

Detection of anti-Nogo receptor autoantibody in the serum of multiple sclerosis and controls

Onoue H, Satoh J-I, Ogawa M, Tabunoki H, Yamamura T. Detection of anti-Nogo receptor autoantibody in the serum of multiple sclerosis and controls.

Acta Neurol Scand 2007; 115: 153–160.

© Blackwell Munksgaard 2006.

Objectives – A myelin-associated neurite outgrowth inhibitor Nogo-A plays a key role in inhibition of axonal regeneration. Axonal damage beginning at the early stage of multiple sclerosis (MS) is responsible for permanent neurological deficits, although its molecular mechanism remains unknown. The aim was to study the prevalence of autoantibodies against Nogo-A and Nogo receptor (NgR) in the serum of MS. **Methods** – The antibodies were identified in the serum of 30 MS patients, 22 patients with non-MS other neurological diseases (OND), and 22 healthy control (HC) subjects by Western blot using recombinant human Nogo-A-specific segment (NAS), the shared segment of Nogo-A and -B (NAB), Nogo-66 (N66), the non-glycosylated form of NgR, the glycosylated NgR (NgR-Fc), and myelin oligodendrocyte glycoprotein (MOG). **Results** – None showed immunoglobulin G (IgG) antibodies against NAS or NAB. In contrast, 30% of MS, 23% of OND and 32% of HC subjects exhibited anti-N66 IgG, while 27% of MS, 27% of OND and 18% of HC showed anti-MOG IgG. None of HC but 33% of MS and 14% of OND showed anti-non-glycosylated NgR IgG. Furthermore, 60% of MS, 18% of OND and 14% of HC showed anti-NgR-Fc IgG. **Conclusions** – Because IgG autoantibodies against N66, NgR and MOG are often detected in the serum of MS and controls, they do not serve as an MS-specific marker.

**H. Onoue¹, J.-I. Satoh^{1,2},
M. Ogawa³, H. Tabunoki²,
T. Yamamura¹**

¹Department of Immunology, National Institute of Neuroscience, NCNP, Tokyo, Japan; ²Department of Bioinformatics, Meiji Pharmaceutical University, Tokyo, Japan; ³Department of Neurology, National Center Hospital for Mental, Nervous and Muscular Disorders, NCNP, Tokyo, Japan

Key words: autoantibodies; multiple sclerosis; Nogo-A; Nogo receptor; Western blot

Jun-ichi Satoh, Department of Bioinformatics, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan
Tel.: +81 42 495 8678
fax: +81 42 495 8678
e-mail: satoj@ncnp.go.jp or satoj@my-pharm.ac.jp

Accepted for publication July 19, 2006

Introduction

Axonal damage beginning at the early stage of multiple sclerosis (MS) is responsible for permanent neurological deficits and progression of clinical disability (1, 2). Although the underlying molecular mechanism remains unknown, the degree of inflammatory demyelination correlates with the extent of axonal damage, suggesting an involvement of proinflammatory mediators in development of axonal degeneration in MS (3). The alternative possibility could be proposed that axonal regeneration is severely impaired in MS lesions, because an extensive accumulation of neurite outgrowth inhibitors and glial scar provides a non-permissive environment for axonal regrowth (4).

Nogo constitutes a family of myelin-associated inhibitors of axonal regeneration (5, 6). The Nogo gene encodes three distinct isoforms named A, B and C, derived by alternative splicing and promoter usage, all of which share a C-terminal 66 amino acid segment named Nogo-66 located between the two transmembrane domains. Nogo-A, the longest isoform, is the major neurite outgrowth inhibitor expressed on oligodendrocytes and myelin sheath and a subpopulation of neurons in the central nervous system (CNS) (7, 8). Nogo-A has at least two discrete regions with neuronal growth-inhibitory activities. One is located in the Nogo-A-specific region that restricts neurite outgrowth, while another is Nogo-66 that induces growth cone collapse, both of which assume different membrane topologies depending on cell types (9). Nogo-66

binds to the Nogo receptor (Nogo-66 receptor-1; NgR), a glycosylphosphatidylinositol-anchored membrane protein (10). In contrast to Nogo-A, NgR is not identified on oligodendrocytes but expressed in a subpopulation of neurons and their axons, including cerebral cortical pyramidal neurons and cerebellar Purkinje cells (10, 11). Signal transduction mediated by NgR depends on its association with a coreceptor p75^{NTR} or TROY in combination with an adaptor molecule LINGO-1 (12–14). Not only Nogo-66 but also myelin-associated glycoprotein and oligodendrocyte-myelin glycoprotein bind to NgR and transduce neurite growth-inhibitory signals via p75^{NTR} by activating RhoA and inhibiting Rac1 (15). *In vivo* blockade of interaction between NgR and its ligands, by neutralizing anti-Nogo-A antibodies, NgR antagonistic peptides, or soluble truncated NgR, induces extensive axonal regeneration and enhances functional recovery after injury in the CNS (16–18). Furthermore, NgR-deficient mice exhibit an enhanced axonal plasticity after ischemic stroke, accompanied by improved functional recovery (19).

Increasing evidence indicates that autoantibodies against CNS antigens play a key role in the immunopathogenesis of MS. The detection of anti-myelin oligodendrocyte glycoprotein (MOG) antibody in the patients with clinically isolated syndrome (CIS) predicts the early conversion of CIS to clinically definite MS (20). The detection of immunoglobulin G (IgG) autoantibody against aquaporin-4 discriminates the patients with neuromyelitis optica from those with the conventional MS (21). Recently, autoantibodies against the Nogo-A segment spanning amino acid residues 1–979 are identified in the serum of relapsing–remitting MS (RRMS) patients, although they show no significant correlation to disease course, duration, relapse rate and disability of the patients (22). More recently, we found that Nogo-A expression is enhanced in surviving oligodendrocytes, while NgR is upregulated in reactive astrocytes at the edge of chronic demyelinating lesions of MS, suggesting that Nogo-A/NgR mediates glial–glial interaction under demyelinating environments (23). Inflammatory demyelination and axonal damage are less severe in Nogo-A-deficient mice affected with experimental autoimmune encephalomyelitis, an animal model of MS (24). These observations propose a possible scenario that the Nogo-A/NgR interaction restricts axonal regeneration in MS brain.

The present study is designed to investigate the prevalence of autoantibodies against a panel of Nogo and NgR fragments in the serum of MS and

controls by Western blot analysis, and to determine the possible association of these antibodies to clinical profiles of MS.

Patients and methods

Study population

The serum samples were obtained from randomly selected 30 MS patients, 22 patients with other neurological diseases (OND) who visited the National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan in 2004, and 22 healthy control (HC) subjects. Written informed consent was obtained from all the subjects. MS was diagnosed following the established criteria (25) by qualified neurologists of the hospital. The MS population was composed of 10 men and 20 women showing the mean age of 41.5 ± 12.2 years, the mean disease duration of 7.1 ± 6.3 years, and the mean Expanded Disability Status Scale (EDSS) score of 3.6 ± 2.0 . The MS group included 17 patients with RRMS during remission, nine with RRMS in acute relapse, three with secondary progressive MS (SPMS) and one with primary progressive MS (PPMS). Among all MS patients, nine were under IFN β treatment, 11 were treated with corticosteroids, and two with azathioprine at the time of blood sampling. The patients received no other immunosuppressive or immunomodulatory drugs. The OND population included 13 men and nine women with the mean age of 57.7 ± 13.8 years, composed of six patients with spinocerebellar degeneration, five with Parkinson's disease, three with myopathy, two with neuropathy, one with myasthenia gravis, Fisher syndrome, adrenoleukodystrophy, spastic paraplegia, epilepsy, or cerebral infarction. The HC population was composed of nine men and 13 women presenting with the mean age of 41.7 ± 12.4 years. After sampling, the serum samples were stored immediately at -30°C before use.

Recombinant human Nogo and NgR fragments

The human Nogo-A gene encoding the Nogo-A-specific segment (NAS; amino acids 186–1004), the segment shared between Nogo-A and Nogo-B (NAB; amino acids 1–185), and the Nogo-66 segment (N66; amino acids 1053–1118), the human NgR gene encoding the full-length NgR following a cleavage of the N-terminal signal peptide (amino acids 27–473), and the human MOG gene encoding the full-length MOG

Table 1 Primers utilized for PCR-based cloning

Genes	Proteins (symbol, amino acid residues)	GenBank accession no.	Sense primers	Antisense primers
RTN4	Nogo-A-specific segment (NAS, 186–1044)	NM_020532	5'-gatgagacccttttgctctct-3'	5'-tcataagtttactcagctctgta-3'
RTN4	Nogo-A/B-shared segment (NAB, 1–185)	NM_020532	5'-atggaagaactggaccagtctct-3'	5'-tcacactgagcccaggagccct-3'
RTN4	Nogo-66 segment (N66, 1053–1118)	NM_020532	5'-agcttaggataatacaagggtgtg-3'	5'-tcaagaatcaactaatcatcaactaa-3'
RTN4R	Nogo receptor without an N-terminal signal peptide (NgR, 27–473)	NM_023004	5'-tgccagggtcctgctatgtctacaat-3'	5'-tcagcaggggccaagcactgtccacag-3'
MOG	Myelin oligodendrocyte glycoprotein without an N-terminal signal peptide (MOG, 30–247)	NM_206809	5'-gggcagttcagagtgataggaccaaga-3'	5'-tcagaagggttctgtagctcttcaagg-3'

The PCR product was cloned into a prokaryotic expression vector pTrcHis-TOPO to produce a fusion protein with an N-terminal Xpress tag in *E. coli*.

following a cleavage of the N-terminal signal peptide (amino acids 30–247) were amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) from cDNA of NTera2-derived human neurons (26) or human brain using a panel of sense and antisense primer sets listed in Table 1. The PCR product was cloned into a prokaryotic expression vector pTrcHis-TOPO (Invitrogen, Carlsbad, CA, USA). After transformation of the vector into *Escherichia coli*, the expression of recombinant proteins was induced by exposing the culture of *E. coli* to isopropyl β -thiogalactoside. All recombinant proteins having an N-terminal Xpress tag for detection were purified from the *E. coli* lysate by isolating the specific bands immunoreactive with monoclonal anti-Xpress antibody (Invitrogen) separated on an 8%, 12% or 15% SDS-PAGE gel. The recombinant proteins were eluted from the gels, precipitated by cold acetone, and dissolved in distilled water containing 0.1% SDS. The protein concentration was determined by a Bradford assay kit (BioRad, Hercules, CA, USA). For control, the pTrcHis-TOPO vector containing a *lacZ* gene fragment (Invitrogen) was transformed into *E. coli* in parallel. Recombinant human NgR (Met1-Ser447)-IgG Fc (Pro100-Lys330) fusion protein (95 kDa), a glycosylated form expressed in a mouse myeloma cell line, was obtained from R&D Systems (Minneapolis, MN, USA).

Western blot analysis

Six different recombinant proteins, including NAS, NgR, LacZ, NAB, MOG and N66, were separated on a single 12% SDS-PAGE gel. After gel electrophoresis, they were transferred onto a nitrocellulose membrane. This membrane is designated the miniarray. Then, the array was incubated at room temperature (RT) overnight with the serum diluted at a concentration of 1:1000 in phosphate-buffered saline containing 5% skim milk. It was followed by incubation at RT for

60 min with horseradish peroxidase (HRP)-conjugated anti-human IgG (160 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with an HRP-conjugated antibody specific for human IgM Fc_{5 μ} fragment (8 ng/ml; Jackson ImmunoResearch, West Grove, PA, USA). To identify autoantibodies against NgR-Fc, the serum was diluted at a concentration of 1:15,000, and either an HRP-conjugated antibody specific for human IgG F(ab')₂ fragment (20 ng/ml; Jackson ImmunoResearch) or a HRP-conjugated antibody against human IgM Fc_{5 μ} fragment (120 ng/ml; Jackson ImmunoResearch) was utilized as a secondary antibody. The specific reaction was visualized at 15 min after incubating the array for 5 min with a chemiluminescent substrate (Pierce, Rockford, IL, USA). The optimal concentration of the serum, secondary antibodies, and incubation time prior to development was determined to obtain the maximum signal/background intensity by preliminary experiments that include varying combinations of serially diluted sera and secondary antibodies, and incubation periods. The signal intensity was graded as (–) none, (+) weak, (++) strong, and (+++) extremely strong.

Statistical analysis

The statistical difference in clinical profiles between the serum autoantibody-positive and negative groups of MS patients was evaluated by Mann-Whitney *U*-test. A *P* value of <0.05 was considered statistically significant.

Results

Miniarray of Nogo and NgR fragments

A panel of recombinant proteins, such as NAS, NgR, LacZ, NAB, MOG and N66, all of which have an N-terminal Xpress tag, were separated on a single 12% SDS-PAGE gel and transblotted onto

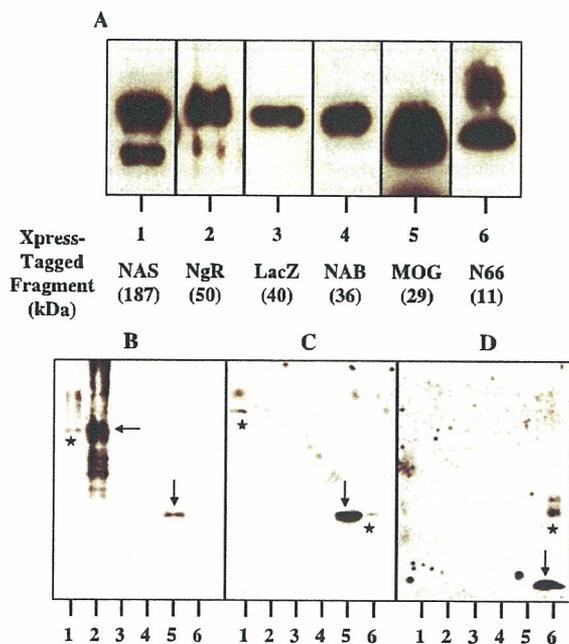


Figure 1. Miniarray of Nogo and NgR fragments. A panel of six different recombinant proteins, all of which have an N-terminal Xpress tag, were purified from *E. coli* transformed by PCR-based cloning strategy using primer sets listed in Table 1. The recombinant proteins, whose amounts range from 60 ng to 11.6 µg per lane, were separated on a single 12% SDS-PAGE gel and transblotted onto the identical nitrocellulose membrane, designated the miniarray. (A) The miniarray immunolabeled with anti-Xpress antibody. The lanes (1–6) represent (1) NAS (187 kDa), (2) NgR (50 kDa), (3) LacZ (40 kDa), (4) NAB (36 kDa), (5) MOG (29 kDa), and (6) N66 (11 kDa). The panels of the miniarray (B–D) represent the serum of (B) SPMS exhibiting both anti-non-glycosylated NgR IgG (lane 2, extremely strong; +++) and anti-MOG IgG (lane 5, weak; +), (C) RRMS in remission exhibiting anti-MOG IgG (lane 5, extremely strong; +++) and (D) HC subject presenting with anti-N66 IgG (lane 6, extremely strong; ++++). The specific bands are indicated by arrows, while non-specific reactions, possibly derived from the cross-reaction with contaminating *E. coli* proteins, are indicated by asterisks.

the identical nitrocellulose membrane, designated the miniarray. Then, the array was immunolabeled with anti-Xpress antibody (Fig. 1A). To verify whether the same amount of recombinant proteins was blotted on each array, the signal intensity of Xpress-immunoreactive bands was normalized between individual arrays onto which different serum samples were exposed. The profile of serum IgG autoantibodies varied from case to case, eliminating the possible cross-reactivity of secondary antibodies with recombinant proteins (Fig. 1B–D). This is further confirmed by the observation that negative controls including all the steps except for incubation with the serum detected no immunoreactive bands (data not shown).

Detection of autoantibodies against MOG, N66, and NgR in the serum of MS and controls

None of the subjects examined showed IgG autoantibodies against NAS or NAB (Table 2). In contrast, 30% of MS patients, 23% of OND patients, and 32% of HC subjects exhibited anti-N66 IgG expressing a specific band with varying intensities (Table 2 and Fig. 2A, lanes 1–7). Furthermore, 27% of MS patients, 27% of OND patients, and 18% of HC subjects showed anti-MOG IgG (Table 2 and Fig. 2B, lanes 1–7). IgG antibody against non-glycosylated NgR was identified in 33% of MS patients and 14% of OND patients, whereas it was undetected in any HC subject (Table 2 and Fig. 3A, lanes 1–7). In several cases, minor bands with lower molecular weights were detected in the blots, which possibly represent immunoreactivity against a small amount of degraded NgR proteins (Fig. 3A). No subjects showed IgG antibody against LacZ fragment tagged with Xpress (Table 2), excluding the possibility that the serum IgG autoantibodies against N66, MOG, and NgR were directed to a potentially immunogenic epitope of the tag. In contrast to the frequent detection of anti-NgR IgG, only two OND patients showed IgM antibody against non-glycosylated NgR (Table 2 and Fig. 3B, lanes 1, 2).

A larger proportion (60%) of MS patients, 18% of OND patients, and 14% of HC subjects showed IgG antibody against glycosylated NgR-Fc (Table 2 and Fig. 3C, lanes 1–7). All progressive MS patients ($n = 4$, Table 2) expressed anti-NgR-Fc IgG. In contrast, no subjects showed anti-NgR-Fc IgM (Table 2). Among RRMS patients positive for anti-NgR-Fc IgG, the patients in remission (12/17) greatly outnumbered those in acute relapse (2/9), while among RRMS patients with anti-MOG IgG, the patients in acute relapse (4/9) outnumbered those during remission (3/17) (Table 2), raising the possibility that anti-NgR-Fc IgG is overexpressed in RRMS patients in remission. However, no significant differences were found between MS patients positive or negative for anti-NgR-Fc IgG in age, sex, disease duration, EDSS score, the number of recent relapses, the number of patients with spinal cord involvement, and the treatment given at blood sampling (Table 3).

Discussion

The present study using recombinant human proteins as target antigens on Western blot showed that a substantial proportion of MS patients, OND patients, and HC subjects exhibited the serum IgG

Table 2 Detection of autoantibodies against Nogo and NgR fragments in the serum of MS and controls

	MS (n = 30)	MS subtypes				OND (n = 22)	HC (n = 22)
		RRMS (total) (n = 26)	RRMS (in remission) (n = 17)	RRMS (in relapse) (n = 9)	SP/PPMS (n = 4)		
NAS IgG	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
NAB IgG	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
N66 IgG	9 (30)	7 (27)	4 (24)	3 (33)	2 (50)	5 (23)	7 (32)
NgR IgG	10 (33)	8 (31)	7 (41)	1 (11)	2 (50)	3 (14)	0 (0)
NgR IgM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (9)	0 (0)
NgR-Fc IgG	18 (60)	14 (54)	12 (71)	2 (22)	4 (100)	4 (18)	3 (14)
NgR-Fc IgM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
MOG IgG	8 (27)	7 (27)	3 (18)	4 (44)	1 (25)	6 (27)	4 (18)
LacZ IgG	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

The number of subjects with immunoreactive bands is shown with the percentage in parenthesis. RRMS, relapsing–remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; OND, non-MS other neurological diseases; HC, healthy controls; NAS, Nogo-A-specific fragment; NAB, Nogo-A and Nogo-B-shared fragment; N66, Nogo-66 fragment; NgR, non-glycosylated Nogo receptor fragment; NgR-Fc, glycosylated Nogo receptor-IgG Fc fusion protein; MOG, myelin-oligodendrocyte glycoprotein.

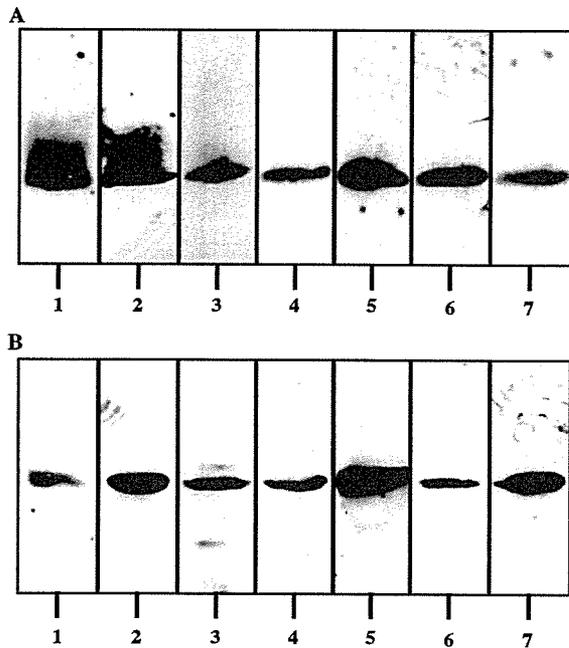


Figure 2. Detection of IgG autoantibodies against N66 and MOG in the serum of MS and controls. The miniarray was incubated with the serum of MS patients, the patients with non-MS neurological diseases (OND) or healthy control (HC) subjects. It was followed by incubation with HRP-conjugated anti-human IgG. (Panel A) Anti-N66 IgG. The lanes (1–7) represent the serum of (1) RRMS in acute relapse, (2) SPMS, (3) Parkinson's disease, (4) adrenoleukodystrophy, (5) HC, (6) HC, and (7) HC. (Panel B) Anti-MOG IgG. The lanes (1–7) represent the serum of (1) RRMS in remission, (2) RRMS in remission, (3) Parkinson's disease, (4) spinocerebellar degeneration, (5) spinocerebellar degeneration, (6) HC, and (7) HC.

autoantibodies against N66, MOG, and NgR. These results agree with a previous study reporting the substantial prevalence of the serum anti-MOG IgG in MS (20). In contrast, no subjects showed

IgG antibodies against NAS or NAB, excluding the possibility that the detection of anti-N66, anti-MOG, and anti-NgR antibodies in our study is simply attributable to non-specific binding. However, our observations do not support a recent study showing that both IgG and IgM autoantibodies against the Nogo-A 1–979 fragment, corresponding to a segment overlapping NAS and NAB in the present study, is often found in the serum of RRMS and OND patients (22). This discrepancy might be derived from following differences between the previous study and our own. First, the study populations were different between both of them. The previous study enrolled 55 patients with RRMS and 38 patients with chronic progressive (CP) MS (22). Ten of them were under treatment with intravenous immunoglobulins (IVIG), which potentially affects the autoantibody profile. In contrast, our study included 26 patients with RRMS, three with SPMS and one with PPMS. None of them received IVIG treatment. Second, the previous study focused on the ELISA-based immunoassay (22), which has more chance of detecting non-specific binding as positive. In contrast, we utilized Western blot throughout the study. Third, the previous study did not include in their assay irrelevant recombinant proteins having the same epitope tag that target fusion proteins have (22), whereas our miniarray included Xpress-tagged LacZ as a negative control.

The present study for the first time showed that 60% of MS patients, 18% of OND patients, and 14% of HC subjects have IgG antibody against glycosylated NgR-Fc. The DNAQLR motif located in the third leucine-rich repeat domain of NgR is the principal epitope recognized by a monoclonal anti-NgR antibody with a capacity to block binding of all NgR ligands (27). The

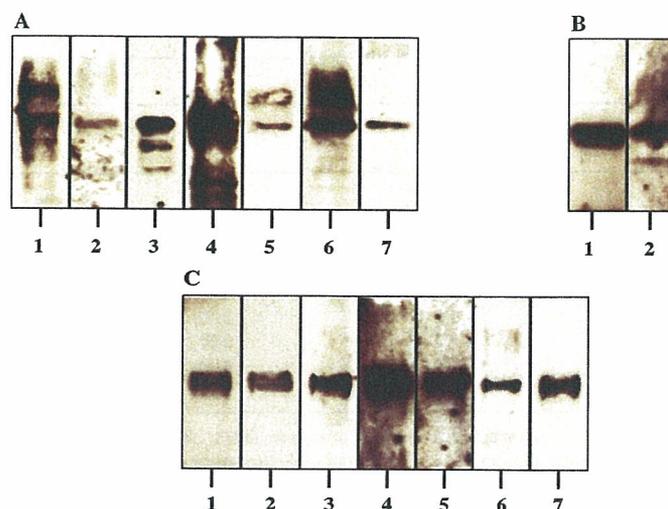


Figure 3. Detection of IgG and IgM autoantibodies against NgR and NgR-Fc in the serum of MS and controls. The miniarray and the NgR-Fc blot were incubated with the serum of MS patients, OND patients or HC subjects. It was followed by incubation with HRP-conjugated anti-human IgG (panel A), IgM $Fc_{\gamma 2\mu}$ (panel B), or IgG $F(ab')_2$ (panel C). (Panel A) IgG antibody against non-glycosylated NgR. The lanes (1–7) represent the serum of (1) RRMS in remission, (2) RRMS in remission, (3) RRMS in remission, (4) SPMS, (5) Parkinson's disease, (6) spinocerebellar degeneration, and (7) Charcot–Marie–Tooth disease CMT1A. (Panel B) IgM antibody against non-glycosylated NgR. The lanes (1,2) represent the serum of (1) spastic paraplegia of unknown etiology and (2) Fisher syndrome. (Panel C) IgG antibody against glycosylated NgR-Fc. The lanes (1–7) represent the serum of (1) RRMS in remission, (2) RRMS in remission, (3) RRMS in remission, (4) spastic paraplegia of unknown etiology, (5) mitochondrial encephalomyopathy, (6) HC, and (7) HC.

Table 3 Clinical characteristics of MS patients positive or negative for IgG antibodies against NgR-Fc in the serum

Clinical characteristics	NgR-Fc IgG-positive MS ($n = 18$)	NgR-Fc IgG-negative MS ($n = 12$)
Age (years) ^a	42.2 ± 10.6	40.4 ± 14.8
Male vs female (number)	8 vs 10	2 vs 10
Disease duration (years) ^a	6.6 ± 5.7	7.9 ± 7.2
EDSS score ^a	3.7 ± 2.2	3.5 ± 1.8
Relapses during the recent one year (number) ^a	0.7 ± 0.9	1.0 ± 1.0
Patients with spinal cord involvement (number and %)	8 (44%)	5 (42%)
Patients in remission, in relapse, and in progression (number)	12 vs 2 vs 4	5 vs 7 vs 0
Patients receiving IFN β , corticosteroids, and azathioprine at blood sampling (number)	5 vs 7 vs 2	4 vs 4 vs 0

^aNo significant differences are found by Mann–Whitney's *U*-test between the two groups of MS in age, disease duration, EDSS score, and relapses during the recent one year. NgR-Fc, glycosylated Nogo receptor-IgG Fc fusion protein; EDSS, Expanded Disability Status Scale.

Nogo-66 region contains several immunogenic epitopes for mouse T and B cells (28). Although immunogenic epitopes of NgR and Nogo-A in the human immune system remain to be clarified, our observations suggest that both NgR and Nogo-66 are more immunogenic than NAS and NAB for activation of human B cells. Among RRMS patients with anti-NgR-Fc IgG, the patients in remission ($n = 12$) outnumbered those in acute relapse ($n = 2$). Therefore, the possibility exists that this antibody might play a role in the maintenance of remission or the protection against relapse in RRMS, by blocking the interaction between Nogo-66 on oligodendrocytes/myelin and

NgR on neurons/axons. This hypothesis should be evaluated by future studies including the larger cohort of MS patients and controls.

Stress-inducing stimuli greatly affect the levels of Nogo-A expression in distinct cell types. Global ischemia enhances Nogo-A expression on the myelin sheath in the adult rat brain (29). Nogo-A expression is enhanced around the lesion site, whereas NgR is maintained at constant levels in the adult mouse and rat spinal cord following injury (7, 11, 30, 31). Kainate-induced seizure elevates Nogo-A mRNA levels in the adult rat hippocampal neurons (32). Nogo-A is induced in hippocampal neurons of the patients with

temporal lobe epilepsy (33). Unexpectedly, we identified the serum IgG autoantibodies against N66, MOG, and NgR-Fc not only in MS but also in non-MS and HC subjects. Our observations indicate that both Nogo-66 and NgR in addition to MOG serve as an autoantigen in the human immune system. Supporting this, a recent study identified anti-MOG IgG in the serum of both RRMS patients and HC subjects at the similar frequency (34). Increasing evidence indicates that Nogo-A, Nogo-B, Nogo-C and NgR are widely distributed in the cells and tissues outside the CNS (35–37). Nogo-A expression is elevated in postmortem and biopsied muscles of amyotrophic lateral sclerosis patients (38, 39). These observations suggest that the Nogo-66/NgR interaction plays an as yet undefined extraneural role, associated with an increased probability of antibody production under pathological conditions, when they are exposed and recognized by the human immune system.

In conclusion, IgG antibodies against N66, NgR, and MOG are frequently detected in the serum of both MS patients and controls. Although the study population is small and heterogeneous, we could definitely conclude that these autoantibodies do not serve as a disease-specific marker for MS.

Acknowledgements

This work was supported by grants from Research on Psychiatric and Neurological Diseases and Mental Health, the Ministry of Health, Labour and Welfare of Japan (H17-kokoro-020), Research on Health Sciences Focusing on Drug Innovation, the Japan Health Sciences Foundation (KH21101), and the Grant-in-Aid for Scientific Research, the Ministry of Education, Culture, Sports, Science and Technology (B18300118), Japan.

References

1. FERGUSON B, MATYSZAK MK, ESIRI MM, PERRY VH. Axonal damage in acute multiple sclerosis lesions. *Brain* 1997;**120**:393–99.
2. TRAPP BD, PETERSON J, RANSOHO RM, RUDICK R, MÖRK S, BÖ L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 1998;**338**:278–85.
3. LASSMANN H. Axonal injury in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2003;**74**:695–97.
4. SCHWAB ME, BARTHOLDI D. Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol Rev* 1996;**76**:319–70.
5. CHEN MS, HUBER AB, VAN DER HAAR ME, et al. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 2000;**403**:434–39.
6. GRANDPRÉ T, NAKAMURA F, VARTANIAN T, STRITTMATTER SM. Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* 2000;**403**:439–44.
7. JOSEPHSON A, WIDENFALK J, WIDMER HW, OLSON L, SPENGER C. Nogo mRNA expression in adult and fetal human and rat nervous tissue and in weight drop injury. *Exp Neurol* 2001;**169**:319–28.
8. WANG X, CHUN S-J, TRELOAR H, VARTANIAN T, GREER CA, STRITTMATTER SM. Localization of Nogo-A and Nogo-66 receptor proteins at sites of axon-myelin and synaptic contact. *J Neurosci* 2002;**22**:5505–15.
9. OERTLE T, VAN DER HAAR ME, BANDTLOW CE et al. Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. *J Neurosci* 2003;**23**:5393–406.
10. FOURNIER AE, GRANDPRÉ T, STRITTMATTER SM. Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* 2001;**409**:341–46.
11. JOSEPHSON A, TRIFUNOVSKI A, WIDMER HR, WIDENFALK J, OLSON L, SPENGER C. Nogo-receptor gene activity: cellular localization and developmental regulation of mRNA in mice and humans. *J Comp Neurol* 2002;**453**:292–304.
12. WANG KC, KIM JA, SIVASANKARAN R, SEGAL R, HE Z. p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* 2002;**420**:74–8.
13. MI S, LEE X, SHAO Z, et al. LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat Neurosci* 2004;**7**:221–28.
14. PARK JB, YIU G, KANEKO S, WANG J, CHANG J, HE Z. A TNF receptor member, TROY, is a coreceptor with Nogo receptor in mediating the inhibitory activity of myelin inhibitors. *Neuron* 2005;**45**:345–51.
15. NIEDERÖST B, OERTLE T, FRITSCHKE J, MCKINNEY RA, BANDTLOW CE. Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. *J Neurosci* 2002;**22**:10368–76.
16. BAREYRE FM, HAUDENSCHILD B, SCHWAB ME. Long-lasting sprouting and gene expression changes induced by the monoclonal antibody IN-1 in the adult spinal cord. *J Neurosci* 2002;**22**:7097–110.
17. FOURNIER AE, GOULD GC, LIU BP, STRITTMATTER SM. Truncated soluble Nogo receptor binds to Nogo-66 and blocks inhibition of axonal growth by myelin. *J Neurosci* 2002;**22**:8876–83.
18. GRANDPRÉ T, LI S, STRITTMATTER SM. Nogo-66 receptor antagonist peptide promotes axonal regeneration. *Nature* 2002;**417**:547–51.
19. LEE J-K, KIM J-E, SIVULA M, STRITTMATTER SM. Nogo receptor antagonism promotes stroke recovery by enhancing axonal plasticity. *J Neurosci* 2004;**24**:6209–17.
20. BERGER T, RUBNER P, SCHAUTZER F et al. Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. *N Engl J Med* 2003;**349**:139–45.
21. LENNON VA, KRZYER TJ, PITTOCK SJ, VERKMAN AS, HINSON SR. IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J Exp Med* 2005;**202**:473–77.
22. REINDL M, KHANTANE S, EHLING R, et al. Serum and cerebrospinal fluid antibodies to Nogo-A in patients with multiple sclerosis and acute neurological disorders. *J Neuroimmunol* 2003;**145**:139–47.
23. SATOH J-I, ONOUE H, ARJIMA K, YAMAMURA T. Nogo-A and Nogo receptor expression in demyelinating lesions of multiple sclerosis. *J Neuropathol Exp Neurol* 2005;**64**:129–38.
24. KARNEZIS T, MANDEMAKERS W, McQUALTER JL, et al. The neurite outgrowth inhibitor Nogo A is involved in autoimmune-mediated demyelination. *Nat Neurosci* 2004;**7**:736–44.

25. McDONALD WI, COMPTON A, EDAN G, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the international panel on the diagnosis of multiple sclerosis. *Ann Neurol* 2001;**50**:121–27.
26. SATOH J-I, KURODA Y. Differential gene expression between human neurons and neuronal progenitor cells in culture: an analysis of arrayed cDNA clones in NTERA2 human embryonal carcinoma cell line as a model system. *J Neurosci Methods* 2000;**94**:155–64.
27. LI W, WALUS L, RABACCHI SA, et al. A neutralizing anti-Nogo66 receptor monoclonal antibody reverses inhibition of neurite outgrowth by central nervous system myelin. *J Biol Chem* 2004;**279**:43780–88.
28. FONTOURA P, HO PP, DEVOSS J, et al. Immunity to the extracellular domain of Nogo-A modulates experimental autoimmune encephalomyelitis. *J Immunol* 2004;**173**:6981–92.
29. ZHOU C, LI Y, NANDA A, ZHANG JH. HBO suppresses Nogo-A, NgR, or RhoA expression in the cerebral cortex after global ischemia. *Biochem Biophys Res Commun* 2003;**309**:368–76.
30. HUNT D, MASON MRJ, CAMPBELL G, CON R, ANDERSON PN. Nogo receptor mRNA expression in intact and regenerating CNS neurons. *Mol Cell Neurosci* 2002;**20**:537–52.
31. HUNT D, CON RS, PRINHA RK, CAMPBELL G, ANDERSON PN. Nogo-A expression in the intact and injured nervous system. *Mol Cell Neurosci* 2003;**24**:1083–102.
32. MEIER S, BRÄUER AU, HEIMRICH B, SCHWAB ME, NITSCH R, SAVASKAN NE. Molecular analysis of Nogo expression in the hippocampus during development and following lesion and seizure. *FASEB J* 2003;**17**:1153–55.
33. BANDTLOW CE, DLASKA M, PIRKER S, CZECH T, BAUMGARTNER C, SPERK G. Increased expression of Nogo-A in hippocampal neurons of patients with temporal lobe epilepsy. *Eur J Neurosci* 2004;**20**:195–206.
34. LAMPASONA V, FRANCIOTTA D, FURLAN R et al. Similar frequency of anti-MOG IgG and IgM in MS patients and healthy subjects. *Neurology* 2004;**62**:2092–94.
35. SATOH J-I, KURODA Y. Cytokines and neurotrophic factors fail to affect Nogo-A mRNA expression in differentiated human neurones: implications for inflammation-related axonal regeneration in the central nervous system. *Neuropathol Appl Neurobiol* 2002;**28**:95–106.
36. OERTLE T, HUBER C, VAN DER PUTTEN H, SCHWAB ME. Genomic structure and functional characterisation of the promoters of human and mouse nogo/rtn4. *J Mol Biol* 2003;**325**:299–323.
37. DODD DA, NIEDERÖST B, BLOECHLINGER S, DUPUIS L, LOEER JP, SCHWAB ME. Nogo-A, -B, and -C are found on the cell surface and interact together in many different cell types. *J Biol Chem* 2005;**280**:12494–502.
38. DUPUIS L, GONZALEZ DE AGUILAR JL, DI SCALA F, et al. Nogo provides a molecular marker for diagnosis of amyotrophic lateral sclerosis. *Neurobiol Dis* 2002;**10**:358–65.
39. JOKIC N, DE AGUILAR J-LG, PRADAT P-F, et al. Nogo expression in muscle correlates with amyotrophic lateral sclerosis severity. *Ann Neurol* 2005;**57**:553–56.

TROY and LINGO-1 expression in astrocytes and macrophages/microglia in multiple sclerosis lesions

J. Satoh*†, H. Tabunoki*, T. Yamamura†, K. Arima‡ and H. Konno§

*Department of Bioinformatics, Meiji Pharmaceutical University, Tokyo, †Department of Immunology, National Institute of Neuroscience, NCNP, Tokyo, ‡Department of Neuropathology, National Center Hospital for Mental, Nervous, and Muscular Disorders, NCNP, Tokyo, and §Department of Neurology, Nishitaga National Hospital, Sendai, Japan

J. Satoh, H. Tabunoki, T. Yamamura, K. Arima and H. Konno (2007) *Neuropathology and Applied Neurobiology* 33, 99–107

TROY and LINGO-1 expression in astrocytes and macrophages/microglia in multiple sclerosis lesions

Nogo constitutes a family of neurite outgrowth inhibitors contributing to a failure of axonal regeneration in the adult central nervous system (CNS). Nogo-A is expressed exclusively on oligodendrocytes where Nogo-66 segment binds to Nogo receptor (NgR) expressed on neuronal axons. NgR signalling requires a coreceptor p75^{NTR} or TROY in combination with an adaptor LINGO-1. To characterize the cell types expressing the NgR complex in the human CNS, we studied demyelinating lesions of multiple sclerosis (MS) brains by immunohistochemistry. TROY

and LINGO-1 were identified in subpopulations of reactive astrocytes, macrophages/microglia and neurones but not in oligodendrocytes. TROY was up-regulated, whereas LINGO-1 was reduced in MS brains by Western blot. These results suggest that the ternary complex of NgR/TROY/LINGO-1 expressed on astrocytes, macrophages/microglia and neurones, by interacting with Nogo-A on oligodendrocytes, might modulate glial–neuronal interactions in demyelinating lesions of MS.

Keywords: LINGO-1, macrophages/ microglia, multiple sclerosis, reactive astrocytes, TROY

Introduction

Axonal damage beginning at the early stage of multiple sclerosis (MS) is responsible for permanent neurological deficits and progression of clinical disability [1]. Although the underlying molecular mechanism remains unknown, the degree of inflammatory demyelination correlates with the extent of axonal damage, suggesting an involvement of proinflammatory mediators in inducing axonal degeneration [2]. However, the alternative possibility could be proposed that axonal regeneration is severely impaired in MS lesions, because an accumulation of glial scar and neurite

growth inhibitors provide a non-permissive environment for regrowth of damaged axons [3].

Nogo constitutes a family of myelin-associated inhibitors of axonal regeneration, composed of three isoforms named A, B and C, all of which share a C-terminal 66-amino-acid segment named Nogo-66 [4]. Nogo-A, the longest isoform, is expressed exclusively on oligodendrocytes and myelin sheath and a subpopulation of neurones [5]. Nogo-66 as well as oligodendrocyte-myelin glycoprotein (OMgp) and myelin-associated glycoprotein (MAG) binds to the Nogo receptor (NgR), a glycosylphosphatidylinositol (GPI)-anchored membrane protein expressed chiefly on a subpopulation of neurones and their axons, including cerebral cortical pyramidal neurones and cerebellar Purkinje cells [6]. NgR transduces inhibitory signals via a coreceptor p75^{NTR} that acts as a displacement factor releasing a small GTP-binding protein RhoA, an

Correspondence: Jun-ichi Satoh, Department of Bioinformatics, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan. Tel: +81 42 495 8678; Fax: +81 42 495 8678; E-mail: satoj@my-pharm.ac.jp

intracellular regulator of the cytoskeleton, from the Rho GDP dissociation inhibitor [6,7]. Neurones lacking p75^{NTR} neither show RhoA activation nor exhibit neurite growth inhibition in the presence of myelin components, suggesting a key role of p75^{NTR} in the NgR signal transduction *in vitro* [6]. However, the role of p75^{NTR} remains unclear in the adult central nervous system (CNS) *in vivo* where NgR is identified in many neurones that exhibit little or no p75^{NTR} expression [8].

Recently, two novel components of the NgR signalling complex, named TROY and LINGO-1, have been identified. TROY is a type I membrane protein belonging to the tumour necrosis factor receptor superfamily that substitutes for p75^{NTR} in the NgR signalling complex to activate RhoA following exposure to myelin-associated neurite growth inhibitors [9,10]. TROY is strongly expressed during development in neural stem cells located in the ventricular and subventricular zones, and is more broadly expressed in various neurones in the adult rodent CNS than p75^{NTR} [9–12]. Overexpression of a truncated form of TROY lacking its intracellular domain blocks neuronal response to myelin-associated inhibitors in a dominant-negative manner [9]. Neurones from TROY-deficient mice are resistant to the suppressive activity of myelin-associated inhibitors [10]. LINGO-1, also a type I membrane protein, consisting of 12 leucine-rich repeats, an immunoglobulin (Ig)-like domain, a transmembrane domain, and a short cytoplasmic tail containing a canonical epidermal growth factor receptor-like tyrosine phosphorylation site, acts as an adaptor that connects NgR (the ligand-binding component) with p75^{NTR} or TROY (the signal transducing component) by forming the trimolecular complex [13,14]. The expression of LINGO-1 is enriched in the limbic system and the neocortex, identified exclusively in subpopulations of neurones in the rodent CNS [13,15]. Coexpression of NgR, TROY and LINGO-1 confers responsiveness to myelin-associated inhibitors in transfected COS-7 cells [13]. Exposure of cultured rat cerebellar granular (CG) neurones to LINGO-1-Fc fusion protein attenuates Nogo-66, OMgp or MAG-mediated neurite outgrowth inhibition [13]. These observations suggest that the NgR/TROY/LINGO-1 complex plays a key role in inhibiting axonal regeneration in the rodent CNS. However, at present, the precise cellular distribution of TROY and LINGO-1 remains to be characterized in the adult human CNS.

Recently, we demonstrated that Nogo-A expression is enhanced in surviving oligodendrocytes, while NgR is

up-regulated in reactive astrocytes and macrophages/microglia in chronic active demyelinating lesions of MS, suggesting a pathological role of Nogo-A/NgR interaction in persistent demyelination and axonal degeneration in MS lesions [16]. In contrast, we found that p75^{NTR} expression is restricted in small regions such as substantia gelatinosa in the spinal cord [16]. The present study by using immunohistochemistry was designed to investigate TROY and LINGO-1 expression in demyelinating lesions of MS.

Materials and methods

Human brain tissues

For immunohistochemistry, 10 micron-thick serial sections were prepared from autopsied brains of five MS patients and 10 non-MS cases. The tissues were fixed with 4% paraformaldehyde or 10% neutral formalin and embedded in paraffin. MS cases included a 29-year-old woman with secondary progressive MS (SPMS) (MS#1), a 40-year-old woman with SPMS (MS#2), a 43-year-old woman with primary progressive MS (PPMS) (MS#3), a 70-year-old woman with SPMS (MS#4) and a 33-year-old man with SPMS (MS#5). Non-MS neurological and psychiatric disease cases included a 47-year-old man with acute cerebral infarction, an 84-year-old man with acute cerebral infarction, a 62-year-old man with old cerebral infarction, a 56-year-old man with old cerebral infarction, a 36-year-old woman with schizophrenia (SCH) and a 61-year-old man with SCH. Neurologically normal control cases included a 79-year-old woman who died of hepatic cancer, a 75-year-old woman who died of breast cancer, a 60-year-old woman who died of external auditory canal cancer and a 74-year-old woman who died of gastric and hepatic cancers.

For Western blot analysis, MS cases included MS#1, #2, #3 and #4. Non-MS cases included a 76-year-old woman with Parkinson's disease (PD#1), a 61-year-old woman with amyotrophic lateral sclerosis (ALS#1), a 74-year-old woman with ALS (ALS#2), a 61-year-old man with ALS (ALS#3), a 66-year-old man with ALS (ALS#4), a 73-year-old man with SCH (SCH#1) and a 77-year-old woman with depression (DEP#1). The *post mortem* interval of the cases ranges from 1.5 h to 10 h prior to freezing the brain tissues. Autopsies were performed either at the National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan or at the Nishitaga National

Hospital, Sendai, Japan. Written informed consent was obtained from all the cases examined. The present study was approved by the Ethics Committee of NCNP.

Human neural cell lines and cultures

Human astrocytes in culture were established from neuronal progenitor cells isolated from the brain of a human foetus at 18.5-week gestation obtained from BioWhittaker, Walkersville, MD, USA [16]. Human neural cell lines were maintained as described previously [17]. Human microglial cell line HMO6 is provided by Dr Seung U. Kim, Division of Neurology, Department of Medicine, University of British Columbia, Vancouver, BC, Canada [18].

Immunohistochemistry

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH 6.0 by autoclave at 125°C for 30 s in a temperature-controlled pressure chamber (Dako, Tokyo, Japan). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block the endogenous peroxidase activity. The tissue sections were then incubated with phosphate-buffered saline (PBS) containing 10% normal rabbit serum or 10% normal goat serum at RT for 15 min to block non-specific staining. The serial sections were incubated in a moist chamber at 4°C overnight with goat anti-TROY antibody (E-19, sc-13711; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-LINGO-1 antibody (#07-678; Upstate, Lake Placid, NY, USA), mouse monoclonal anti-p75^{NTR} antibody (clone ME20.4; Sigma, St Louis, MO, USA) or the antibodies against cell type-specific markers described previously [16]. After washing with PBS, the tissue sections were labelled at RT for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibodies (Nichirei, Tokyo, Japan), followed by incubation with a colourizing solution containing diaminobenzidine tetrahydrochloride (DAB) and a counterstain with haematoxylin. For negative controls, the step of incubation with primary antibodies was omitted, or the tissue sections were incubated with a negative control reagent (Dako) instead of primary antibodies.

In some experiments, tissue sections were initially stained with mouse anti-GFAP monoclonal antibody (GA5; Nichirei), then followed by incubation with alkaline phosphatase-conjugated secondary antibody (Nichirei)

and colourized with New Fuchsin substrate. After inactivation of the antibody by autoclaving the sections in 10 mM citrate sodium buffer, pH 6.0, they were relabelled with anti-TROY antibody (sc-13711) or anti-LINGO-1 antibody (#07-678), followed by incubation with peroxidase-conjugated secondary antibody (Nichirei) and colourized with DAB substrate. Immunoreactivity was graded as intense, intermediate, weak or undetectable.

Western blot analysis

To prepare total protein extract, frozen brain tissues prepared from the frontal cerebral cortex or the cerebellar cortex were homogenized in RIPA lysis buffer composed of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (Roche Diagnostics, Tokyo, Japan), followed by centrifugation at 13 400 g for 20 min at RT. The supernatant was collected for separation on a 12% SDS-PAGE gel. The protein concentration was determined by a Bradford assay kit (Bio-Rad, Hercules, CA, USA). After gel electrophoresis, the protein was transferred onto nitrocellulose membranes, and immunolabelled at RT overnight with anti-TROY antibody (sc-13711), anti-LINGO-1 antibody (#07-678), mouse monoclonal anti-NgR antibody (clone 188428; R&D Systems, Minneapolis, MN, USA), anti-p75^{NTR} antibody (ME20.4) or goat anti-heat shock protein HSP60 antibody (N-20, sc-1052; Santa Cruz Biotechnology) for the internal control. Then, the membranes were incubated at RT for 30 min with HRP-conjugated anti-mouse, rabbit or goat IgG (Santa Cruz Biotechnology). The specific reaction was visualized by using a chemiluminescent substrate (Pierce, Rockford, IL, USA). After the antibodies were stripped by incubating the membranes at 50°C for 30 min in stripping buffer composed of 62.5 mM Tris-HCl, pH 6.7, 2% SDS and 100 mM 2-mercaptoethanol, the membranes were processed for relabelling several times with different antibodies.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

DNase-treated total cellular RNA was processed for cDNA synthesis using oligo(dT)₁₂₋₁₈ primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Then, cDNA was amplified by polymerase chain reaction (PCR) using HotStar Taq DNA polymerase (Qiagen,

Valencia, CA, USA) and a panel of sense and antisense primer sets following: 5'-tcccatgggtggtgacaacatctc-3' and 5'-gaatgagactggactggaacagcc-3' for a 159-bp product of TROY; 5'-cctcctacccttctacacagtt-3' and 5'-gtgtcggttcgctggcttcaact-3' for a 175-bp product of LINGO-1; 5'-agcagccaggtgtgtgtacatacag-3' and 5'-cgccgaacctgtaaacatgatgg-3' for a 154-bp product of NgR; 5'-gaccacactctctgtccagagaga-3' and 5'-atatgacacctgtgtggaggag-3' for a 142-bp product of p75^{NTR}; and 5'-ccatgttcgtcatgggtgtgaccca-3' and 5'-gccatagaggcaggatgatgttc-3' for a 251-bp product of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene as an internal control. The amplification program consisted of an initial denaturing step at 95°C for 15 min, followed by a denaturing step at 94°C for 1 min, an annealing step at 60°C for 40 s and an extension step at 72.9°C for 50 s for 38 cycles, except for G3PDH amplified for 32 cycles. For the positive control, total RNA of the human frontal cerebral cortex (Clontech, Mountain View, CA, USA) was processed in parallel for RT-PCR.

Results

The constitutive expression of TROY and LINGO-1 mRNA in various human neural cell lines and primary cultures

First, the expression of TROY and LINGO-1 mRNA was studied in human neural cell lines and cultures by RT-PCR analysis. High levels of NgR and TROY transcripts were identified in all cell types, including cultured astrocytes, neuronal progenitor cells, NTERA2 teratocarcinoma-derived neurones (NTERA2N), Y79 retinoblastoma, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, U-373MG astrocytoma, HeLa cervical carcinoma, HepG2 hepatocellular carcinoma and HMO6 microglial cell line, along with in the human cerebral cortex (Figure 1a,b, lanes 1, 3–12). LINGO-1 and p75^{NTR} transcripts were also detected in various cell lines, although LINGO-1 mRNA levels were fairly low in U-373MG and HeLa, and p75^{NTR} mRNA levels were extremely low in HMO6 cells (Figure 1c,d, lanes 1, 3–12). The levels of G3PDH mRNA, a housekeeping gene, were almost constant among the cells examined (Figure 1c, lanes 1, 3–12), while no products were amplified when total RNA was processed for PCR without inclusion of the reverse transcription step, confirming that a contamination of genomic DNA was excluded (Figure 1a–e, lane 2).

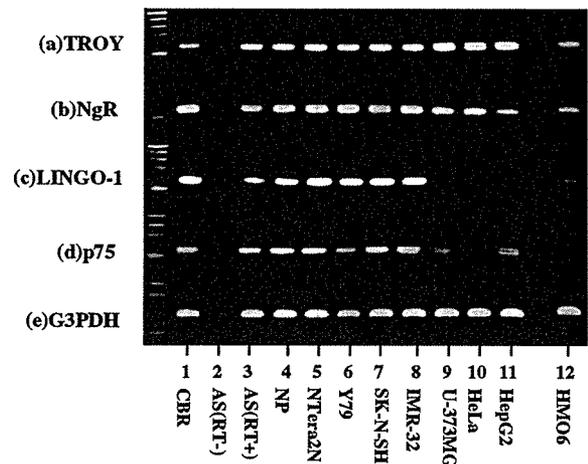


Figure 1. TROY and LINGO-1 mRNA expression in human neural cells. The expression of (a) TROY, (b) NgR, (c) LINGO-1, (d) p75^{NTR} and (e) G3PDH (an internal control) mRNA was studied in human neural cells by RT-PCR analysis. The lanes (1–12) represent: (1) the frontal cerebral cortex (CBR), (2) cultured astrocytes (AS) without inclusion of the reverse transcription step (RT-), (3) cultured astrocytes (AS) with inclusion of the reverse transcription step (RT+), (4) cultured neuronal progenitor (NP) cells, (5) NTERA2 teratocarcinoma-derived neurones (NTERA2N), (6) Y79 retinoblastoma, (7) SK-N-SH neuroblastoma, (8) IMR-32 neuroblastoma, (9) U-373MG astrocytoma, (10) HeLa cervical carcinoma, (11) HepG2 hepatocellular carcinoma and (12) HMO6 microglial cell line. The DNA size marker (100-bp ladder) is shown on the left.

These results indicate that a panel of human neural cells in culture express both NgR/TROY/LINGO-1 and NgR/p75^{NTR}/LINGO-1 complexes.

Neurones, reactive astrocytes and macrophages/microglia but not oligodendrocytes expressed TROY and LINGO-1 in MS and control brains

In the next step, the expression of TROY and LINGO-1 proteins was studied in MS and non-MS brains by immunohistochemistry. A subpopulation of neurones in the cerebral cortex, brainstem and spinal cord expressed constitutively variable intensities of immunoreactivity for TROY or LINGO-1 in both MS and non-MS brains (Figure 2a,b). An intermediate or weak immunolabelling of TROY or LINGO-1 was identified in a subset of reactive hypertrophic astrocytes in chronic active demyelinating lesions of MS (Figure 2c,d) and ischaemic lesions of acute cerebral infarction (not shown), although the percentage and intensity of immunopositive cells/total hypertrophic reactive astrocytes was much greater and stronger in

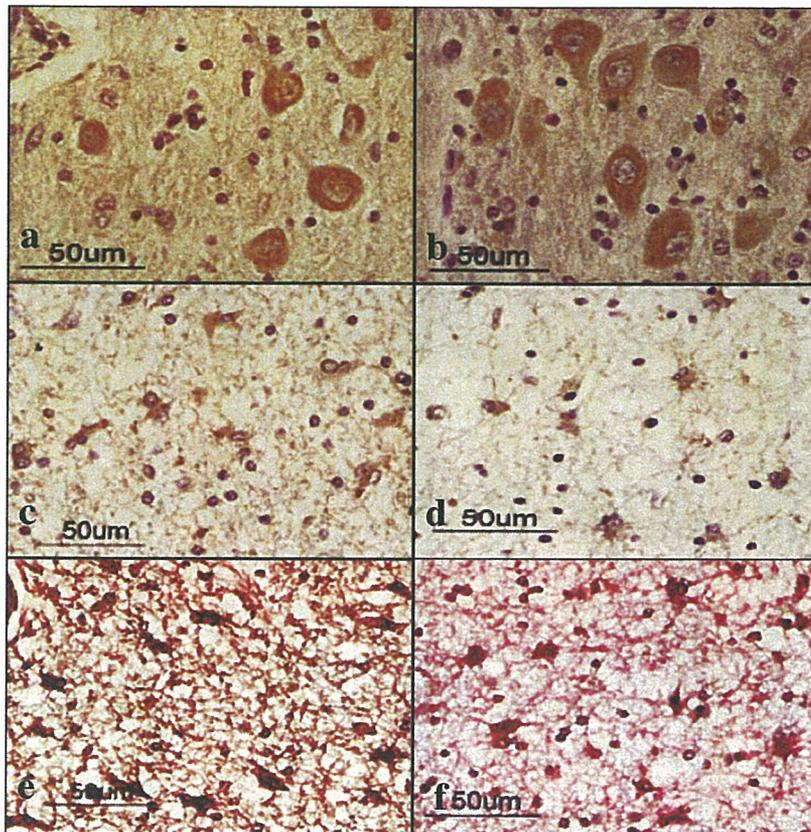


Figure 2. TROY and LINGO-1 immunoreactivities are identified on neurones and reactive astrocytes in multiple sclerosis (MS) brains. The expression of (a,c,e) TROY and (b,d,f) LINGO-1 was studied in MS brains by immunohistochemistry. The panels represent (a) TROY immunoreactivity in the pontine base of MS#1, (b) LINGO-1 immunoreactivity in the pontine base of MS#1, (c) TROY immunoreactivity in chronic active demyelinating lesions in the frontal cerebral cortex of MS#2, (d) LINGO-1 immunoreactivity in chronic active demyelinating lesions in the frontal cerebral cortex of MS#3, (e) TROY (brown) and GFAP (red) double immunolabelling of chronic active demyelinating lesions in the frontal cerebral cortex of MS#2, and (f) LINGO-1 (brown) and GFAP (red) double immunolabelling of chronic active demyelinating lesions in the frontal cerebral cortex of MS#3. Neurones (a,b) and reactive hypertrophic astrocytes (c-f) express TROY and LINGO-1.

TROY (> 80%) than in LINGO-1 (< 10%), suggesting that not all TROY-expressing astrocytes coexpress substantial levels of LINGO-1. Double immunolabelling verified that a population of the cells expressing TROY or LINGO-1 immunoreactivity was comprised of GFAP⁺ astrocytes (Figure 2e,f). The great majority of macrophages and microglia expressed an intense/intermediate immunoreactivity for TROY and LINGO-1 in MS and non-MS brain lesions (Figure 3a,b). In contrast, both TROY and LINGO-1 was neither detectable in surviving oligodendrocytes remaining in demyelinating lesions nor in oligodendrocytes in the normal appearing white matter of MS (Figure 3c,d) and non-MS brains (not shown). p75^{NTR} expression was restricted in small regions such as

substantia gelatinosa in the spinal cord (Figure 3e), as described previously [16].

Up-regulated expression of TROY in MS brains

Finally, the expression of TROY and LINGO-1 proteins was studied in brain tissues of four MS and seven non-MS cases by Western blot analysis. TROY protein levels were much higher in four MS cases MS#1, #2, #3 and #4 (Figure 4a, lanes 1–7), when compared with five non-MS cases PD#1, ALS#1, #2 and #3, and SCH#1 (Figure 4a, lanes 8–14 and 17), or comparable to two non-MS cases ALS#4 and DEP#1 (Figure 4a, lanes 16 and 19), when standardized against the levels of HSP60, an internal standard detected

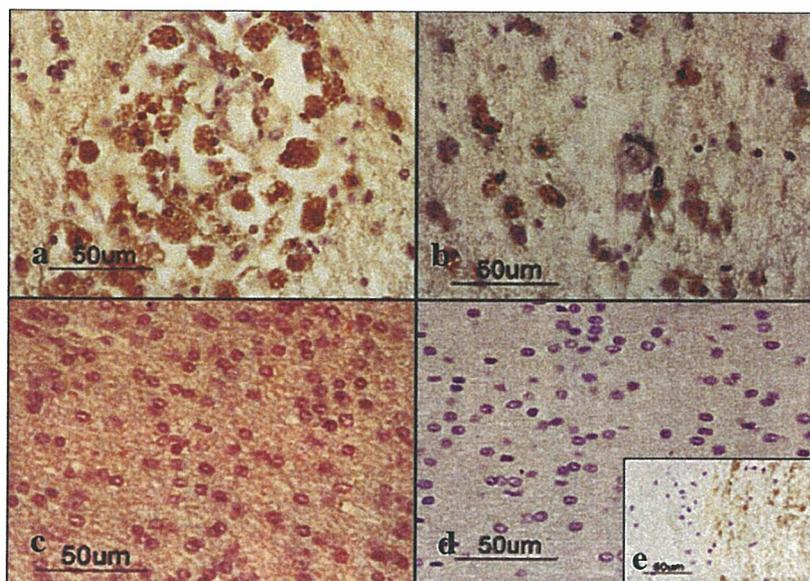


Figure 3. TROY and LINGO-1 immunoreactivities are identified on macrophages/microglia but not on oligodendrocytes in multiple sclerosis (MS) brains. The expression of (a,c) TROY, (b,d) LINGO-1 and (e) p75^{NTR} was studied in MS brains by immunohistochemistry. The panels represent (a) TROY immunoreactivity in chronic active demyelinating lesions in the frontal cerebral cortex of MS#3, (b) LINGO-1 immunoreactivity in chronic active demyelinating lesions in the parietal cerebral cortex of MS#3, (c) TROY immunoreactivity in the normal appearing white matter of the frontal cerebral cortex of MS#1, (d) LINGO-1 immunoreactivity in the normal appearing white matter of the frontal cerebral cortex of MS#1, and an inset, (e) p75^{NTR} immunoreactivity in the substantia gelatinosa of the spinal cord of MS#2. Macrophages/microglia (a,b) but not oligodendrocytes (c,d) express TROY and LINGO-1.

in corresponding blots, which appeared to be almost constant among the samples (Figure 4e, lanes 1–19). In contrast, LINGO-1 protein levels were reduced in all MS cases and the cerebellum of the case of DEP#1, compared with other cases (Figure 4c, lanes 1–7 and 19). The pattern of p75^{NTR} protein expression (Figure 4d, lanes 1–19) was generally similar to that of TROY (Figure 4a, lanes 1–19), except for one case of MS MS#2 that expressed a high level of TROY but a trace of p75^{NTR} (Figure 4a,d, lane 2). In contrast, NgR protein levels varied among the cases and the regions examined, although there existed a trend for higher levels of NgR in the cerebellum vs. lower levels in the cerebrum (Figure 4b, lanes 1–19).

Discussion

By immunohistochemistry, the present study for the first time demonstrated that the expression of TROY and LINGO-1 was more widespread than p75^{NTR} in the brains of MS and non-MS cases, identified in subpopulations of reactive astrocytes, macrophages/microglia and neurones but not in oligodendrocytes in chronic active demyelinat-

ing lesions of MS and ischaemic lesions of cerebral infarction. The expression of TROY and LINGO-1 in astrocytes and microglia, the findings previously unreported [9–15], was verified by immunohistochemistry of brain tissue sections and RT-PCR analysis of human astrocytes in culture and the microglia cell line HMO6. Non-neuronal distribution of TROY and LINGO-1, both of which constitute pivotal components of the NgR signalling complex [9,10,13], is not so surprising. Previous studies showed that TROY mRNA was detected in glioma and embryonal carcinoma cells [11], and recently, we reported that Nogo-A expression is enhanced in surviving oligodendrocytes, while NgR is up-regulated in reactive astrocytes and macrophages/microglia in chronic active demyelinating lesions of MS, suggesting an active role of NgR signalling in non-neuronal cell types [16]. Our observations put forth the hypothesis that the ternary complex of NgR/TROY/LINGO-1 expressed on astrocytes, macrophages/microglia and neurones, by interacting with Nogo-A expressed on oligodendrocytes, plays some role in regulating glial-neuronal and glial–glial interactions in active demyelinating lesions of MS.

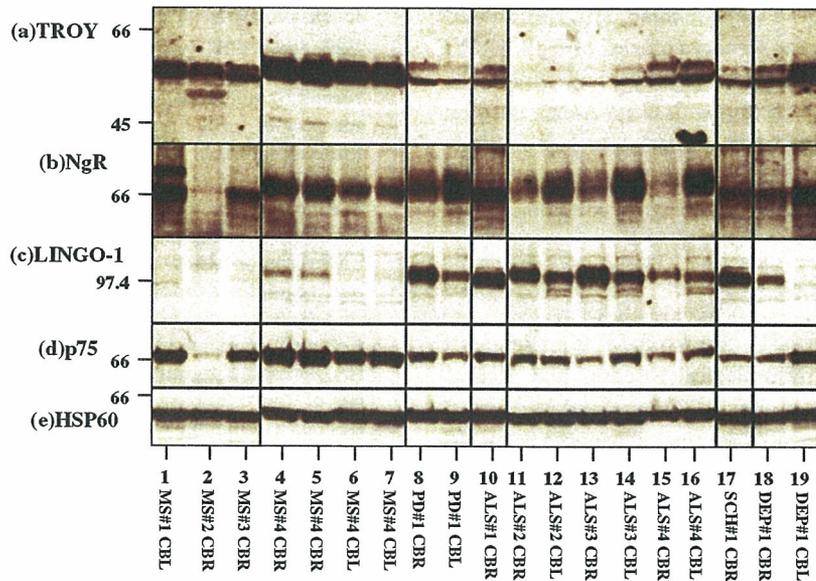


Figure 4. TROY and LINGO-1 protein expression in MS and non-MS brains. The expression of (a) TROY (55 kDa), (b) NgR (67 kDa), (c) LINGO-1 (97 kDa), (d) p75^{NTR} (68 kDa) and (e) HSP60 (60 kDa, an internal control) proteins was studied by Western blot analysis in brain homogenates prepared from either the frontal cerebral cortex (CBR) or the cerebellar cortex (CBL) of four MS and seven non-MS cases. Sixty micrograms of total protein separated on a 12% SDS-PAGE gel was transferred onto nitrocellulose membranes, and processed for relabelling several times with different antibodies. The lanes (1–19) represent (1) MS#1 CBL, (2) MS#2 CBR, (3) MS#3 CBR, (4) MS#4 CBR, (5) a different part of MS#4 CBR, (6) MS#4 CBL, (7) a different part of MS#4 CBL, (8) PD#1 CBR, (9) PD#1 CBL, (10) ALS#1 CBR (11) ALS#2 CBR, (12) ALS#2 CBL, (13) ALS#3 CBR, (14) ALS#3 CBL, (15) ALS#4 CBR, (16) ALS#4 CBL, (17) SCH#1 CBR, (18) DEP#1 CBR and (19) DEP#1 CBL. PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; SCH, schizophrenia; DEP, depression. The position of molecular weight marker is indicated on the left.

By Western blot analysis, we found that TROY levels were elevated, whereas LINGO-1 levels were reduced in MS brains, although the sample size of the present study was too small, i.e. seven samples of four MS cases vs. 12 samples of seven non-MS cases, to obtain definitive conclusions. In contrast, immunohistochemical studies showed that both TROY and LINGO-1 immunoreactivities were enhanced in subpopulations of astrocytes and macrophages/microglia in MS brains. Because a subset of neurones expressed variable levels of TROY and LINGO-1, the discrepancy in the results between Western blot and immunohistochemistry is in part attributable to the varying degree of axonal loss in the lesions examined. Recent studies indicate that LINGO-1 expression is regulated by neuronal activity [19]. The expression of LINGO-1 mRNA is strongly up-regulated, while NgR mRNA levels are decreased in the dentate gyrus of rat brain following treatment with brain-derived neurotrophic factor or kainic acid, suggesting a role of LINGO-1 in activity-dependent neuronal plasticity responses [19]. Reduced neuronal activity causes irreversible axonal damage in demyelinat-

ing lesions of MS [20]. Recent studies indicate that LINGO-1 is expressed on rat oligodendrocytes where it negatively regulates differentiation and myelination competence of oligodendrocytes [21]. Expression of dominant-negative LINGO-1, LINGO-1 RNA-mediated interference, or an exposure to soluble LINGO-1-Fc enhances differentiation of rat oligodendrocytes in culture, accompanied by down-regulation of RhoA activity [21]. Furthermore, LINGO-1 knockout mice have greater numbers of myelinated axons [21]. These observations do not apparently agree with our present and previous studies showing that LINGO-1, along with NgR and TROY, was undetectable on oligodendrocytes in the brains of any cases examined [16]. This discrepancy is attributable in part to the differences between the previous study [21] and our own in the species and age, developing rat vs. adult human, or to the methods, cell cultures vs. immunohistochemistry, or both.

In conclusion, the expression of TROY and LINGO-1 was identified in subpopulations of reactive astrocytes, macrophages/microglia and neurones but not in oligodendrocytes in chronic active demyelinating lesions of MS

and ischaemic lesions of cerebral infarction. These observations suggest that the ternary complex of NgR/TROY/LINGO-1 expressed on astrocytes, macrophages/microglia and neurones, by interacting with Nogo-A expressed on oligodendrocytes, might play a regulatory role in glial-neuronal and glial–glial interactions under demyelinating conditions of MS and other pathological conditions.

Acknowledgements

This work was supported by grants to J.-I.S. from Research on Psychiatric and Neurological Diseases and Mental Health, the Ministry of Health, Labour and Welfare of Japan (H17-020), Research on Health Sciences Focusing on Drug Innovation, the Japan Health Sciences Foundation (KH21101), and the Grant-in-Aid for Scientific Research, the Ministry of Education, Culture, Sports, Science and Technology, Japan (B18300118). All autopsied brain samples were obtained from Research Resource Network (RRN), Japan. The authors thank Dr Seung U. Kim, Division of Neurology, Department of Medicine, University of British Columbia, Vancouver, BC, Canada for providing us with HMO6.

References

- 1 Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mörk S, Bö L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 1998; **338**: 278–85
- 2 Lassmann H. Axonal injury in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2003; **74**: 695–7
- 3 Domeniconi M, Filbin MT. Overcoming inhibitors in myelin to promote axonal regeneration. *J Neurol Sci* 2005; **233**: 43–7
- 4 Chen MS, Huber AB, van der Haar ME, Frank M, Schnell L, Spillmann AA, Christ F, Schwab ME. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 2000; **403**: 434–9
- 5 Wang X, Chun S-J, Treloar H, Vartanian T, Greer CA, Strittmatter SM. Localization of Nogo-A and Nogo-66 receptor proteins at sites of axon-myelin and synaptic contact. *J Neurosci* 2002; **22**: 5505–15
- 6 Wang KC, Kim JA, Sivasankaran R, Segal R, He Z. p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* 2002; **420**: 74–8
- 7 Yamashita T, Tohyama M. The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nat Neurosci* 2003; **6**: 461–7
- 8 Josephson A, Trifunovski A, Widmer HR, Widenfalk J, Olson L, Spenger C. Nogo-receptor gene activity: cellular localization and developmental regulation of mRNA in mice and humans. *J Comp Neurol* 2002; **453**: 292–304
- 9 Park JB, Yiu G, Kaneko S, Wang J, Chang J, He Z. A TNF receptor family member, TROY, is a coreceptor with nogo receptor in mediating the inhibitory activity of myelin inhibitors. *Neuron* 2005; **45**: 345–51
- 10 Shao Z, Browning JL, Lee X, Scott ML, Shulga-Morskaya S, Allaire N, Thill G, Levesque M, Sah D, McCoy JM, Murray B, Jung V, Pepinsky RB, Mi S. TAJ/TROY, an orphan TNF receptor family member, binds Nogo-66 receptor 1 and regulates axonal regeneration. *Neuron* 2005; **45**: 353–9
- 11 Kojima T, Morikawa Y, Copeland NG, Gilbert DJ, Jenkins NA, Senba E, Kitamura T. TROY, a newly identified member of the tumor necrosis factor receptor superfamily, exhibits a homology with Edar and is expressed in embryonic skin and hair follicles. *J Biol Chem* 2000; **275**: 20742–7
- 12 Hisaoka T, Morikawa Y, Kitamura T, Senba E. Expression of a member of tumor necrosis factor receptor superfamily, TROY, in the developing mouse brain. *Dev Brain Res* 2003; **143**: 105–9
- 13 Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, Crowell T, Cate RL, McCoy JM, Pepinsky RB. LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat Neurosci* 2004; **7**: 221–8
- 14 Chen Y, Aulia S, Li L, Tang BL. AMIGO and friends: an emerging family of brain-derived, neuronal growth modulating, type I transmembrane proteins with leucine-rich repeats (LRR) and cell adhesion molecule motifs. *Brain Res Rev* 2006; **51**: 265–74
- 15 Carim-Todd L, Escarceller M, Estivill X, Sumoy L. LRRN6A/LERN1 (leucine-rich repeat neuronal protein 1), a novel gene with enriched expression in limbic system and neocortex. *Eur J Neurosci* 2003; **18**: 3167–82
- 16 Satoh J, Onoue H, Arima K, Yamamura T. Nogo-A and Nogo receptor expression in demyelinating lesions of multiple sclerosis. *J Neuropathol Exp Neurol* 2005; **64**: 129–38
- 17 Satoh J, Kuroda Y. Cytokines and neurotrophic factors fail to affect Nogo-A mRNA expression in differentiated human neurones: implications for inflammation-related axonal regeneration in the central nervous system. *Neuropathol Appl Neurobiol* 2002; **28**: 95–106
- 18 Nagai A, Nakagawa E, Hatori K, Choi HB, McLarnon JG, Lee MA, Kim SU. Generation and characterization of immortalized human microglial cell lines: expression of cytokines and chemokines. *Neurobiol Dis* 2001; **8**: 1057–68
- 19 Trifunovski A, Josephson A, Ringman A, Brené S, Spenger C, Olson L. Neuronal activity-induced regulation of Lingo-1. *Neuroreport* 2004; **15**: 2397–400
- 20 Kurnellas MP, Nicot A, Shull GE, Elkabes S. Plasma membrane calcium ATPase deficiency causes neuronal pathology in the spinal cord: a potential mechanism for

- neurodegeneration in multiple sclerosis and spinal cord injury. *FASEB J* 2005; **19**: 298–300
- 21 Mi S, Miller RH, Lee X, Scott ML, Shulag-Morskaya S, Shao Z, Chang J, Thill G, Levesque M, Zhang M, Hession C, Sah D, Trapp B, He Z, Jung V, McCoy JM, Pepinsky RB.

LINGO-1 negatively regulates myelination by oligodendrocytes. *Nat Neurosci* 2005; **8**: 745–51

Received 25 June 2006

Accepted after revision 3 September 2006

Neuropathology**Original Article****Revised Manuscript ID NEU-06-0072****Full Title: Human astrocytes express aquaporin-1 and aquaporin-4 *in vitro* and *in vivo*****Jun-ichi Satoh,^{1,2} Hiroko Tabunoki,¹ Takashi Yamamura,² Kunimasa Arima³ and Hidehiko Konno⁴**

¹Department of Bioinformatics, Meiji Pharmaceutical University, Tokyo, ²Department of Immunology, National Institute of Neuroscience, NCNP, Tokyo, ³Department of Neuropathology, National Center Hospital for Mental, Nervous, and Muscular Disorders, NCNP, Tokyo, and ⁴Department of Neurology, Nishitaga National Hospital, Sendai, Japan

Correspondence: Jun-ichi Satoh, MD, Department of Bioinformatics, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan.

Tel. and Fax: +81-42-495-8678; E-mail: satoj@my-pharm.ac.jp

Running Title: Human astrocytes express AQP1 and AQP4

ABSTRACT

Aquaporins (AQPs) constitute an evolutionarily conserved family of integral membrane water transport channel proteins. Previous studies indicate that AQP1 is expressed exclusively in the choroid plexus epithelium, while AQP4 is localized on the vascular foot of astrocytes in the central nervous system (CNS) under physiological conditions. To investigate a role of AQPs in the pathophysiology of neurological diseases involving astrogliosis, we studied the expression of AQP1 and AQP4 in cultured human astrocytes and brain tissues of multiple sclerosis (MS), cerebral infarction, and control cases. By RT-PCR and Western blot analysis, cultured human astrocytes coexpressed both AQP1 and AQP4 mRNA and proteins, where AQP4 levels were elevated by exposure to $IFN\gamma$ but neither by $TNF\alpha$ nor $IL-1\beta$, whereas AQP1 levels were unaffected by any cytokines examined. By Western blot analysis, AQP1 and AQP4 proteins were detected in brain homogenates of MS and non-MS cases, where both levels were correlated with those of GFAP. By immunohistochemistry, astrocytes with highly-branched processes surrounding blood vessels, along with glial scar, expressed intensely AQP1 and AQP4 in MS and ischemic brain lesions, whereas neither macrophages, neurons nor oligodendrocyte cell bodies were immunopositive. These immunohistochemical results indicate that the expression not only of AQP4 but also of AQP1 was enhanced in MS and ischemic brain lesions, located predominantly in astrocytes, suggesting a pivotal role of astrocytic AQPs in maintenance of water homeostasis in the CNS under pathological conditions.

Key words: astrocytes, AQP1, AQP4, cerebral infarction, multiple sclerosis.

INTRODUCTION

The aquaporins (AQPs) constitute a family of integral channel proteins that facilitate the rapid transport of water across cell membranes in response to osmotic gradients.^{1,2} Presently, at least 13 mammalian members have been identified, which are widely expressed in various fluid-transporting epithelial and endothelial cells. AQP1, AQP2, AQP4, AQP5 and AQP8 are chiefly water selective, while AQP3, AQP7, AQP9 and AQP10, named aquaglyceroporins, transport glycerol in addition to water. Among these, AQP1, AQP4 and AQP9 are expressed in the central nervous system (CNS).^{1,2} AQP1, initially identified in red blood cells and renal proximal tubular epithelium, is found to be selectively and constitutively expressed in the apical surface of choroid plexus epithelium (CPE) in the CNS.³ The AQP1 water channel is composed of a dumbbell-shaped homotetramer of 28-kDa subunits, each of which contains 6 transmembrane domains and an extracellular epitope that defines the Colton blood group antigen on red blood cells. AQP1 serves as both a water channel and a cGMP-gated ion channel on CPE.⁴ AQP1-null individuals negative for the Colton antigens who have homozygous mutations in the AQP1 gene exhibit a defective urinary concentrating ability and reduced pulmonary vascular permeability following fluid overload, suggesting a major role of AQP1 in kidney and pulmonary physiology.^{5,6} In mice, a targeted disruption of the AQP1 gene caused a decrease in cerebrospinal fluid (CSF) production and intracranial pressure, indicating a primary role of AQP1 in secretion of CSF in the CNS.⁷ Although AQP1 is expressed widely in vascular endothelial cells outside the CNS, its levels are very low in brain endothelial cells and no expression is identified in primary cultures of rodent astrocytes.⁸

AQP4 is the predominant form of AQPs enriched in the CNS.⁹ It is constitutively expressed in the astroglial end-feet at the perivascular and the subpial membranes where it regulates brain water homeostasis by acting as a key constituent of the blood-brain barrier (BBB) and the blood-CSF barrier.¹⁰ Alpha-syntrophin, an adapter protein