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Identification of target antigens of naturally occurring autoantibodies in cerebrospinal fluid

Akio Kimura [a,*], Nobuaki Yoshikura [a], Akihiro Koumura [a], Yuichi Hayashi [a], Yuko Kobayashi [b], Issei Kobayashi [b], Takeo Yano [c], Takashi Inuzuka [a]

[a] Department of Neurology and Geriatrics, Gifu University Graduate School of Medicine, Gifu City, Gifu, Japan
[b] Life Science Research Center, Center for Molecular Biology and Genetics, Mie University, Tsu City, Mie, Japan
[c] Graduate School of Regional Innovation Studies, Mie University, Tsu City, Mie, Japan

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ABSTRACT

Naturally occurring autoantibodies have natural physiologic functions related to normal cell processes. However, the repertoire of naturally occurring autoantibodies against neuronal antigens in CSF is unclear. The purpose of this study was to identify naturally occurring autoantibodies against neuronal antigens in CSF from patients with various neurologic diseases by proteomics-based analysis. The CSF samples were collected from 77 patients with various neurologic disorders. The antigen source for 2-dimensional immunoblotting was the SH-SY5Y human neuroblastoma cell line. There were 8 spots recognized in CSF from more than one-fourth of the 77 patients including all patient groups and these spots were recognized in intravenous immunoglobulin preparations. These antigen spots were identified as heat shock 105-kDa/110-kDa protein 1, isoform CRA_b, 78-kDa glucose-regulated protein, heat shock cognate 71-kDa protein, tubulin beta chain, vimentin (2 spots), and 60-kDa heat shock protein, mitochondrial; we could not identify the protein name corresponding to 1 of the 8 spots. In summary, there were 6 proteins identified that were main target antigens that reacted with naturally occurring autoantibodies in CSF from patients with varied neurologic disorders; the functions of autoantibodies against the identified antigens are unknown and may be clarified with further studies.

Biological significance
Naturally occurring autoantibodies may have important functions in tissue homeostasis. In this study, we identified 6 common target antigens that reacted with autoantibodies in cerebrospinal fluid (CSF) from patients, independent of disease type. These findings may clarify the importance of naturally occurring autoantibodies in CSF and the use of these antibodies potentially may be a novel therapy for various neurologic disorders.

1. Introduction

Antineuronal antibodies are present in serum and cerebrospinal fluid (CSF) of patients with several neurologic diseases. Some antineuronal antibodies are specific and useful diagnostic markers of neurologic diseases. Antineuronal antibodies against intracellular proteins such as Hu and Yo are markers of paraneoplastic neurologic syndromes [1,2]. Antineuronal antibodies against cell surface or synaptic proteins such as N-methyl-D-aspartate receptor and leucine-rich, glioma-inactivated 1 are markers of autoimmune encephalopathies [3,4]. Although these antineuronal antibodies are disease-specific antibodies, the presence of brain-reactive autoantibodies is ubiquitous in human serum [5]. These naturally occurring autoantibodies are present in the serum of healthy individuals without overt immunization or infection [6].

Naturally occurring autoantibodies have natural physiologic functions related to normal cell processes, either stimulating cell processes or removing cellular debris [7–9]. Naturally occurring autoantibodies bind to apoptotic cell membranes and inhibit the proinflammatory properties of lupus autoantibody immune complexes [10]. Higher naturally occurring autoantibody levels against apoptosis-associated antigens correlate with protection from cardiovascular events and renal disease in patients with systemic lupus erythematosus [11]. In addition, the use of naturally occurring autoantibodies is a potentially important therapeutic technique for various neurologic and malignant diseases [12–15]. However, the repertoire of naturally occurring autoantibodies against neuronal antigens in CSF is unclear.

The purpose of this study was to identify naturally occurring autoantibodies against neuronal antigens in CSF from patients with various neurologic diseases by proteomics-based analysis using SH-SY5Y

Abbreviations: CSF, cerebrospinal fluid; IVIg, intravenous immunoglobulin; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline containing 0.1% Tween 20.

* Corresponding author at: Department of Neurology and Geriatrics, Gifu University Graduate School of Medicine, Gifu, 1-1 Yanagido, Gifu 501-1194, Japan.
E-mail address: kimura1@gifu-u.ac.jp (A. Kimura).

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human neuroblastoma cells as the antigen source. As a result, we identified 6 common neuronal antigens that reacted with autoantibodies in CSF from patients, independent of disease type, and in intravenous immunoglobulin (IVIg) preparations. These findings may clarify the importance of naturally occurring autoantibodies in CSF.

2. Materials and methods

2.1. Patients and cerebrospinal fluid samples

The CSF samples were collected from 17 patients who had functional or psychogenic neurologic disorders (14 patients with somatization disorder and 3 patients with functional headache), 20 patients who had multiple sclerosis, 20 patients who had viral meningitis, and 20 patients who had Alzheimer's disease. All CSF samples from the 20 multiple sclerosis patients were taken during the relapse phase. This study was approved by the institutional review board of Gifu University Graduate School of Medicine, Gifu City, Japan.

2.2. Preparation of neuronal antigens

We investigated all the target spots corresponding to proteins that reacted with autoantibodies in the CSF samples of all 77 patients by 2-dimensional electrophoresis, followed by Western blot. We used the human neuroblastoma SH-SY5Y cell line (DS Pharma Biomedical, Osaka, Japan) as the antigen source for 2-dimensional immunoblot. The reason for using SH-SY5Y cells as the antigen source was that they were suitable to obtain a large, uniform quantity of neuronal antigens for 2-dimensional immunoblot with high reproducibility and reliability [16]. The cultured cells were homogenized in a lysis buffer (5 M urea, 2 M thiourea, 0.2% sodium dodecyl sulfate [SDS], 0.1 M dithiothreitol, 2% Pharmalyte [pH, 3 to 10] [GE Healthcare, Buckinghamshire, UK]) containing proteinase inhibitor mixtures (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany) and centrifuged at 18,800 $\times g$ for 40 min. The resulting supernatant was used in all experiments.

2.3. 2-Dimensional electrophoresis and immunoblot

Proteins in the prepared human neuroblastoma cultured cell extract were separated by 2-dimensional electrophoresis performed in an immobilized pH gradient, isoelectric focusing, and SDS-polyacrylamide gel electrophoresis system according to a previous report [17]. The first dimension immobilized pH gradient and isoelectric focusing were carried out on a reswollen immobilized pH gradient gel (Immobiline DryStrip; pH range, 4 to 7; nonlinear; length, 13 cm) (GE Healthcare, Buckinghamshire, UK). The extracted protein (maximum, 100 μg) was applied on the immobilized pH gradient gel. The horizontal electrophoresis system (CoolPhoreStar-IPG-IEF-Type-PX, Anatech, Tokyo, Japan) was maintained at 20 °C, and isoelectric focusing was performed as follows: step 1 (500 V for 1.5 h); step 2 (700 V for 45 min); step 3 (1000 V for 45 min); step 4 (1500 V for 45 min); step 5 (2000 V for 45 min); step 6 (2500 V for 45 min); step 7 (3000 V for 45 min); and step 8 (3500 V for 8 h). After isoelectric focusing, the immobilized pH gradient gel was equilibrated with the SDS treatment solution followed by carbamoylmethylation in an iodoacetamide-containing buffer. An equilibrated gel strip was placed on top of a 12.5% polyacrylamide gel. The SDS-polyacrylamide gel electrophoresis was run vertically in a Tris–glycine buffer system using an electrophoresis apparatus (ERICA-S, DRC, Tokyo, Japan) at a constant voltage of 300 V for 2 h.

After electrophoresis, the SDS-polyacrylamide gels were stained (SyproRuby, Bio-Rad Laboratories, Hercules, CA, USA) or used for protein transfer onto polyvinylidene difluoride (PVDF) membranes. Separated proteins were electrophoretically transferred to a PVDF membrane at a constant voltage of 32 V for 3 h in a buffer transfer tank equipped with a cooling device (ERICA-S, DRC). The membrane was incubated in a blocking solution (5% skim milk in 1× Tris-buffered saline containing 0.1% Tween 20 [TBST]) for 1 h at room temperature, and reacted with the patient's CSF diluted (1:200) with 1% skim milk in 1× TBST overnight in a cold room. The PVDF membrane after blotting also was incubated with a blocking solution overnight in a cold room, and reacted with the IVIg preparations diluted (1:2000) with 1% skim milk in 1× TBST for 1 h at room temperature. Thereafter, the membrane was washed 5 times with 1× TBST and reacted with peroxidase-conjugated goat antihuman immunoglobulin (Ig) A, G, and M antibodies (Life Technologies, Carlsbad, CA, USA) diluted (1:3000) with 1% skim milk in 1× TBST for 1 h at room temperature. After 6 washes, the membrane was incubated with the Western blot detection reagent (ECL-Plus, GE Healthcare) for 20 min and scanned using a luminescent image analyzer (LAS-4000 mini, Fujifilm, Kanagawa, Japan). The antibody-reactive protein spots were matched with the stained protein spots (SyproRuby, Bio-Rad Laboratories) using image analysis software (Adobe Photoshop 6.0, Adobe Systems, San Jose, CA, USA).

2.4. In-gel digestion and mass spectrometry

Proteins were detected by staining (SyproRuby, Bio-Rad Laboratories). For mass spectrometric identification, a target protein spot on the stained 2-dimensional electrophoresis gel was excised (FluoroPhoreStar 3000, Anatech) and digested with trypsin. Peptide fragments were analyzed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF mass spectrometry (MALDI-TOF/TOF-MS) (4800 Plus MALDI TOF/TOF Analyzer, AB SCIEX, Framingham, MA, USA). Up to 10 of the most intense ion signals per spot position, characterized by an S/N >10, were selected as precursors for MS/MS acquisition. Proteins were identified from tandem mass spectrometry (MS/MS) spectra using protein identification software (ProteinPilot 4.0, AB SCIEX, Framingham, MA, USA).

2.5. 1-Dimensional electrophoresis and immunoblot using human recombinant proteins

To validate and determine whether identified proteins were unique targets of the naturally occurring autoantibodies, we performed 1-dimensional immunoblot analysis. The commercially available full-length human recombinant proteins (HSP 105/110 and GRP78 recombinant proteins, ProSpec-Tany TechnoGene, Ness Ziona, Israel; HSPA8 recombinant protein, StressMarq, Victoria, BC, Canada; β-tubulin, vimentin, and HSP60 recombinant proteins, Abnova, Taipei, Taiwan) were loaded onto a 4% to 12% bis–Tris minigel (Invitrogen, Waltham, MA, USA). Electrophoresis was performed (Invitrogen XCell Surelock Mini-Cell, Invitrogen) with SDS running buffer (NuPAGE MOPS, Invitrogen) according to instructions from the manufacturer (200 V for 50 min). Separated proteins were electrophoretically transferred to a PVDF membrane at a constant voltage of 30 V for 3 h in a buffer transfer tank equipped with a cooling device (Mini PROTEAN, Bio-Rad Laboratories). The membrane was incubated in the blocking solution (5% skim milk in TBST) for 1 h at room temperature, and reacted with the mixed patient CSF samples from 5 patients from each patient group according to disease, diluted (1:100) with 1% skim milk in 1× TBST overnight in a cold room. The 2-dimensional electrophoresis and immunoblot method was repeated.

2.6. Statistical analysis

We assessed the differences in positivity of autoantibodies against each detected spot between the patient groups. Average values were reported as mean ± standard deviation (SD). The statistical difference was determined by chi-square test for independent evaluation. We also assessed the differences in the number of antigen spots that reacted with individual CSF between the patient groups according to disease. Data were analyzed by nonparametric analysis of variance. Differences with $P \leq .05$ were considered significant.

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