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Detection of autoantibody against extracellular epitopes of *N*-methyl-D-aspartate receptor by cell-based assay

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ABSTRACT

The concept of anti-*N*-methyl-D-aspartate receptor (NMDAR) encephalitis, a severe, potentially lethal, treatment-responsive disorder, mediated by autoantibodies against NMDAR was proposed. Because paraneoplastic anti-NMDAR encephalitis has a better prognosis after tumor resection and immunotherapy, rapid quantitative systems for detecting functional autoantibodies against extracellular epitopes of NMDAR are necessary. To detect autoantibodies recognizing extracellular epitopes of NMDAR, we stably expressed mutant NMDAR that decreases Ca^{2+} permeability on a heterologous cell surface without any antagonist. Serum and CSF samples from patients were analysed using the cells expressing mutant NMDAR subunits by immunocytochemistry and on-cell Western analysis using live cells stably expressing mutant NMDAR. Furthermore, we were able to express mutant GluRζ1(NR1, GluN1) subunit of NMDAR alone on the cell surface and obtained direct evidence of the presence of autoantibodies recognizing extracellular epitopes of GluRζ1 and the induction of internalization by autoantibodies in serum and CSF from patients. The specificity of on-cell Western analysis was improved at 37 °C. The combination of this rapid quantitative assay using our on-cell Western analysis, detailed analysis of extracellular epitopes of NMDAR, and internalization assay of NMDAR will be valuable for the diagnosis, evaluation of clinical treatments, and follow-up of anti-NMDAR encephalitis.

1. Introduction

The *N*-methyl-D-aspartate receptor (NMDAR) is critically involved in normal neural network formation, synaptic plasticity, and higher brain functions such as learning and memory (Bliss and Collingridge, 1993; Komuro and Rakic, 1993). A highly active NMDAR is composed of multiple glutamate-binding GluRε (NR2, GluN2) subunits and glycine/D-serine-binding GluRζ1 (NR1, GluN1) subunit (Mori and Mishina, 1995). The hyperactivation of NMDAR has been shown to mediate acute neuronal death and chronic neurodegeneration (Lancelot and Beal, 1998). Furthermore, the hypoactivation of NMDAR is involved in psychiatric states (Gunduz-Bruce, 2009). Recently, the concept of anti-NMDAR encephalitis, a severe, potentially lethal, treatment-responsive disorder, mediated by autoantibodies against NMDAR has been proposed by Dalmau et al. (2008). Some mechanisms underlying the pathogenic effects of autoantibodies on NMDAR were proposed including (1) attenuation of NMDAR function by receptor internalization and degradation of NMDAR by anti-NMDAR antibodies associated with paraneoplasms, such as ovarian tumors (Dalmau et al., 2008; Hughes et al., 2010) and (2) neuronal damage and death caused by complement-mediated immune reactions observed in systemic lupus erythematosus (DeGiorgio et al., 2001; Kowal et al., 2006). These functional autoantibodies should recognize extracellular epitopes of NMDAR. Because paraneoplastic anti-NMDAR encephalitis has a better prognosis after tumor resection and immunotherapy (corticosteroids, intravenous immunoglobulins, or plasma exchange) (Dalmau et al., 2007), rapid quantitative evaluation systems for detecting autoantibodies against extracellular epitopes of NMDAR are necessary.

Abbreviations: CHO, Chinese hamster ovary; CSF, cerebrospinal fluid; DAPI, 4′,6-diamidio-2-phenylindole; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorter; HEK, human embryonic kidney; NMDAR, NMDA receptor; PBS, phosphate-buffered saline; PBST, PBS containing 0.1% Tween-20.

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Table 1
Clinical features of samples.

Sample	Serum				CSF			
	Patients (n = 18)		Controls (n = 13)		Patients (n = 10)		Controls (n = 6)	
Median age (range)	24.6 (5–65)		32.9 (6–66)		23.2 (7–65)		18.5 (1–60)	
Men:women	1:17		5:8		1:9		2:4	
Clinical diagnosis	Encephalitis associated with OT	9	Limbic encephalitis	2	Encephalitis associated with OT	6	Unexplained encephalitis	3
	Limbic encephalitis	7	Unexplained encephalitis	5	Limbic encephalitis	3	Cerebellar ataxia	1
	Unexplained encephalitis	1	Cerebellar ataxia	1	Small cell lung cancer	1	Autism	1
	Small cell lung cancer	1	Healthy	5			Refractory epilepsy	1

Abbreviation: OT = ovarian teratoma.

To establish such evaluation systems for detecting autoantibodies recognizing extracellular epitopes of NMDAR, the establishment of cells stably expressing each NMDA receptor subunit and functional NMDAR is desirable. However, Ca^{2+} influx through NMDAR activated by glutamate and glycine present in culture medium is toxic to non-neurons (Anegawa et al., 1995). Inducible expression system of NMDAR and antagonists of NMDAR are used for the stable expression of NMDAR (Uchino et al., 1997; Varney et al., 1996). For the cell surface expression of NMDAR, both the GluRε and GluRζ1 subunits of NMDAR are necessary (McIlhinney et al., 1998; Meddows et al., 2001). The epitope of GluRζ1 recognized by autoantibodies in patients has been mapped between the amino acid residues 25-380 of human NR1 by in vitro enzyme-linked immunosorbent assay (ELISA) with a mutant subunit (NR1d4) expressed and purified from cells as an antigen (Dalmau et al., 2008). The NR1d4 subunit can assemble with the NR2 subunit in the cytoplasm; however, this complex cannot be expressed on the cell surface (Meddows et al., 2001). The treatment of autoantibodies in patients induces the reduction of the NR1 subunit on cultured neurons (Dalmau et al., 2008; Hughes et al., 2010); however, these findings cannot exclude the possibility of the presence of antibodies recognizing the NR2 subunit and cell surface molecules associated with NMDAR. Thus, the presence of autoantibodies recognizing extracellular epitopes of NMDAR expressed on the cell surface is controversial. Furthermore, reported cell-based assays using the transient expression of NMDAR subunits in heterologous cells and the preparation of primary cultured neurons are time-consuming and less quantitative. Thus, cells stably expressing NMDAR are desirable. Here, we constructed and stably expressed mutant NMDAR that decreases Ca^{2+} permeability (Burnashev et al., 1992; Yamakura et al., 1993) on a heterologous cell surface without any NMDAR antagonist, and analysed the presence and function of autoantibodies against NMDAR.

2. Materials and methods

2.1. Serum and cerebrospinal fluid (CSF) samples

The clinical features of subjects are summarized in Table 1. Frozen serum and CSF samples were used in this study. The serum and CSF samples were first examined by the immunocytochemical analysis as described below and determined for the positivity and negativity for anti-NMDAR autoantibodies. The subjects positive for the autoantibodies and those negative for them are referred to as the patients and controls, respectively, and the samples from these subjects were used for on-cell Western analysis as described below. The experiments were undertaken with the understanding of the subjects and written informed consent was obtained from of each subject; the study conformed with The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the *British Medical Journal* (18 July 1964). This study was approved by the Research Ethics Committees of the University of Toyama (Approval No. 23-18) and Shizuoka Institute of Epilepsy and Neurological Disorders (Approval No. 2006-2).

2.2. Construction of mutant NMDAR subunit expression vectors

We used mouse NMDAR GluRε2 (NR2B, GluN2B) and GluRζ1 (NR1, GluN1) subunits in this study. The following expression vectors were constructed using standard recombinant DNA techniques. The DNA fragments obtained by polymerase chain reaction were confirmed by DNA sequencing. The 5.1 kb *Eco*RI fragment containing the entire coding sequence of the GluRε2-N589R mutant from pBKSAε2-N589R (Yamakura et al., 1993) was inserted into the *Eco*RI site of the mammalian expression vector pCAGGS (Niwa et al., 1991) to yield the vector pCAG-GluRε2-N589R. The DNA fragment containing the open reading frame of the GluRε2-N589R mutant was fused in-frame to the coding sequence of DsRed derived from pDsRed-Monomer-C1 (Clonetech, Mountain View, CA, USA) and cloned into pCAGGS to yield the vector pCAG-GluRε2-N589R-DsRed. There is no additional amino acid sequence at the fusion site between GluRε2-N589R and DsRed. The DNA fragment containing the open reading frame of the GluRζ1-N598R mutant (Yamakura et al., 1993) was fused in-frame to the coding sequence of enhanced green fluorescent protein (EGFP) derived from pEGFP-N1 (Clontech) and cloned into pCAGGS to yield pCAG-GluRζ1-N598R-EGFP. There was a linker amino acid sequence (PVAT; the one-letter amino acid sequence derived from the multiple-cloning-site sequence of pEGFP-N1) at the fusion site between GluRζ1-N589R and EGFP.

2.3. Cell culture, DNA transfection, and cell sorting

Human embryonic kidney (HEK) 293T cells and Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum. Expression vectors were transfected into HEK293T cells and CHO cells using Trans-IT 293 and Trans-IT CHO transfection reagents (Mirus, Madison, WI, USA), respectively, as instructed in the manufacturer's protocol. For the isolation of HEK 293T transformants stably expressing mutant NMDAR subunits, the DNA-transfected cells were sorted four times on the basis of high-fluorescence-intensity signals of EGFP (excitation at 488 nm and emission at 530 nm) and DsRed (excitation at 488 nm and emission at 585 nm) using a fluorescence-activated cell sorter (FACS) (FACSAria II flow cytometer, Becton Dickinson Biosciences, San Jose, CA, USA).

2.4. Western blotting

HEK 293T cells stably expressing the mutant NMDAR subunits were grown for 36–48 h, washed with phosphate-buffered saline (PBS, pH 7.4), homogenized in mammalian protein extraction reagent (M-PER, Thermo SCIENTIFIC, Kanagawa, Japan), and centrifuged at 14,000 rpm for 10 min. The resulting supernatant was obtained. The extracted protein samples were heat-denatured and electrophoresed in 7.5% sodium dodecyl sulfate-polyacrylamid gel; separated products were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Tokyo, Japan). The PVDF membrane was blocked with 2.5% skim milk in PBS containing 0.1% Tween-20 (PBST) for 1 h. The membrane was incubated with a rabbit anti-GluRε2-N antibody (1:200; it recognizes the extracellular amino-terminal region of the GluRε2 subunit, Watanabe et al., 1998), or a mouse monoclonal anti-NMDAR1 antibody (1:200; BD Pharmingen, San Diego, CA, USA; it recognizes the amino acids 660–811 of the rat NR1 subunit located on the extracellular region between the transmembranes M3 and M4; we referred to it as the anti-GluRζ1-M3/M4 antibody in this report) overnight at 4 °C. The next day, the membrane was washed in PBST and incubated with a secondary antibody (1:5000 goat anti-rabbit IgG conjugated to HRP, BIO-RAD, Tokyo, Japan; or 1:5000 donkey anti-mouse IgG conjugated to HRP, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. The membrane was washed in PBST, and protein bands were detected using an ECL Plus Western Blotting Detection System (GE Healthcare) with a luminoimaging analyzer (ImageQuant LAS-4000, FUJIFILM, Tokyo, Japan).

2.5. Immunocytochemistry and confocal microscopy

HEK293T cells were seeded on a polyethyleneimine-coated 35 mm glass-based dish (IWAKI, Chiba, Japan) at 2.5×10^5 cells/dish. About 24 h later, the cells were transfected with the plasmids pCAGGS-GluRζ1-N598R-EGFP (0.2 μg) and pCAGGS-GluRε2-N589R (2 μg), or pCAGGS-GluRζ1-N598R-EGFP alone (2 μg). The cells were grown for 36–48 h, washed in PBS, and blocked in PBS containing 5% goat serum or in PBS containing 5% rabbit serum at room temperature for 30 min. Then, the cells were incubated with a serum sample (1:200), a CSF sample (1:10), a rabbit anti-GluRε2-N antibody (1:250), or a mouse anti-GluRζ1-M3/M4 antibody (1:250) overnight at 4 °C. The next day, the cells were washed in PBS and incubated with a secondary antibody, namely, goat anti-human IgG conjugated to Alexa Fluor 647 (1:500), goat anti-rabbit IgG conjugated to Alexa Fluor 647(1:500), or rabbit anti-