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Short communication

Antibodies against Wnt receptor of muscle-specific tyrosine kinase in myasthenia gravis

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ABSTRACT

Muscle-specific tyrosine kinase (MuSK) antibodies are detected in a proportion of myasthenia gravis (MG) patients who are negative for acetylcholine receptor (AChR) antibodies and have prominent bulbar weakness and crises. In the MuSK ectodomains, the immunoglobulin-like 1 and 2 domains (Ig1/2) mediate the agrin–Lrp4–MuSK signaling and the cysteine-rich domain (CRD) mediates the Wnt–MuSK–Dishevelled signaling; both contribute to AChR clustering. Immunoblotting against recombinant proteins showed MuSK Ig1/2 antibodies in 33 anti-AChR-negative MG patients; 10 patients of them (30%) were additionally positive for MuSK CRD antibodies. The result suggests that MuSK antibodies have heterogeneity in their binding to functional domains of MuSK.

1. Introduction

Myasthenia gravis (MG) is a disease of neuromuscular junction (NMJ) which is mainly caused by an immune response to the nicotinic acetylcholine receptor (AChR) in skeletal muscle. A proportion of MG patients are negative for AChR antibodies, and instead have antibodies (largely IgG4 and partially IgG1) against the muscle-specific tyrosine kinase (MuSK) (Vincent et al., 2008) with the clinical features including prevalence in women, prominent bulbar involvement, crises and anticholinesterase nonresponsiveness (Pasnoor et al., 2010; Guptill et al., 2011). MuSK is stimulated from "inside" the muscle cells by Dok7 (Yamanashi et al., 2012), and from the "outside" by Lrp4 (agrin receptor) with and without agrin (Weatherbee et al., 2006; Kim et al., 2008; Zhang et al., 2008); MuSK thereby contributes to AChR stabilization and clustering through various intracellular kinase cascades (Wu et al., 2010). Also, MuSK contains the receptor for Wnts which belong to a family of secreted glycoproteins and interact with the muscle-expressed Dishevelled (Dvl) that is essential for the noncanonical Wnt signaling cascade contributing to AChR clustering (Luo et al., 2002; Korkut and Budnik, 2009; Wu et al., 2010); this pathway also regulates a retrograde signaling to nerve terminals (Luo et al., 2002). In the MuSK ectodomains, its first and second immunoglobulin-like domains (Ig1/2) mediate the agrin–Lrp4 signaling (Stiegler et al., 2006) and its cysteine-rich domain (CRD) mediates the Wnt signaling (Stiegler et al., 2009). In view of these, we studied the MG patients focusing on the antibodies against the MuSK CRD in association with the antibodies against the MuSK Ig1/2 domains.

2. Patients and methods

2.1. Patients

Serum samples were obtained from 43 anti-AChR-negative patients, aged from 6 to 80 years at onset (13 men and 30 women), with generalized MG defined by the Myasthenia Gravis Foundation of America (MGFA) classification (grades from IIa to V) (Jaretzki et al., 2000). The diagnosis was based on fatigable muscle weakness with electrophysiological evidence of decrementing compound muscle action potentials to low-rate repetitive nerve stimulation or increased jitter on single-fiber electromyography; a positive response to edrophonium injection was considered as a finding in favor of the diagnosis. Thirty-three patients, aged from 6 to 80 years at onset (10 men and 23 women), of these 43 patients were positive for MuSK antibodies (ranged from 5.32 to 131.40 nM. control, <0.05 nM) determined by the standard radioimmunoassay (RIA) (McConville et al., 2004); the remaining 10 patients negative for standard RIA-determined MuSK antibodies, aged from 20 to 65 years at onset (3 men and 7 women) were also studied as MG diagnosed by clinical and electrophysiological features (MGFA-graded from IIa to IIIa). Control sera were obtained from 10 healthy volunteers aged from 22 to 43 years (5 men and 5 women) and from 10 disease controls with MG, aged from 18 to 70 years at onset (2 men

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and 8 women) and MGFA-graded from IIb to IIIb who were positive for AChR antibodies (from 5.6 to 77.0 nM. control, <0.2 nM) and negative for standard RIA-determined MuSK antibodies (<0.05 nM).

2.2. Recombinant expression of MuSK Ig1/2 domains and CRD

DNA fragments coding from human MuSK Ig1/2 domain-contained amino acid residues (22–212) (Stiegler et al., 2006) and CRD-contained amino acid residues (313–494) (Stiegler et al., 2009) were generated by PCR method using human MuSK cDNA (OriGene Technol., USA) as a template. Expression constructs were generated in pcDNA3.3-TOPO (Invitrogen, USA) with DNA fragments ligated to the synthetic mouse trypsin prepro sequence followed by 7 histidines tagged at the N-terminus. Human embryonic kidney (HEK) 293F cells (Invitrogen, USA) were transiently transfected with these constructs according to the instructions (FreeStyle™293 Expression System, Invitrogen, USA), and the cultured supernatants were harvested for 4 days after transfection. Clarified supernatants were directly loaded onto a HisTrap HP column (GE Healthcare, USA) equilibrated with 0.1 M Tris–Cl, 0.5 M NaCl, 20 mM Imidazole, pH 7.4. After the column was washed with the equilibration buffer, Ig1/2 and CRD proteins were eluted with linear gradient of Imidazole. Purity of Ig1/2 and CRD proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, USA) and Western blotting using mouse anti-human monoclonal antibodies (ab86456 for Ig1/2 and ab55549 for CRD. Abcam, USA).

2.3. Western blot analysis

Either Ig1/2 domains or CRD of MuSK at an amount of 5 μg/lane was subjected to 12.5% SDS-PAGE under reducing conditions with dithiothreitol. Proteins were transfected onto a polyvinylidene difluoride membrane and were then cut into strips. The antibody detection was performed by using Immun-Blot® Assay Kit (Bio-Rad Lab., USA). In brief, after blocking with Tris buffered saline containing 3% gelatin for 1 h, each strip was incubated with the serum sample which was diluted from 1:100 to 1:1000 and with a 1000-fold-diluted each mouse anti-human monoclonal antibody (used for the confirmation of purified recombinant protein) at room temperature for 1 h. After 30 min-incubation with 3000-fold-diluted goat anti-human IgG or anti-mouse IgG conjugated with alkaline phosphatase (AP), specific reactivity was estimated by the intensity with which each strip was stained with AP color development solution. The 22 kDa and 38 kDa immunostained bands were visualized for MuSK Ig1/2 and CRD monoclonal antibodies, respectively. When the serum sample showed the immunostained band at the same migration position as that proved with the test monoclonal antibody in the serum dilutions more than 1:500, the result was judged as antibody-positive.

3. Results

In the study collecting the serum samples from 43 anti-AChR-negative MG patients, 10 patients were negative for MuSK antibodies determined by both the standard RIA and the present study (group 3 in Table 1). All of the remaining 33 patients positive for MuSK antibodies determined by RIA were positive for MuSK Ig1/2 antibodies (groups 1 and 2 in Table 1); 10 patients of them (30%) were additionally positive for MuSK CRD antibodies (group 1 in Table 1). None was positive for MuSK CRD antibodies alone. Ten disease controls (anti-AChR-positive MG; group 4 in Table 1) and 10 healthy volunteers were all negative for MuSK Ig1/2 and MuSK CRD antibodies as well as for standard RIA-determined MuSK antibodies. The disease severity (MGFA grades) and clinical symptoms in each group showed that a trend was noted toward severe severity in anti-MuSK-positive MG (groups 1 and 2 in Table 1). However, there was no significant correlation between the positive (group 1 in Table 1)/negative (group 2 in Table 1) results from MuSK CRD antibody determination and the MGFA grades (Wilcoxon test, $p = 0.0747$). Representative immunoblots are shown in Fig. 1: C corresponding to group 1 and D corresponding to group 2 in Table 1. No immunostained band for MuSK Ig1/2 and MuSK CRD was seen with the serum from an anti-AChR-positive MG patient (B corresponding to group 4 in Table 1). The antibody specificity was confirmed by the same migration positions as those of purified, CBB-stained recombinant proteins (A in Fig. 1).

Table 1
Clinical and immunological profiles of 53 myasthenia gravis (MG) patients.

Groups (numbers of patients)	1 (10)	2 (23)	3 (10)	4 (10)
Age at onset (years)	22–75	6–80	20–65	18–70
Gender	F 8/M 2	F 15/M 8	F 7/M 3	F 8/M 2
Antibodies against				
• MuSK Ig1/2 domains (immunoblot)	Positive	Positive	Negative	Negative
• MuSK CRD (immunoblot)	Positive	Negative	Negative	Negative
• Full-length of MuSK extracellular segment, determined by standard RIA (control, <0.05 nM)	6.08–131.40 nM	5.32–45.75 nM	<0.05 nM	<0.05 nM
• AChR, determined by standard RIA (control, <0.2 nM)	<0.2 nM	<0.2 nM	<0.2 nM	5.6–77.0 nM
MG severity (MGFA grades)				
IIa	0	3	4	0
IIb	0	7	2	3
IIIa	0	2	4	6
IIIb	4	1	0	1
IVa	0	0	0	0
IVb	0	0	0	0
V	6	10	0	0
Clinical symptoms				
Facial/bulbar weakness	10	20	2	4
Respiratory crisis	5	10	0	0
Neck weakness	5	2	0	0
Ophthalmoplegia	9	21	10	10
Limb weakness	6	13	10	7

F: female. M: male. MuSK: muscle-specific tyrosine kinase (Ig1/2 domains: immunoglobulin-like 1 and 2 domains; CRD: cysteine-rich domain). AChR: acetylcholine receptor. RIA: radioimmunoassay. MGFA: Myasthenia Gravis Foundation of America. Figures in MG severity and clinical symptoms indicate numbers of the patients subject to each item.

4. Discussion

This brief report shows that MuSK antibodies in a part of MG patients recognize not only the main immunogenic site(s) (Ig1/2 domains) responsible for the agrin signaling (McConville et al., 2004) but also the CRD responsible for the Wnt signaling (Stiegler et al., 2009). However, it remains to study as to whether the antibody heterogeneity could correlate with disease activity or characteristics.

Recent studies highlight Wnt involvement in critical aspects of the NMJ function and structure (Korkut and Budnik, 2009; Wu et al., 2010). Wnts 11 (Jing et al., 2009; Zhang et al., 2012), 9a (Zhang et al., 2012), 4 (Strochlic et al., 2012) and 3 (Henriquez et al., 2008), which are released from neurons or derived from muscles, bind to MuSK CRD and contribute to the AChR clustering via the Dvl-mediated noncanonical Wnt signaling cascade (Luo et al., 2002; Korkut and Budnik, 2009; Wu et al., 2010; Zhang et al., 2012). Therefore, the Wnt–Dvl signaling via MuSK CRD converges upon the agrin–Lrp4 signaling via MuSK Ig1/2 domains, and both contribute to AChR clustering. Therefore, our results suggest that MuSK CRD antibodies could be involved in impairment of the Wnt–MuSK interaction, suggesting a diversity to MuSK-implicated pathophysiology in MG.

Reportedly, the inhibited MuSK–Dvl interaction decreases the frequency of spontaneous synaptic currents (SSC) in association