Association of Cerebrospinal Fluid Levels of Lateral Olfactory Tract Usher Substance (LOTUS) With Disease Activity in Multiple Sclerosis

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**IMPORTANCE** Although multiple sclerosis (MS) is generally considered an autoimmune demyelinating disorder of the central nervous system, axonal degeneration through Nogo receptor-1 signaling was recently recognized as an important pathological feature. Our previous identification of lateral olfactory tract usher substance (LOTUS), an endogenous Nogo receptor-1 antagonist, prompted us to analyze the relationship between LOTUS levels of cerebrospinal fluid and the clinical course of MS to evaluate whether LOTUS could be a useful biomarker for MS.

**OBJECTIVE** To examine variations in LOTUS concentrations in the cerebrospinal fluid of patients with MS in accordance with their clinical course.

**DESIGN, SETTING, AND PARTICIPANTS** Cerebrospinal fluid samples were obtained retrospectively from normal controls (NCs; n = 27) and patients with MS (n = 40), amyotrophic lateral sclerosis (n = 22), and multiple system atrophy (n = 10) between January 1, 2008, and January 1, 2014. Patients with MS were divided into relapsing-remitting MS (RRMS; n = 30) and secondary progressive MS (n = 10). Patients with RRMS were further divided into relapse and remission groups.

**MAIN OUTCOMES AND MEASURES** The LOTUS concentration in cerebrospinal fluid was quantitatively detected by immunoblotting using a specific LOTUS antibody and the concentrations compared in accordance with the patients’ clinical course, such as remission and relapse groups in RRMS and secondary progressive MS.

**RESULTS** The mean (SD) cerebrospinal fluid LOTUS concentration in the relapse group of RRMS (9.3 [3.6] μg/dL) was lower than that of NCs (19.2 [4.7] μg/dL; \(P < .001\)) whereas the level in the remission group of RRMS (19.6 [5.8] μg/dL) was similar to that of NCs. The LOTUS concentration in SPMS (6.7 [1.4] μg/dL; \(P < .001\)) was lower than that of NCs and the remission group of RRMS. The LOTUS levels in other neurodegenerative diseases, such as amyotrophic lateral sclerosis and multiple system atrophy, were normal.

**CONCLUSIONS AND RELEVANCE** Variations in LOTUS concentrations were correlated with disease activity in MS. Therefore, LOTUS concentration may be useful as a possible biomarker for MS. Low LOTUS concentrations may be possibly involved in Nogo receptor-1 signaling, which may induce axonal degeneration in the relapse phase of RRMS and secondary progressive MS.


Published online December 1, 2014.
Multiple sclerosis (MS) is characterized by repeated relapses and remissions in early stages (relapsing-remitting MS [RRMS]) in most patients. Occasionally, RRMS later transforms into secondary progressive MS (SPMS), which is characterized by a persistent progressive phase without apparent relapses. Although MS is generally considered an autoimmune demyelinating disorder of the central nervous system, axonal degeneration is a recently recognized important pathological feature of RRMS. In particular, SPMS is characterized by widespread and prominent axonal degeneration. The mechanism of axonal degeneration in MS involves myelin-derived axonal growth inhibitors, such as Nogo and its receptor, Nogo receptor-1 (NgR1). Activation of NgR1-mediated signal-derived axonalgrowthinhibitors,suchasNogoanditsreceptor,mechanism of axonal degeneration in MS involves myelin-degenerative disorders. Also examined in CSF samples from patients with other neurodegenerative diseases with their clinical course. The LOTUS concentrations were measured by blocking Nogo-NgR1 interactions. Here, we examined LOTUS protein as an endogenous NgR1 antagonist that prevents Nogo-NgR1 binding. Lateral olfactory tract usher substance (LOTUS) as an endogenous NgR1 antagonist that may be an endogenous inhibitor of axonal degeneration in MS by blocking Nogo-NgR1 interactions. Here, we examined LOTUS in healthy human cerebrospinal fluid (CSF) and variations in LOTUS concentrations in the CSF of patients with MS in accordance with their clinical course. The LOTUS concentrations were also examined in CSF samples from patients with other neurodegenerative disorders.

Methods

Participants

All patients enrolled in this study underwent neurological evaluation at our hospital and were diagnosed with MS, other diseases, or were found to be normal. Samples of CSF were obtained retrospectively from 62 patients with MS, 22 patients with amyotrophic lateral sclerosis (ALS), 10 patients with multiple system atrophy (MSA), and 27 normal controls (NCs) between January 1, 2008, and January 1, 2014. All patients with MS fulfilled the McDonald criteria and were initially divided into the RRMS group (52 patients) or the SPMS group (10 patients). Secondary progressive MS was defined as progressive deterioration of disability for at least 6 months with an increase of at least 1 Expanded Disability Status Scale point during the last 2 years, with or without superimposed exacerbations following an initial RRMS course. The 52 patients of the RRMS group were further divided into a relapsing group (36 patients) or remitting group (16 patients) based on clinical findings and clinical history in medical records by trained neurologists. Twenty of the 36 patients in the relapsing group had test results that were positive on gadolinium-enhanced magnetic resonance imaging and were analyzed as patients with relapse in our study. The patients in the remitting group were defined as keeping remission state without exacerbation and relapse for at least 4 months and no abnormal lesions on gadolinium-enhanced magnetic resonance imaging based on clinical findings in the remission phase. Ten of the 16 patients clinically judged to be in a remission state met these criteria and were used in analysis. The periods of remission state in the remitting group were 4 to 44 months (15.8 months on average).

Written informed consent was obtained from each participant according to the research ethics of our hospital. This study was approved by the ethics committee of the Yokohama City University Medical Hospital.

Procedures

After the lumbar punctures, CSF samples were frozen at −80°C. Samples were centrifuged at 15,000g for 10 minutes to remove cells and debris. Samples of CSF (7.5 μL/well) were separated and transferred to polyvinylidene difluoride membranes. The membrane was incubated with primary antibody against mouse LOTUS (0.25 μg/mL) for 1 hour and then incubated with horseradish peroxidase–labeled donkey antibodies against rabbit IgG (1:1000; GE Healthcare) for 1 hour. An enhanced chemiluminescence substrate was used and signals were quantified using an image analyzer (ImageQuant 400; GE Healthcare). The Myc-His 6-tagged LOTUS protein was purified as previously described and determined the concentration based on a bovine serum albumin standard. Standard curves were generated by analyzing the density of the concentration of Myc-His 6-tagged LOTUS and the concentration of LOTUS in the CSF was calculated using this standard curve.

We performed the additional experiments 3 times using some of the same samples to verify intra-assay reproducibility. Furthermore, we performed additional experiments using a different kind of antibody (which is commercially available; R & D System) 3 times and under blinded conditions to determine inter-assay variability.

Statistical Analysis

Statistical analysis was performed with the Kruskal-Wallis test followed by the Mann-Whitney U test for pairwise analysis as appropriate, using GraphPad Prism version 6.0 (GraphPad Software). A P value less than .05 was considered to be statistically significant.

Results

We identified LOTUS protein in human CSF with anti-LOTUS immunoblotting and confirmed LOTUS expression using peptide blocking. The patients’ characteristics in this study are summarized in the Table. We compared the CSF LOTUS concentration between NCs and patients with RRMS. The mean (SD) LOTUS concentration in patients in the relapse group of RRMS (9.3 [3.6] μg/dL) was lower than that in NCs (19.2 [4.7] μg/dL; P < .001; Figure). The LOTUS concentration in the remission group of RRMS (9.6 [5.8] μg/dL) was similar to that in NCs (19.2 [4.7] μg/dL; Figure). The LOTUS concentration in the relapse group of RRMS (9.3 [3.6] μg/dL) was lower than that in the remission group of RRMS (19.6 [5.8] μg/dL; P < .001; Figure). Intra-assay and inter-assay experiments yielded the same results.

Next, we compared the LOTUS concentration between patients with SPMS and patients with RRMS. The LOTUS concentration in patients with SPMS (6.7 [1.4] μg/dL) was lower than that in the remission group of RRMS (19.6 [5.8] μg/dL; P < .001; Figure) and similar to that in the relapse group of...
NodifferenceamongtheCSFLOTUSconcentrationsinpatientswithALS,patientswithMSA, and NCs was found (Figure). We found a significant difference between relapse in patients with RRMS and NCs (P < .001; Kruskal-Wallis test followed by the Mann-Whitney U test for pairwise analysis) and between relapse and remission in RRMS (P < .001). No significant difference was found among remission in patients with RRMS, ALS, and MSA and NCs. A significant difference was found between SPMS and NCs but no significant difference was found between SPMS and relapse in RRMS. The horizontal lines represent the mean LOTUS concentration in each group and each symbol are data from an individual patient or NC.

The LOTUS concentration during the remission period was examined in the 3 patients with RRMS who maintained a remission state and recovered to NC levels following a relapse. Patient 1’s LOTUS concentration changed from 11.4 μg/dL at relapse to 16.4 μg/dL, 5 months after relapse; patient 2’s LOTUS concentration changed from 8.6 μg/dL at relapse to 14.6 μg/dL, 25 months after relapse; and patient 3’s LOTUS concentration changed from 5.1 μg/dL at relapse to 18.0 μg/dL, 26 months after relapse (eFigure in the Supplement).

### Discussion

Activation of NgR1 signaling or upregulation of Nogo expression in demyelinating lesions may be a main cause of axonal degeneration in the progressive phase of MS. We previously reported that LOTUS completely suppresses Nogo binding to NgR1 in mice. Accordingly, LOTUS may be an endogenous inhibitor of axonal degeneration by blocking the NgR1 function in MS. Here, the LOTUS concentration was 19.2 (4.7) μg/dL in healthy human CSF and drastically decreased in the CSF of patients with RRMS in the relapse phase (9.3 [3.6] μg/dL). A decrease in LOTUS may activate NgR1 signaling by attenuating

### Table. Demographics and Clinical Characteristics of Patients and NCs

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients With RRMS Relapse (n = 20)</th>
<th>Patients With RRMS Remission (n = 10)</th>
<th>Patients With SPMS (n = 10)</th>
<th>Patients With ALS (n = 22)</th>
<th>Patients With MSA (n = 10)</th>
<th>NCs (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), y</td>
<td>31.7 (9.4)</td>
<td>37.9 (14.7)</td>
<td>44.1 (7.3)</td>
<td>66.2 (12.2)*</td>
<td>65.1 (9.3)*</td>
<td>44.4 (20.5)</td>
</tr>
<tr>
<td>Sex, No. (%)</td>
<td>5 (25.0)</td>
<td>8 (80.0)</td>
<td>3 (30.0)</td>
<td>13 (59.1)</td>
<td>7 (70.0)</td>
<td>15 (55.6)</td>
</tr>
<tr>
<td>Disease duration, mean (SD), y</td>
<td>4.3 (3.8)</td>
<td>7.4 (3.1)</td>
<td>13.9 (7.5)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDSS score, mean (SD)</td>
<td>2.3 (1.0)</td>
<td>2.1 (0.8)</td>
<td>5.4 (1.5)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF total protein level, mean (SD), mg/dL</td>
<td>29.1 (6.5)</td>
<td>35.9 (15.4)</td>
<td>33.0 (11.5)</td>
<td></td>
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<tr>
<td>IgG index, mean (SD)</td>
<td>0.89 (0.38)*</td>
<td>0.77 (0.21)</td>
<td>0.81 (0.15)*</td>
<td></td>
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<tr>
<td>Positive IgG index of those tested, No. (%)</td>
<td>11 (55.0)</td>
<td>3 (30.0)</td>
<td>6 (77.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6 (23.0)</td>
</tr>
<tr>
<td>Positive OGB of those tested, No. (%)</td>
<td>9 (47.4)</td>
<td>2 (25.0)</td>
<td>5 (55.6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>LOTUS, mean (SD), μg/dL</td>
<td>9.3 (3.6)*</td>
<td>19.6 (5.8)</td>
<td>6.7 (1.4)*</td>
<td>19.2 (3.4)</td>
<td>19.3 (4.1)</td>
<td>19.2 (4.7)</td>
</tr>
</tbody>
</table>

Abbreviations: ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; LOTUS, lateral olfactory tract usher substance; MSA, multiple system atrophy; NCs, normal controls; OGB, oligoclonal IgG bands; RRMS, relapsing-remitting multiple sclerosis, SPMS, secondary progressive multiple sclerosis.

*Patients with ALS (P < .02) and MSA (P < .001) differed significantly from NCs.

*Patients with RRMS relapse (P < .04) and SPMS (P < .03) differed significantly from NCs.

*Patients with RRMS relapse (P < .001) and SPMS (P < .001) differed significantly from NCs (RRMS relapse; P < .001, SPMS; P < .001).
its antagonistic action on NgR1 function and may trigger the pathological cascade that leads to axonal degeneration. However, in RRMS, a decrease in LOTUS in the relapse phase was presumably transient with minimum axonal damage and recovered to the NC level in the remission phase along with improvement in clinical findings.

Maintenance of a lower concentration of LOTUS (6.7 [1-4] µg/dL) was found in SPMS, which is characterized by a progressive clinical course and shows continuous severe axonal degeneration. We speculate that LOTUS is a possible endogenous inhibitor of axonal degeneration and that a long-term decrease in LOTUS may be 1 of the causative mechanisms for persistent axonal degeneration in the progressive phase of MS.

Furthermore, fluctuations in the CSF concentration of LOTUS may be a novel biomarker of disease activity in terms of ongoing axonal degeneration. Although magnetic resonance imaging is useful for determining a definite diagnosis or detecting relapse lesions in RRMS, the availability of this technique is generally limited in hospitals. Furthermore, conventional CSF biomarkers, such as oligoclonal bands and the IgG index, are controversial as diagnostic tools for acute relapses in MS; the sensitivity of these biomarkers varies according to race/ethnicity and country. In European populations, more than 90% of patients with MS have positive test results for oligoclonal bands and show an increase in the IgG index while these are found in only 30% to 60% of patients with MS in Asian populations, including Japanese.12,13 Accordingly, a smaller proportion of the patients with positive oligoclonal bands and IgG Index was observed in this study cohort. Moreover, these biomarkers are not directly related to the pathogenesis of MS. Therefore, a new CSF biomarker is required for accurate diagnosis, appropriate therapy, comprehension of pathogenesis, and the development of new therapeutic strategies in MS. Lateral olfactory tract usher substance is a neuronal molecule and our data showing a good correlation between variations in LOTUS concentrations and disease dynamics strongly suggest that LOTUS may be a useful biomarker for disease activity and prognosis for both patients with RRMS and patients with SPMS.

From a clinical point of view, our findings provide new avenues for LOTUS as a useful biomarker and for understanding the pathogenesis of MS. However, our study has limitations, including the small number of patients and absence of longitudinal follow-up of CSF LOTUS levels. A large-scale longitudinal multicenter cohort study is required to confirm our results.

Conclusions

The CSF LOTUS concentrations in the relapse group of RRMS and SPMS were lower than that of NCs, whereas the level in the remission group of RRMS was similar to that of NCs. Variations in LOTUS concentrations were correlated with disease activity in MS. Therefore, LOTUS concentration may be useful as a possible biomarker for MS.

REFERENCES