Experimental Neurology 297 (2017) 158–167

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Immunization of mice with LRP4 induces myasthenia similar to MuSK-associated myasthenia gravis

Shuuichi Mori[a], Norio Motohashi[a], Rumi Takashima[a], Masahiko Kishi[b], Hiroshi Nishimune[c], Kazuhiro Shigemoto[a,*]

[a] Department of Geriatric Medicine, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan
[b] Department of Internal Medicine, Toho University Sakura Medical Center, Chiba, Japan
[c] Department of Anatomy and Cell Biology, University of Kansas School of Medicine, Kansas City, KS, United States

ARTICLE INFO

Keywords:
Myasthenia gravis
Animal model
Neuromuscular junction
Lrp4
MuSK

ABSTRACT

Since the first report of experimental animal models of myasthenia gravis (MG) with autoantibodies against low-density lipoprotein receptor-related protein 4 (LRP4), there have not been any major reports replicating the pathogenicity of anti-LRP4 antibodies (Abs). Recent clinical studies have cast doubt on the specificity and pathogenicity of anti-LRP4 antibodies for MG, highlighting the need for further research. In this study, we purified antigens corresponding to the extracellular region of human LRP4 stably expressed with chaperones in 293 cells and used these antigens to immunize female A/J mice. Immunization with LRP4 protein caused mice to develop myasthenia having similar electrophysiological and histological features as are observed in MG patients with circulating Abs against muscle-specific kinase (MuSK). Our results clearly demonstrate that active immunization of mice with LRP4 proteins causes myasthenia similar to the MG induced by anti-MuSK Abs. Further experimental and clinical studies are required to prove the pathogenicity of anti-LRP4 Abs in MG patients.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease characterized by ptosis, fatigue, and muscle weakness, caused by the association of pathogenic antibodies (Abs) with postsynaptic membrane proteins at neuromuscular junctions (NMJs). Analyses of patient sera showed that many MG patients produce autoantibodies against acetylcholine receptor (AChR, ~85% of MG patients) or muscle-specific kinase (MuSK, ~8% of MG patients) (Binks et al., 2016; Gilhus and Verschuuren, 2015). Abs against low-density lipoprotein receptor (LDLR)-related protein 4 (LRP4) were found in 2% to 54% of MG patients who were seronegative for anti-AChR and anti-MuSK Abs, as well as in sera from MG patients with Abs against AChR (0 to 7.5% of AChR-MG patients) or MuSK (2.7 to 7.9% of MuSK-MG patients) (Higuchi et al., 2011; Pevzner et al., 2012; Zhang et al., 2012; Zisimopoulou et al., 2014). The variable frequency with which anti-LRP4 Abs were found in MG patients was likely influenced by differences in research methods among the groups (Binks et al., 2016; Evoli and Iorio, 2015).

LRP4 is a single-pass transmembrane protein of the LDLR family. LRP4 mutant mice display severe defects in NMJ formation, which are strikingly similar to those observed in MuSK mutant mice and which lead to neonatal lethality (DeChiara et al., 1996; Weatherbee et al., 2006). As a functional receptor for the nerve-derived heparin sulfate proteoglycan agrin, LRP4 forms a complex with MuSK at the NMJ, where it plays a critical role in agrin-stimulated MuSK activation and NMJ formation (Kim et al., 2008; Zhang et al., 2008). Based on its similarity to MuSK in terms of NMJ formation and its localization to NMJs, LRP4 is considered a candidate autoantigen in seronegative MG (Higuchi et al., 2011).

The experimental autoimmune myasthenia gravis (EAMG) model of AChR-MG and MuSK-MG has been used to understand the pathogenicity of autoantibodies and the mechanisms underlying MG symptoms of muscle weakness and atrophy (Conti-Fine et al., 2006; Mori and Shigemoto, 2013; Phillips et al., 2014). Recently, active immunization with recombinant proteins of the LRP4 ectodomain and passive transfer of anti-LRP4 Abs produced in immunized rabbits were shown to cause mild myasthenia in mice (Shen et al., 2013). However, the electromyography (EMG) results of these LRP4-MG mice were not characteristic of features found in animal models or patients with AChR-MG or MuSK-MG. Furthermore, anti-LRP4 Abs were detected in two out of 16 patients with neuromyositis optica without MG symptoms and 24 out of 204 patients with amyotrophic lateral sclerosis (Tzartos et al., 2014; Zhang et al., 2012). These findings cast doubt on the Ab specificity and pathogenicity for MG, and demonstrate the need for further studies to

* Corresponding author at: Department of Geriatric Medicine, Tokyo Metropolitan Institute of Gerontology, Sakaecho 35-2, Itabashi-ku, Tokyo 173-0015, Japan.
E-mail address: kazshige@tmig.or.jp (K. Shigemoto).

http://dx.doi.org/10.1016/j.expneurol.2017.08.006
Received 27 June 2017; Received in revised form 11 August 2017; Accepted 11 August 2017
Available online 18 August 2017

determine whether anti-LRP4 Abs are pathogenic in MG (Binks et al., 2016; Evoli and Iorio, 2015).

There have been no reports replicating the pathogenicity of anti-LRP4 Abs in animals since the first EAMG models of LRP4 were developed. In this study, we generated EAMG models by actively immunizing mice with recombinant proteins of human LRP4 ectodomain expressed with chaperones. Immunized mice exhibited myasthenia with the characteristic EMG decrement that has been observed in other EAMG models and MG patients. Pathohistological examination of NMJs and in-vitro electrophysiological studies using hemidiaphragms with the phrenic nerve were performed to evaluate the pathological roles of anti-LRP4 Abs in mice.

2. Methods

2.1. Preparation of recombinant LRP4 protein

The cDNAs encoding human LRP4, human receptor-associated protein (RAP), and human mesoderm development (MESD) were obtained from Open Biosystems (GE Healthcare, UK). The whole region of the extracellular domain (1–1688 aa) of the LRP4 cDNA with a C-terminal Myc/His-tag sequence was inserted into the pEBMulti-Neo expression vector (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Full-length RAP and MESD cDNAs were inserted into the pEBMulti-Hyg expression vector (Wako Pure Chemical Industries, Ltd.). Human embryonic kidney Expi293F cells were transfected by using the ExpiFectamine 293 transfection kit (Life Technologies, Grand Island, NY) at a ratio of plasmid DNA: ExpiFectamine 293 reagent of 1:2.7. Plasmid samples consisted of equal amounts of LRP4, RAP, and MESD plasmids.

Recombinant LRP4 protein expression levels were determined by immunoblot analysis with an anti-Myc Ab (Life Technologies). Secreted LRP4 protein was purified from conditioned medium (CM) by using Ni-Sepharose beads (GE Healthcare) and concentrated through an Amicon centrifugal filter device (Millipore, Billerica, MA). Recombinant protein concentrations were determined by SDS-PAGE analysis with Coomassie blue staining, with bovine serum albumin (BSA) as the standard.

2.2. Immunization of mice with recombinant LRP4 protein

All animal procedures were carried out according to basic guidelines of animal experiments by the Ministry of Health, Labor and Welfare, and approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontrogy. Female A/J mice (Japan SLC) were used after 4 months of age. On day 0, mice were anesthetized with a mixture of medetomidine, midazolam, and butophanol, followed by subcutaneous injection in the hind footpads with 10 μg of LRP4 emulsified with complete Freund's adjuvant (Sigma-Aldrich). All mice were boosted with 10 μg of recombinant LRP4 emulsified with incomplete Freund's adjuvant (Sigma-Aldrich) on day 14. Mice that did not lose body weight (BW) after the second injection received a third injection of the same dose of LRP4 emulsified with incomplete adjuvant on day 35. Control mice were injected with equal volumes of PBS emulsified with adjuvant in the same manner on days 0, 14, and 35.

Thirty-three mice (11 control mice, 22 LRP4-injected mice) were used in this study. Mice were weighed and inspected for general health daily. When LRP4-immunized mice exhibited gait disturbance and lost > 20% of their BW (based on the value at the time of the second or third injection), inspection was terminated and mice were analyzed for EAMG. Blood samples were collected from the tail vein (for detection of anti-LRP4 antibodies) or the heart (for AChR clustering and MuSK phosphorylation assays) under anesthesia at the end of the experimental period. Six mice immunized with LRP4 were excluded from the analysis because they exhibited a significant increase in BW after the third immunization, and their anti-LRP4 Ab titers were significantly lower than those of mice exhibiting myasthenic weakness.

2.3. Muscle strength measurements

Muscle strength was evaluated by an inverted screen test, performed with a wire cage lid apparatus (O'Hara & Co., Ltd., Tokyo, Japan). The mouse was placed on a wire mesh, which was then smoothly inverted and held above a soft surface. The time that the mouse hung from the wire lid before falling was recorded. Mice were scored according to this time, as follows (Cossins et al., 2004): 1, 0–10 s; 2, 11–25 s; 3, 26–60 s; 4, 61–100 s; 5, 101–119 s; and 6, 120 + s.

2.4. ELISA assay to detect anti-LRP4 antibodies

Wells of an ELISA plate were coated with recombinant human LRP4 protein (150 ng/well) diluted in Tris-buffered saline (TBS). After washing with 0.1% Tween-20 in TBS (TBST), plates were blocked with 4% BlockAce (Dainippon Pharmaceutical, Osaka, Japan) and incubated for 2 h with mouse serum (diluted 1:1000). Plates were washed, incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies (diluted 1:3000; GE Healthcare), washed again, and reacted with *o*-phenylenediamine dihydrochloride peroxidase substrate (Sigma-Aldrich). The colorimetric reaction was stopped by the addition of 2 N HCl. Absorbance values at 490 nm were determined. Subclasses of anti-LRP4 antibodies were determined by using a Mouse Typer Sub-Isotyping Kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. For isotyping analysis, serum from LRP4-immunized mice was diluted 1:3000.

2.5. Cell-based assay to detect anti-LRP4 antibodies

Full-length human LRP4 cDNA fused to mCherry cDNA at its C terminus was inserted into the pMX plasmid. Retroviral packaging Plat-E cells, cultured in 10% FCS/DMEM, were transfected with the LRP4-mCherry expression plasmid by using FuGENE6 (Promega Corp., Madison, WI). Two days after transfection, indirect immunofluorescence staining of the transfected cells was performed. Cells were incubated with mouse serum (diluted 1:50) for 1 h at 37 °C, washed with serum-free DMEM, and fixed with 2% paraformaldehyde (PFA) in PBS for 15 min. Cells were washed with PBS, blocked with 1% BSA in PBS for 30 min, and incubated with Alexa Fluor 488-labeled goat anti-mouse IgG Abs (1 μg/ml; Life Technologies) for 1 h. After washing, the cells were mounted with Fluoromount/Plus (Diagnostic Biosystems, Pleasanton, CA). A Leica DMI6000 microscope (Leica Microsystems, Buffalo Grove, IL) was used to obtain fluorescence images.

2.6. EMG

Changes in compound muscle action potential (CMAP) were determined as described previously (Mori et al., 2012a). Trains of 10 impulses were applied at 3, 10, 20, and 40 Hz with a 10–30 s pause between trains. Decrement was calculated as the percent amplitude change between the first CMAP and the smallest CMAP evoked by a train of 10 impulses.

2.7. In-vitro electrophysiology

Membrane potentials (MPs) and miniature endplate potentials (MEPPs) were recorded by using a specimen composed of the left phrenic nerve and hemidiaphragm muscle, as described previously (Mori et al., 2012b). To measure evoked endplate potentials (EPPs), 1 μM μ-conotoxin GIIIB (Peptide Institute, Osaka, Japan) was applied to suppress muscle contraction. The phrenic nerve was stimulated by a suction electrode with supramaximal voltage at 0.7 Hz. Amplitudes of EPPs and MEPPs were standardized to MP of − 75 mV. Quantal content was calculated as described previously (Mori et al., 2012a).