGAM1 as the target antigen of autoantibodies in sera from the MS patients by proteomics-based analysis. Western blotting analysis using the human PGAM1 recombinant protein

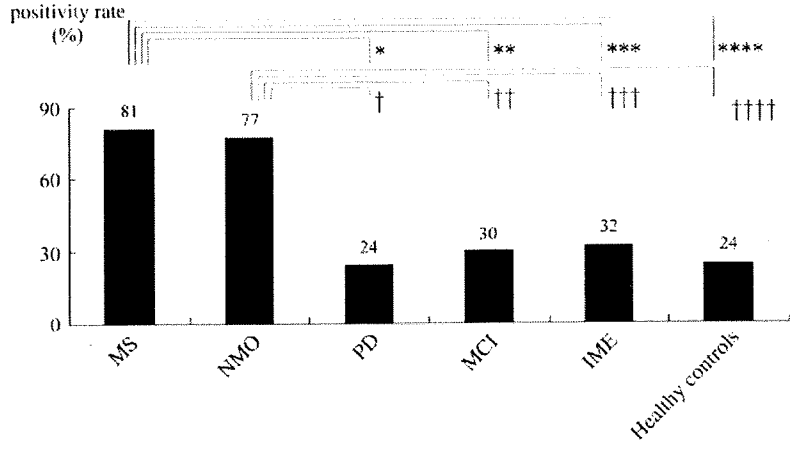

Fig. 3. Prevalence of antibodies against human phosphoglycerate mutase 1 (PGAM1) full-length recombinant protein. MS, patients with multiple sclerosis; NMO, patients with neuromyelitis optica; PD, patients with Parkinson's disease; MCI, patients with multiple cerebral infarctions; IME, patients with infectious meningoencephalitis. *$P<0.001$, **$P<0.003$, ***$P<0.005$, ****$P<0.002$, †$P<0.008$, ††$P<0.03$, †††$P<0.04$, and ††††$P<0.02$.

showed that the prevalence of the anti-PGAM1 antibody is much higher in not only patients with MS, but also those with patients with NMO, than in those with other neurological diseases and in healthy controls. To the best of our knowledge, this is the first study that elucidated the relationships between the anti-PGAM1 antibody and CNS autoimmune diseases. Lu et al. (2008) reported that the prevalence of the anti-PGAM1 antibody is much higher in patients with autoimmune hepatitis (AIH) than in those with other hepatic diseases and in healthy subjects. AIH is a rare liver disease and is characterized by hypergammaglobulinemia even in the absence of cirrhosis, characteristic autoantibodies, and a favorable response to immunosuppressive treatment (Zachou et al., 2004; Zolfino et al., 2002). Although the etiology of this disease is as yet unknown, the presence of several circulating autoantibodies such as the anti-nuclear antibody, anti-smooth muscle antibody, anti-liver kidney microsome type 1 antibody, and anti-liver cytosol type 1 antibody, which are serological markers for diagnostic criteria (Alvarez et al., 1999), suggests the important role of humoral mechanisms in AIH. There are several reports on MS patients with the complication of AIH (Pulicken et al., 2006; Takahashi et al., 2008; Ferrò et al., 2008). de Seze et al. (2005) reported that the prevalence of AIH seems to be about tenfold higher in patients with MS than in the general population. The anti-PGAM1 antibody can be generated in an immunological background common to both autoimmune CNS diseases and AIH.

Phosphoglycerate mutase is a glycolytic enzyme that catalyzes the interconversion of 3- and 2-phosphoglycerate with 2, 3-bisphosphoglycerate as the primer of the reaction (Fothergill-Gilmore and Watson, 1989). In mammalian tissues, PGAM exists in three isozymes, composed of homodimeric and heterodimeric combinations of two different subunits, type M (muscle form, PGAM2) and type B (brain form, PGAM1). The homodimer MM form is mainly expressed in the muscle; the BB form in the brain, kidney and liver; and the heterodimer MB form in the heart (Omenn and Cheung, 1974; Zhang et al., 2001). A previous study showed that PGAM1 is induced after hypoxia, which would occur in patients with cerebral infarction (Takahashi et al., 1998). In this study, the positivity rate of the anti-PGAM1 antibody in patients with MCI is not significantly higher than those in patients with other neurological diseases and in healthy controls. This finding suggests that an immunological background is important for production of the anti-PGAM 1 antibody.

In conclusion, the results of this study suggest that the anti-PGAM1 antibody is not only a marker of AIH but also a nonspecific marker of CNS autoimmune diseases. However, further studies are required to assess the presence of the anti-PGAM1 antibody in a large cohort of patients, including those with other autoimmune-mediated diseases, and controls.

Acknowledgments

We greatly thank Toshikazu Minohata of Shimadzu Corporation for technical assistance with the amino acid sequence analysis performed using an MS/MS and PSD mode of MALDI-TOF-MS (AXIMA Performance).

This research was partially supported by a Japanese Health and Labor Sciences Research Grant for Research on Psychiatry and Neurological Diseases and Mental Health (H18-026), and a Grant-in-Aid for Scientific Research (C) (1690486) from the Japan Society for the Promotion of Science (JSPS).

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Biochemistry and Neurobiology of Prosaposin: A Potential Therapeutic Neuro-Effector

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Abstract: Prosaposin, a 66 kDa glycoprotein, was identified initially as the precursor of the sphingolipid activator proteins, saposins A-D, which are required for the enzymatic hydrolysis of certain sphingolipids by lysosomal hydrolases. While mature saposins are distributed to lysosomes, prosaposin exists in secretory body fluids and plasma membranes. In addition to its role as the precursor, prosaposin shows a variety of neurotrophic and myelinotrophic activities through a receptor-mediated mechanism. In studies *in vivo*, prosaposin was demonstrated to exert a variety of neuro-efficacies capable of preventing neuro-degeneration following neuro-injury and promoting the amelioration of allodynia and hyperalgesia in pain models. Collective findings indicate that prosaposin is not a simple house-keeping precursor protein; instead, it is a protein essentially required for the development and maintenance of the central and peripheral nervous systems. Accumulating evidence over the last decade has attracted interests in exploring and developing new therapeutic approaches using prosaposin for human disorders associated with neuro-degeneration. In this review we detail the structure characteristics, cell biological feature, *in vivo* efficacy, and neuro-therapeutic potential of prosaposin, thereby providing future prospective in clinical application of this multifunctional protein.

1. INTRODUCTION

Prosaposin, a 66kDa glycoprotein, is a multifunctional protein and was first identified as the precursor protein of the sphingolipid activator proteins, saposins A-D [1, 2]. Saposins are 12-15 kDa heat-stable, small glycoproteins which activate sphingolipid hydrolases in lysosomes. They are generated from the common precursor, prosaposin, through limited proteolytic processing [3]. Prosaposin has been found in a secretory form in multiple body fluids, especially seminal plasma, human milk and cerebrospinal fluid [4, 5] and as a plasma membrane integral constituent [6, 7]. Prosaposin was also expressed in rat brains at birth and gradually increases in content when synaptogenesis begins to take place [8, 9]. Prosaposin mRNA expression during development is tissue-specific and abundant in brain and dorsal root ganglia during embryonic development [10, 11]. This distribution in intracellular and extracellular pools suggests that prosaposin may have physiological roles in its intact form, especially during central nervous system (CNS) development. The physiological significance of prosaposin is evidenced by death during fetal development or early childhood as a consequence of total saposin deficience in humans due to mutations in the prosaposin gene [12-17]. Pronounced generalised neurolysosomal storage combined with a severe neural depletion and hypomyelination are the typical neuropathological phenotype observed in the patients [12-16]. Accumulation of sphingolipids including glucocerebroside, lactosylceramide, globotriaosyl ceramide, globotetraosyl, sulphatides, and ceramides is evident in a variety of the patients' tissues. The lipid accumulation might include the accumulation of neurocytotoxic lipids such as psychosines that potentially cause neurodystrophy. On the other hand, it is unclear whether the lipid accumulation is as the consequence of mature saposin deficiency or prosaposin deficiency. Similar clinical features were also demonstrated in prosaposin knock-out mice [18, 19]. The severity of the CNS impairment together with the clinical and pathological features of prosaposin deficiency in humans underscores the crucial involvement of prosaposin in nervous system development.

An experiment *in vitro* using various cholinergic neuroblastomas demonstrated that prosaposin has versatile neurotrophic activities capable of promoting neurite outgrowth extension, choline acetyltransferase activity and neural cell survival [20]. The neurotophic region was pinpointed to a 12-amino acid sequence in the domain for saposin C and chemically synthesized peptides encompassing the neurotrophic region were shown to mimic the biological activity of prosaposin [21]. One of the unique characteristics of prosaposin is that it acts on both neurons and glial cells. On Schwann cells and oligodendrocytes, prosaposin acts as a myelinotrophic protein to increase sulfatide contents and to prevent cell death through a G protein-coupled receptor-

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1871-5249/09 $55.00+.00

mediated mechanism [22-24]. Recent studies with models of neuro-injury have shown the neuro-efficacy of prosaposin and its peptide mimetic, prosaptide. The present review summarizes the basic biochemical features and neuropharmacological properties of prosaposin to explore the potential of prosaposin as a future neurotherapeutic for treatment of patients with neuro-degenerative disorders.

2. DISCOVERY OF PROSAPOSIN

Since the first sphingolipid activator protein (sulfatide activator protein, saposin B) was discovered in 1964 [25], five activator proteins have been identified to date: four saposins (A-D) and GM2-activator protein [2]. GM2 activator protein, which promotes the lysosomal hydrolysis of GM2 ganglioside by β-hexosaminidase A, is coded by a gene located on chromosome 5 and is genetically distinct from the other four activators. In contrast all 4 saposins are coded by a single gene located on chromosome 10 [26-29]. Discovery of saposin deficiencies in humans substantiated the essential role of saposins in lysosomal sphingolipid hydrolysis *in vivo* [30-36]. Subsequently, the gene for the sulfatide activator protein was isolated from a human hepatoma cDNA library [37, 38]. Surprisingly, the clone was found to encode a 70 kDa protein with four highly homologous domains. The sequnce included the sulfatide activator protein (saposin **B**), the glucocerebrosidase activator (saposin C), and two additional putative saposin domains as illustrated in Fig. (**1**) [26]. Subsequently, the two putative saposins were isolated from Gaucher spleen and identified as saposins A and D [39-41], and the precursor protein was termed "prosaposin". Prosaposin was later isolated in an intact form from human milk and seminal plasma and characterized by biochemical and cell biological approaches as described below. It is noted that the mammalian and chicken prosaposin gene share such common structure [42]. A brief summary of properties and function of saposin proteins is provided in Table **1**.

3. BIOCHEMISTRY OF PROSAPOSIN

i. Structure of Saposin Proteins

In this section, we look into the structures of saposin proteins. While the molecular and structural nature of saposins had been well characterized, there is a lack of substantial information on the structure of prosaposin. Therefore, it is important to observe the structures of individual saposins in order to get insight into the molecular structure of prosaposin.

Amino acid sequencing of human prosaposin isolated from milk and seminal plasma indicated that the N-terminal amino acid of prosaposin corresponds to the glycine in the 17th position counting from the N-terminal methionine of pre-prosaposin. [5, 43]. This indicates that the first 16-amino acids are cleaved as the signal peptide to generate prosaposin. In addition, deglycosylation of prosaposin yielded a 54 kDa protein, indicating that about 12 kDa of carbohydrate is linked to the protein backbone as N-glycan [5]. Further structural details of prosaposin remains uncovered at this point; however, saposins have been well characterized. Therefore, it is reasonable to assume that the structural features of saposins contribute to the structure of prosaposin.

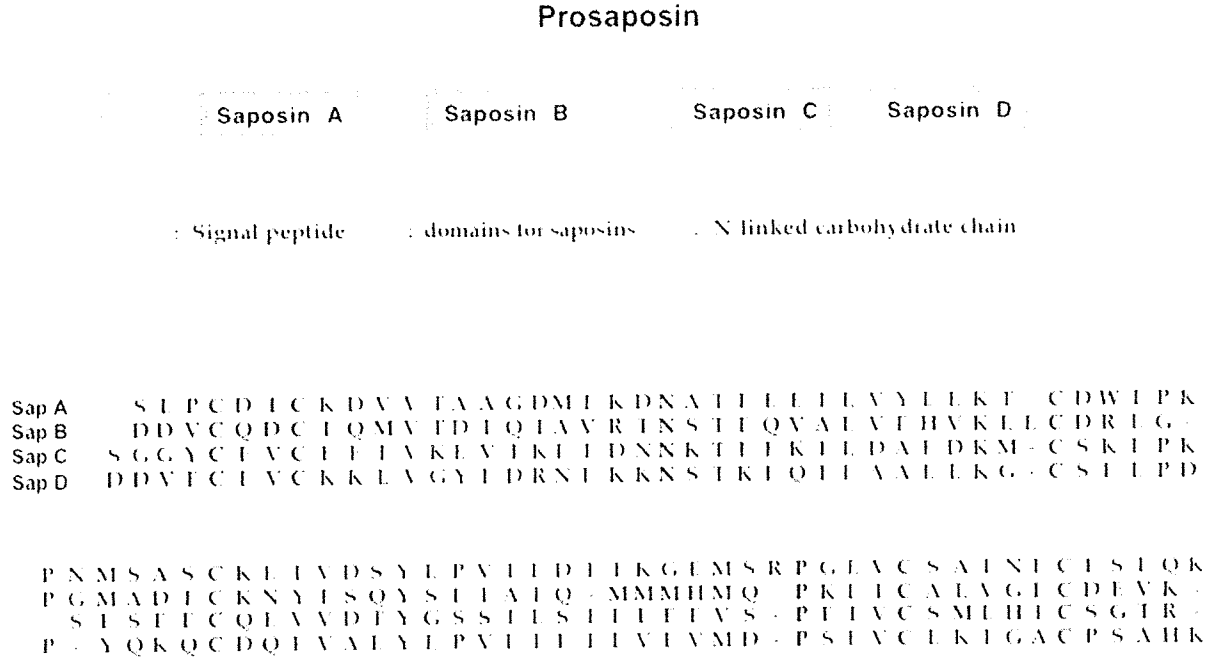

Fig. (1). Structure of prosaposin.
[*Top*] The structure of prosaposin was illustrated. [*Bottom*] Amino acid sequences of individual saposins. Boxes indicate structurally significant amino acids and shaded areas indicate other conserved amino acids.

Table 1. Properties and Function of Saposin Proteins

Saposin Protein	Molecular Weight	Distribution	Activator for	Deficiency in Man (Clinical symptoms)	Reference
Saposin A	15 kDa	lysosomes	glucocerebrosidase and galactocerebrosidase	globoid cell dystrophy-like	[34, 36]
Saposin B	12 kDa	lysosomes	arylsulfatase A, α-galactosidase, and β-galactosidase	metachromatic leukodystrophy-like	[30-32, 35]
Saposin C	12 kDa	lysosomes	glucocerebrosidase and galactocerebrosidase	Gaucher's disease-like	[33]
Saposin D	12 kDa	lysosomes	sphingomyelinase and ceramidase	not yet found	---
Prosaposin	66 kDa	body fluids and neural membranes	retains all activator activities except arylsulfatase A	severe neuro-degeneration with hypomyelination	[12-16]

Each saposin consists of about 80 amino acid residues, in which structurally important amino acid residues are placed at nearly identical positions. As shown in Fig. (**1**), all six cysteines, a proline essential for the turn and helix structure and an asparagine which serves as a N-glycosylation site [two in saposin A, one each in saposins B, C, and D] are all consistently placed across the four proteins. All six cysteine residues are involved in the formation of intra-molecular disulfide linkages, which together with the proline residue make those structures compact and rigid. Such structural features could be responsible for the saposin's extreme heat-stability and resistance to most proteases. The N-linked carbohydrate structure of each saposin is highly heterogeneous [44]. For example, the carbohydrate content of saposins from Gaucher spleen is about 20% in saposins B, C, and D and about 40% in saposin A. On SDS-PAGE, saposin A shows a higher molecular heterogeneity compared to the other saposins. This could be due to its higher carbohydrate content and micro-heterogeneity present in the N-linked carbohydrate chains at two different N-glycosylation sites. It has been demonstrated *in vitro* demonstrated that carbohydrate removal from saposin B has no effect on the stability and activity of saposin B; however, reduced, deglycosylated saposin B refolds aberrantly [45]. This might implicate a potential role for the N-glycan of saposins in folding, although its precise role remains unknown.

On CD spectroscopy, each saposin is α-helical-rich and highly structured [26]. The α-helical content of each saposin is maximal at the optimal pH for lysosomal enzymes stimulation, which might indicate that α-helical formation is involved in both their interaction with lipids and in overall activator function. Based on a secondary structural prediction, a triple helix structure for saposins A-C was proposed. In those helical coils, hydrophobic amino acids are likely to face inside the helixes, creating a hydrophobic pocket [26]. In contrast, saposin B has a unique structural feature with a higher β-sheet content compared to other saposins. In a separate analysis, saposins A-D were shown to have a polypeptide motif that shares common locations of six cysteines and several hydrophobic residues with acid sphingomyelinase and surfactant protein B [46]. It is interesting to note that sphingomyelinase and surfactant protein B are also known to interact with lipids. The study proposed that three intra-domain disulfide linkages could create a common amphipathic structural framework for diverse functions. This disulfide-linked α-helical structure is likely to be responsible for various functions; however, the structural basis for the structure-function relationship remains unclear.

Recent crystallography studies exposed further details on the structure of saposins [47, 48]. Fig. (**2**) shows the closed, lipid-free conformations of saposins A, C and D. The three-dimensional structures of saposin A, C and D reveal compact and mutually similar structures with four α-helixes and two intra-disulfide linkages. Very recently, further details on the crystal structures of human saposins C and D were proposed[49]. In those models, saposins C and D were shown to

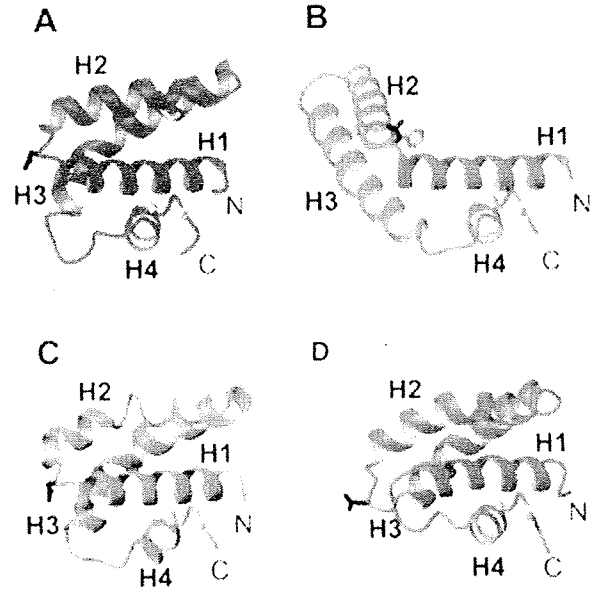

Fig. (2). Three-dimensional structures of saposin proteins.
The figure shows a ribbon diagram of **[A]** saposin A, **[B]** saposin B, **[C]** saposin C and **[D]** saposin D. The conserved Asn side chains which are N-glycosylated are shown with black arrow. The side chain of ^{24}Phe in saposin B is indicated by open arrow. It is noted that each saposin has three intra-molecular disulfide bonds: two between H1 and H4, one between H2 and H3.

form a homo-dimer but in a different way than previously understood. This likely creates their unique specificity difference in lipid recognition and membrane interactions. In addition, the study suggested that the ^{10}Lys and ^{17}Arg of saposin D are involved in initial association with polar lipid head-groups in biological membranes. On the other hand, saposin B has shown to form an unusual dimer structure with a large hydrophobic pocket surrounding a phosphatidylethanolamine [PE] molecule. The linkage between helixes H1 and H2 in saposin B is different from saposins A, C and D by the insertion of ^{24}Phe, which packs against the relatively small side chains of ^{17}Ala and ^{18}Val. The absence of ^{24}Phe and the larger side chains at positions 17 and 18 may enable collapse of the structure, as demonstrated in the structures of saposin A, C and D. It remains to be determined if the bound PE is responsible for these conformational discrepancies, or if saposin B adopts the open structure in the absence of lipid.

In order to gain insight further into the structure of prosaposin, it is also important to pay attention to some differences in activator function observed between prosaposin and mature saposins. One of the striking differences is the lipid binding nature. Saposin B binds to sulfatide to form a water soluble complex and promotes the enzymatic hydrolysis of sulfatide by arylsulfatase A. On the other hand, prosaposin showed virtually no sulfatide binding and no sulfatide activator activity [5, 50]. It has also been noted that prosaposin activates the enzymatic hydrolysis of GM1 ganglioside by lysosomal β-galactosidase similar to saposin B [50]. These findings implicate a structural difference between saposin B and the saposin B domain in the precursor protein. The structure created by the dimer formation might lend saposin B a unique lipid binding characteristic that is not shared with prosaposin.

ii. Prosaposin Localization

An immunochemical determination of prosaposin was attempted in various fluid samples from rats [51, 52]. The highest concentration of prosaposin was detected in fluid from efferent ducts of the rat testis while less was detectable in seminiferous tubules, testicular lymph, and serum. Prosaposin concentration in human tissues and body fluids were also determined immunochemically [5]. While prosaposin was barely detectable in brain, liver, kidney, testis, and placenta, body fluids such as milk, seminal plasma, and cerebrospinal fluid contained significant amounts as summarized in Table **2**. Prosaposin also occurs in neural membranes [6]. Subcellular fractionation studies have demonstrated the occurrence of membrane-bound prosaposin in the plasma membranes of rat neuroblastoma NS20Y cells. In addition to neural surface membranes, prosaposin was also found on the surface of neurites and in dendrites, axons, and cell bodies of subcortical and spinal cord neurons in human brain.

Table 2. Prosaposin Distribution in Various Body Fluids

Species	Sample	Concentration
		μγ/μλ
Rat	Serum	1.5
	Testis efferent duct fluid	109
	Seminiferous tubule fluid	60
	Testicular lymph fluid	7.1
	Caput epididymidis fluid	51.7
	Cauda epididymidis fluid	12.2
Human	Cerebrospinal fluid	10-35
	Milk	7
	Seminal plasma	90

iii. Prosaposin as the Precursor of Lysosomal Saposins.

Maturation of Saposin in Cultured Cells

The human prosaposin gene is translated to a pre-precursor protein in ribosomes and then post-translationally modified. Most of the precursor is processed into mature saposins and then distributed to lysosomes. The other small portion is localized to secreted fluids and membranes in the unprocessed precursor form. Pulse-chase studies, using cultured human skin fibroblasts, were conducted to understand details on the biosynthesis of saposin B and C [53, 54]. The studies demonstrated that both activator proteins were initially generated as a 65-68 kDa protein, which is converted further to a 70-73 kDa form within 30 min of chase. This is the first observation to predict the existence of the large precursor for the lysosomal activator proteins. The 70-73 kDa protein was secreted partly into the culture medium and another portion was processed to mature saposin. In the case of saposin B, the 70-73 kDa precursor was rapidly processed into mature 8-13 kDa saposin B via the formation of 52 and 35 kDa intermediates. Those intermediates could represent the trisaposin and disaposin species. On the other hand, saposin C [9-12 kDa] was generated via the formation of a 50 kDa intermediate. In both cases, mature saposins became detectable within 1.5 hours of chase. It has been postulated that the lysosomal targeting of prosaposin in human fibroblasts is predominantly mediated by the mannose-6-phosphate [M6P] receptor-dependent uptake mechanism [55]. Importantly, the 70-73 kDa intermediate was almost completely retained in the cells with about half being released by M6P. Several lines of evidence also implicate that the prosaposin trafficking is only partially dependent on the M6P receptor-mediated uptake mechanism. For example, murine embryonic fibroblasts deficient in M6P receptor efficiently internalized prosaposin [56]. An independent study demonstrated that the low-density lipoprotein [LDL] receptor is involved in cellular prosaposin uptake [57]. Although the precise role of the LDL receptor-mediated prosaposin uptake is still unclear, that mechanism might be important in prosaposin sorting to determine its multiple functional fates.

Prosaposin Processing In Vitro

During the course of prosaposin purification from human seminal plasma, we found and isolated proteolytically degraded prosaposin fragments with the molecular sizes of 48 kDa and 29 kDa [5]. Based on N-terminal sequence analysis and SDS-PAGE followed by immuno-blot analysis,

the 48 kDa and 29 kDa proteins are a trisaposin containing domains for saposin B-D and a disaposin containing domains for saposin C and D, respectively. This proteolytic degradation occurred maximally occurred at pH 3.5 and was inhibited completely by pepstatin, a specific inhibitor for lysosomal cathepsin D. This implicated cathepsin D as the primary protease responsible for prosaposin processing to mature saposins. This was substantiated by an *in vitro* study using purified prosaposin and cathepsin D [3]. In that study, cathepsin D cleaved prosaposin into two fragments each containing domains for saposins A-C and B-D [trisaposins]. Subsequently, the two fragments were cleaved into disaposins containing domains for saposins A and B and saposins C and D. Ultimately, the cathepsin D digestion generated four fragments that contain each saposin domain and part of the inter-domain sequences. No further processing occurred with cathepsin D. The final processing to generate mature saposins was achieved by incubating each fragment with isolated rat lysosomes. Thus other lysosomal protease activity is required for the *in vitro* maturation of saposin. Interestingly, gangliosides inhibited the cathepsin D-mediated proteolytic processing of prosaposin. Since gangliosides form a tightly bound complex with prosaposin [see Section **iv**], this finding indicates that prosaposin may be protected from lysosomal proteolysis by forming a complex with ganglioside. Complex formation of prosaposin with ganglioside might be important in prosaposin sorting to determine its extracelluar functionality. Further study is required to substantiate such a possibility.

iv. Lipid-Prosaposin/Saposin Interaction

The lipid binding properties of the sulfatide activator protein [saposin B] have been studied in detail [58]. Saposin B forms water-soluble complexes with sulfatide and various other glycospingolipids including GM1 and globotriaosylceramide. It has also been demonstrated to act as a glycosphingolipid transfer protein between liposomes. The carbohydrate chain length of lipids and the acyl residue in the ceramide moiety were shown to be significant factors for the lipid interaction nature of the activator protein. Although saposin B possesses the ability to interact with a variety of sphingolipids, it stimulates the enzymatic degradation of only GM1 and sulfatide derivatives. This study further demonstrated that sulfatide and ganglioside GM1 with short acyl chains did not require saposin B for their enzymatic hydrolyses by arylsulfatase A or β-galactosidase. This finding suggests that the activator protein could interact with its activating hydrolyases at their lipid binding sites but not at the catalytic sites to assist the enzymatic lipid hydrolysis. Further study is required to elucidate the action of saposin as a sphingolipid activator protein.

The lipid binding nature of prosaposin and saposins A-D have been further examined using various glycosphingolipids [50]. The study demonstrated that prosaposin and all saposins formed stable complexes with 13 different gangliosides; however, kinetic data suggested that the lipid binding mode of saposins A and B was distinct from that of the other group, saposins C and D. For example, saposins A and B bind to ganglioside GM1 in a single binding mode with high affinity, while saposins C and D show a two phase binding with high-affinity with much lower capacity and lower affinity. The ganglioside-binding of prosaposin showed an intermediate between these two groups of saposins. Importantly, prosaposin binds to a-series gangliosides with higher affinity, in particular, neurogenic gangliosides such as GQ1b and GT1b. On the other hand, b-series gangliosides, O-acetylated gangliosides and gangliosides with shorter carbohydrate chains were bound with lower affinity. In addition to ganglioside, saposin B forms water-soluble complexes with sulfatide. As mentioned earlier, the dimerization followed by PE acquisition might give saposin B this unique lipid binding nature [48]. The study also demonstrated that prosaposin and the other saposins also promoted the transfer of ganglioside GM1 from donor liposomes to acceptor human erythrocyte ghost membranes. This finding, together with its extracellular localization, suggests that prosaposin may need to be active as a ganglioside binding and transfer protein to be involved in neuronal differentiation and synaptogenesis *in vivo*.

4. CELL BIOLOGY OF PROSAPOSIN

i. Prosaposin as Neurotrophic Protein

Prosaposin was identified as a neurotrophic factor capable of promoting neurite outgrowth, cell survival, and choline acetyltransferase [ChAT] activity in various cholinergic neuroblastoma cells in culture [20]. The functional domain required for neurotrophic activity of prosaposin was pinpointed to a 12-amino acid stretch [^{18}LIDNNKTEKEI^{29}L] in the N-terminal portion of the saposin C domain as shown in Fig. (**3**) [21]. Nanomolar concentrations of a 22-mer peptide encompassing this region stimulated neurite outgrowth and ChAT activity, and prevented cell death in neuroblastoma cells and primary cerebellar granular neurons in culture [21, 59]. The neurotrophic region containing the two adjacent asparagines was a β-turn flanked by α-helical regions. This is also the most hydrophilic region within the protein. The adjacent asparagine is necessary for the neurotrophic activity of the sequence, since the amino acid-replacement of ^{21}N with D abolishes its neurotrophic potential [21]. An independent study similarly verified the importance of the residues 22-31 for the neuritogenic effect of saposin C [60].

ii. Cell Biological Function and Action Mode of Prosaposin

In vitro, a low nanomolar concentration of prosaposin was enough to activate extracellular signal-regulated protein kinase [ERK] and sphingosine kinase [SK] to generate sphingosine-1 phosphate [S1P], thus eliciting cell proliferation and cell death prevention [61-63]. In several cell types, ERK-activated signaling by a G-protein-dependent mechanism is an absolute requirement for triggering proliferative response. Our study demonstrated that prosaposin treatment induced PC12 entry into the S phase of the cell cycle, which required ERK phosphorylation and thus was potentially a G-protein-dependent mechanism [62]. It has been shown that external stimuli modulate lipid enzyme activities such as sphingomyelinase, ceramidase and sphingosine kinase to regulate cellular sphingolipid turnover. It is well-known that the balance between cellular levels of ceramide [cell death inducer] and S1P [cell death inhibitor] is critical in maintaining cellular homeostasis. Prosaposin could be a key modulator in the ceramide-S1P rheostat via the ERK pathway, thus