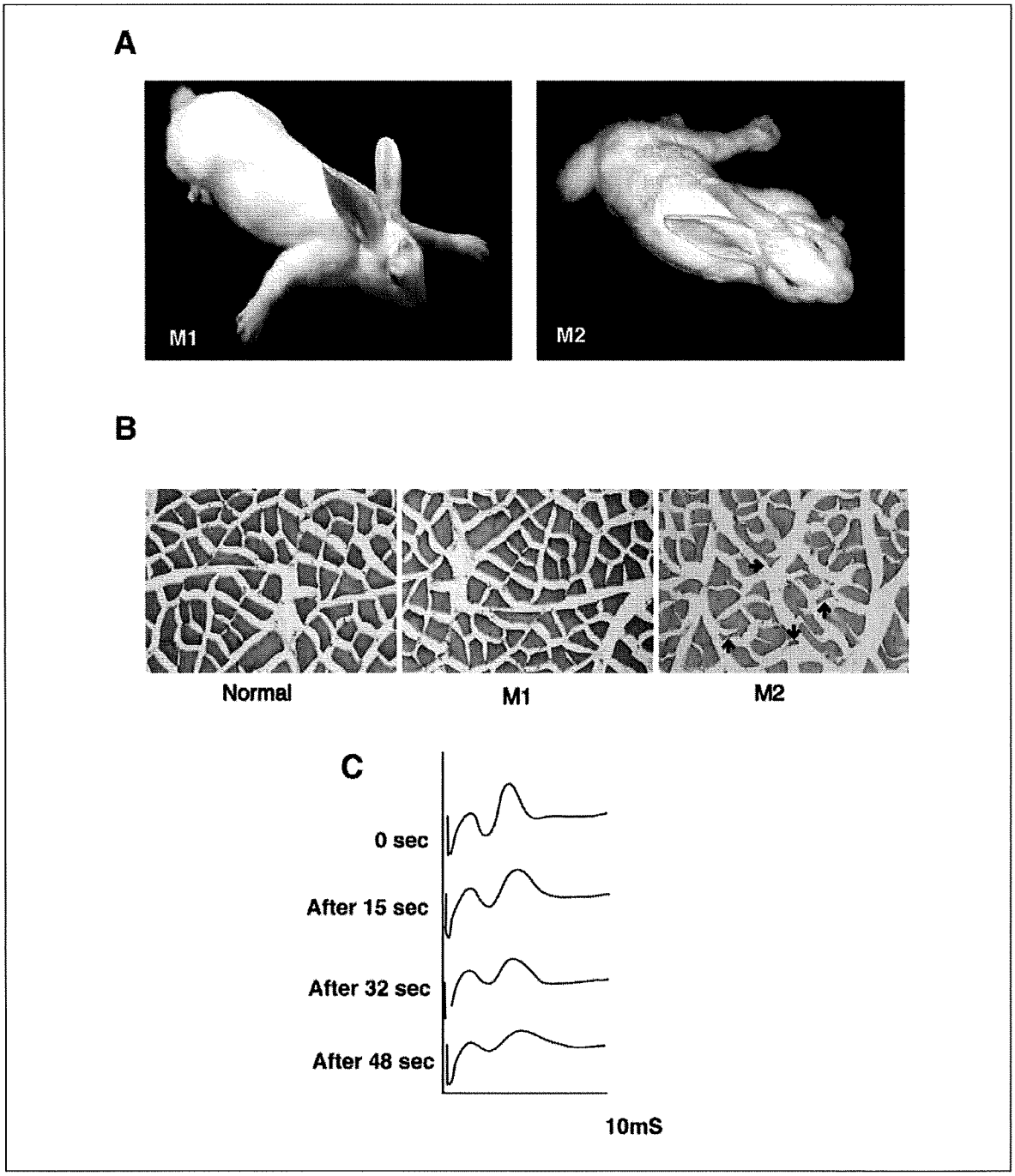

Figure 1. Rabbits manifest myasthenia gravis (MG)-like paresis after immunization with MuSK protein. (A) Two rabbits representative of four animals with outcomes manifested myasthenic weakness after immunization with the recombinant MuSK protein. After three injections of MuSK protein, M1 and M2 rabbits manifested flaccid weakness within three and nine weeks respectively. M2 rabbit developed severe exhaustion with muscle weakness. (B) Cross-sections from the soleus muscles of two paretic (M1 and M2) and a normal rabbits (Normal) were stained with H&E. Muscle fibers in M1 paretic rabbit showed only subtle changes in shape and smallness, whereas an atrophy of muscles fibers in M2 paretic rabbit was observed as small angular fibers (indicated by arrows). Scale bar, 50 mm. (C) Electromyograms recorded from M1 paretic rabbit. The retro-auricular branch of the facial nerve was continuously by a constant current stimulator delivered square-wave pulses of 0.1 msec during at 20 Hz, and the compound muscle action potential (CMAP, second peak observed on the oscilloscope screen recorded at the indicated time-points during stimulation) shows a decremental pattern, consistent with MG.

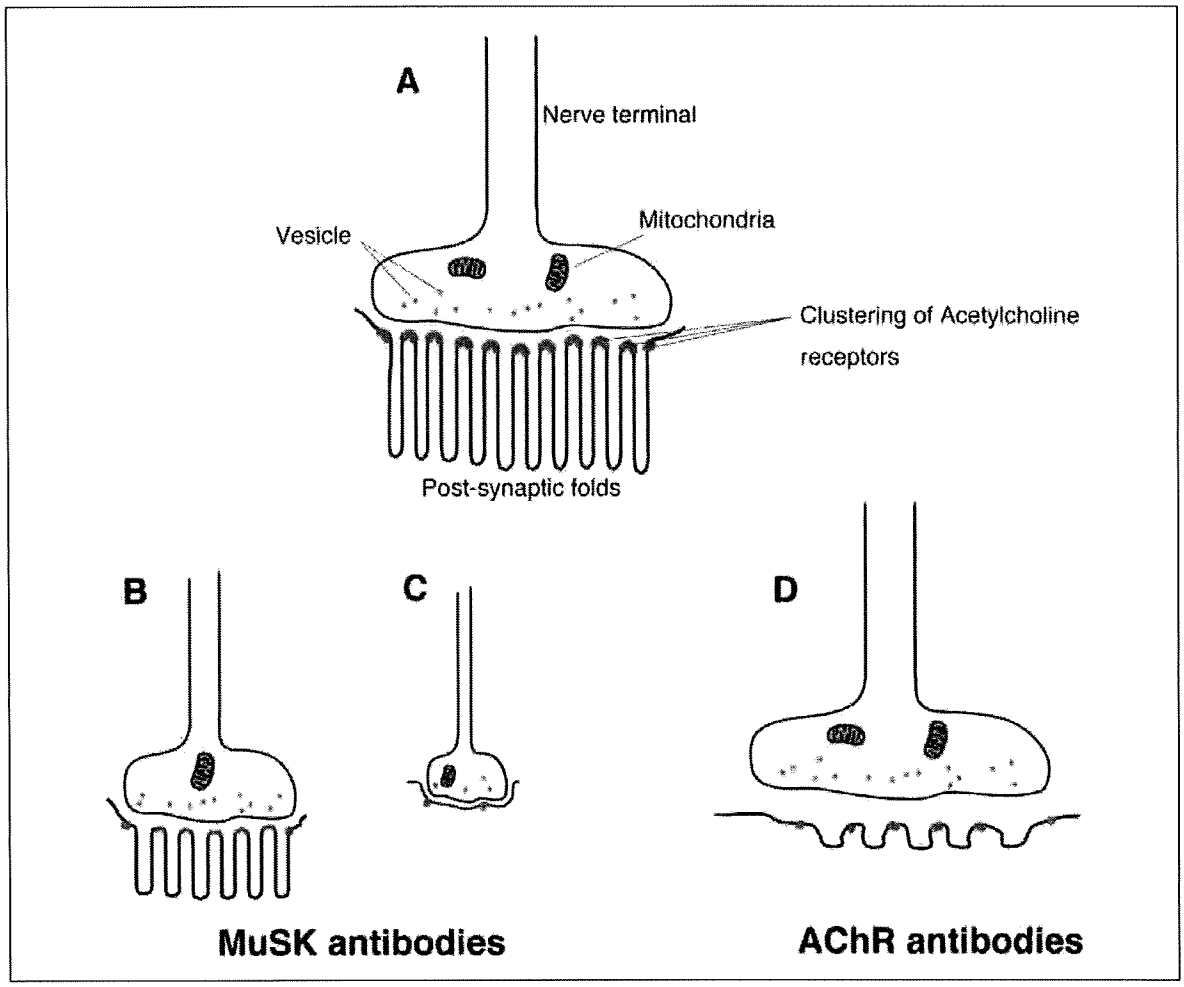

Figure 2. Schematic appearances of NMJs observed in normal volunteers and patients. A: Normal NMJ. AChRs are concentrated at the peaks of abundant, well-preserved and intricately twisted junctional folds. B and C: NMJ in EAMG induced by MuSK antibodies, CMS with MuSK or Dok-7 mutations. Small NMJ in both pre- and post-synaptic structures. (B) Attenuation of AChR and reduced twisting of synaptic folds at the post-synaptic membrane without widened synaptic space. (C) Disappearance of post-synaptic folds with preserved synaptic space. D: NMJ in MG patients with AChR antibodies. Complement-mediated lysis of post-synaptic membranes. The myasthenic junction has a reduced number of AChR, simplified synaptic folds and a widened synaptic space with a normal nerve terminal.

by agrin on C2C12 cells, indicating that complement-mediated mechanisms are not necessarily required for such inhibition (unpublished data). We also noted that MuSK-specific antibodies strongly inhibited AChR clustering induced by all known agrin-independent pathways as well as by agrin itself (16).

We then determined whether the expression of AChR at NMJ was reduced in soleus muscles of paretic compared to normal rabbits. Using fluorescence microscopy and a digital camera, we examined and recorded the size and optical densities of AChR clusters stained with the rhodamine-conjugated AChR agonist, α-bungarotoxin (α-BTX). The images were measured with NIH image analysis software for comparison with unprocessed digitized NIH images (16). The areas and intensity of AChR fluorescence in muscles of these paretic rabbits were significantly reduced compared with those in normal rabbits. In addition, the structure of NMJ in our paretic rabbits, as well as the size and branching of the motor terminals, were significantly reduced. Electron microscopic observations of NMJ in rabbits with EAMG induced by injection of MuSK protein demonstrated that the normally convoluted synaptic folds (Fig. 2A) underwent a significant simplification of structure (Fig. 2B and C) but no destruction (Fig. 2D). Within these intricately twisted synaptic folds, the high density of voltage-gated sodium channels contained in the membranes' depths amplify the end-plate current,

thus enhancing neuromuscular transmission and muscle contraction (23). Any reduction in the size and branching of the motor terminals contributes to decreases in ACh output. Moreover the simplification of post-synaptic structure increases of the threshold for generating muscle fiber action potential. These structural abnormalities in NMJ, including those in both pre- and post-synaptic structures, thus impair neuromuscular transmission in the EAMG rabbits (16, 22). Intriguingly, similar abnormalities of NMJ structure were also observed in rats with reduced expression of MuSK, as noted by RNA interference (15), in a patient with congenital myasthenic syndromes (CMS) caused by MuSK mutations and also in mice expressing the MuSK missense mutation by electroporation experiments (24). MuSK knock-out mice also displayed presynaptic defects in addition to postsynaptic ones, indicating that MuSK is required for retrograde signals, so far unidentified, to maintain the pre-synaptic structure in mature NMJ.

Resemblance of clinical features between MuSK MG and CMS with Dok-7 mutations

Recently a MuSK-interacting protein called Dok-7 was discovered (25) and identified as a member of the Dok family of cytoplasmic proteins. Dok-7 is postulated to have three main functional domains: a pleckstrin homology (PH) domain, essential for membrane association; a phosphotyrosine-binding (PTB) domain involved in the Dok-7 induced activation of MuSK; and a large C-terminal domain containing multiple tyrosine residues. Dok-7 knock-out mice showed marked disruption of neuromuscular synaptogenesis that was indistinguishable from the features found in MuSK-deficient mice. Thus, Dok-7 is essential for neuromuscular synaptogenesis through its interaction with MuSK.

Mutations in the Dok-7 protein cause a genetic form of limb-girdle myasthenia (also classed as CMS) (26). Some clinical features in these patients resemble those in the severe type of MG accompanied by MuSK antibodies (27). Proximal muscles are usually more affected than those in distal regions, as evident in MuSK MG patients, and ptosis is often present. Limb-muscle weakness is comparatively less severe. Previous studies showed no reduction of AChR clustering with significant changes in NMJ of MuSK MG patients (20), but further structural analysis of NMJ is required in muscles where severe weakness occurs commonly. The weakness and atrophy are not observed uniformly in muscles of these patients, although both MuSK and Dok-7 are essential for the formation of NMJ during the embryonic stage (25). Of course, one of the major distinctions between acquired MuSK MG and CMS with the Dok-7 mutation is the timing when weakness begins. The CMS patients typically have difficulty in walking after reaching that normal motor milestone during early childhood, whereas the onset of weakness for MG patients, in most instances, occurs in adulthood. Interestingly, AChR clustering and post-synaptic folds are reduced with small motor terminals as observed at NMJ in CMS with Dok-7 mutations. AChR clustering and post-synaptic folds are reduced with small motor terminals as observed at NMJ in CMS with Dok-7 mutations (28). The effect of Dok-7 mutations on post-synaptic structures may also be an alteration of retrograde signaling to the pre-synaptic nerve terminals resulting in a reduced NMJ size in these patients (Figure 2). Dok-7, along with MuSK, is required not only for synaptogenesis but also for the maintenance of NMJ.

Conclusions

We now believe that MuSK antibodies cause MG in humans. Using an experimental model for myasthenia revealed that MuSK antibodies mediate the pathogenesis of this syndrome in rabbits and mice (14, 16, 22). In most cases, the symptoms take more than three months to manifest themselves in animals. Moreover, the symptoms are somewhat difficult to induce experimentally by passive transfer of MuSK antibodies from MG patients into animal hosts. The mechanisms employed by these antibodies include multiple events during which MuSK functions stall in their process of regulating synapse formation and maintenance. MuSK antibodies against compound antigenic determinants in the extracellular domain may engage in their pathogenic activities through antigenic modulation and/or restraint of MuSK functions, and the consequences of these effects range from a partial to entire loss of MuSK function without the involvement of complement-mediated damage. The point that MuSK antibodies in MG patients are mainly of the IgG4 subclass, which does not activate complement, may be relevant here. These diverse possibilities reflect the complexity of clinical features seen in patients ranging from typical MG and throughout its many variants.

MG has long served as model for studying the pathogenesis and treatment of generalized autoimmune disease. In fact, understanding of MG's pathogenesis has enhanced comprehension of all synaptic functions. Now, the EAMG model with MuSK antibodies will facilitate further progress in resolving the pathogenic basis of MG and CMS at the molecular level and identifying beneficial treatment strategies. Additional areas of relevance are the many physical conditions in which muscles shrink or atrophy, as in patients with cancer or AIDS, termed cahexia, when limbs are immobilized following injury, or even during atrophy from aging, termed sarcopenia. Understanding the molecular basis of NMJ maintenance promises to provide new targets for innovative therapeutics to create healthy, enduring muscles.

Acknowledgments

We thank Ms. P. Minick for excellent editorial assistance. This study was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan, by a grant from the Health Science Research Grants for Research on Psychiatric and Neurological Diseases and Mental Health from the Ministry of Health, Labor, and Welfare, Japan and by a grant from the Kato Memorial Trust for Nambyo Research. We are also grateful to the stuff of the Integrated Center for Science of Ehime University for assistance with animal care and sequence analysis.

References

1. Conti-Fine BM, Milani M, Kaminski HJ. *Myasthenia gravis: past, present, and future.* J Clin Invest 2006;116:2843-54.
2. Vincent A, Lang B, Kleopa KA. *Autoimmune channelopathies and related neurological disorders.* Neuron 2006;52:123-38.
3. Patrick J, Lindstrom J. *Autoimmune response to acetylcholine receptor.* Science 1973;180:871-2.
4. Evoli A, Tonali PA, Padua L, et al. *Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis.* Brain 2003;126:2304-11.
5. Hoch W, McConville J, Helms S, et al. *Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies.* Nat Med 2001;7:365-8.
6. Sanders DB, El-Salem K, Massey JM, et al. *Clinical aspects of MuSK antibody positive seronegative MG.* Neurology 2003;60:1978-80.
7. Vincent A, Bowen J, Newsom-Davis J, et al. *Seronegative generalised myasthenia gravis: clinical features, antibodies, and their targets.* Lancet Neurol 2003;2:99-106.
8. Yeh JH, Chen WH, Chiu HC, et al. *Low frequency of MuSK antibody in generalized seronegative myasthenia gravis among Chinese.* Neurology 2004;62:2131-2.
9. Ohta K, Shigemoto K, Fujinami A, et al. *Clinical and experimental features of MuSK antibody positive MG in Japan.* Eur J Neurol 2007;14:1029-34.
10. Ohta K, Shigemoto K, Kubo S, et al. *MuSK Ab described in seropositive MG sera found to be Ab to alkaline phosphatase.* Neurology 2005;65:1988.
11. Ohta K, Shigemoto K, Kubo S, et al. *MuSK antibodies in AChR Ab-seropositive MG vs. AChR Ab-seronegative MG.* Neurology 2004;62:2132-3.
12. Kummer TT, Misgeld T, Sanes JR. *Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost.* Curr Opin Neurobiol 2006;16:74-82.
13. Strochlic L, Cartaud A, Cartaud J. *The synaptic muscle-specific kinase (MuSK) complex: new partners, new functions.* Bioessays 2005;27:1129-35.
14. Jha S, Xu K, Maruta T, et al. *Myasthenia gravis induced in mice by immunization with the recombinant extracellular domain of rat muscle-specific kinase (MuSK).* J Neuroimmunol 2006;175:107-17.
15. Kong XC, Barzaghi P, Ruegg MA. *Inhibition of synapse assembly in mammalian muscle in vivo by RNA interference.* EMBO Rep 2004;5:183-8.
16. Shigemoto K, Kubo S, Maruyama N, et al. *Induction of myasthenia by immunization against muscle-specific kinase.* J Clin Invest 2006;116:1016-24.
17. Akaaboune M, Grady RM, Turney S, et al. *Neurotransmitter receptor dynamics studied in vivo by reversible photo-unbinding of fluorescent ligands.* Neuron 2002;34:865-76.
18. Ruff RL. *Neuromuscular Junction Physiology and Pathophysiology.* In: Kaminski, ed. *Myasthenia Gravis and Related Disorders.* New Jersey: Humana Press Inc.
19. Lindstrom J. *Is "seronegative" MG explained by autoantibodies to MuSK?* Neurology 2004;62:1920-1.
20. Shiraishi H, Motomura M, Yoshimura T, et al. *Acetylcholine receptors loss and postsynaptic damage in MuSK antibody-positive myasthenia gravis.* Ann Neurol 2005;57:289-93.
21. Vincent A. *Unravelling the pathogenesis of myasthenia gravis.* Nat Rev Immunol 2002;2:797-804.
22. Shigemoto K, Sachiho K, Chen J, et al. *Experimentally induced myasthenia gravis with muscle-specific kinase.* Ann N Y Acad Sci 2008 (in press).
23. Wood SJ, Slater CR. *Safety factor at the neuromuscular junction.* Prog Neurobiol 2001;64:393-429.
24. Chevessier F, Faraut B, Ravel-Chapuis A, et al. *MUSK, a new target for mutations causing congenital myasthenic syndrome.* Hum Mol Genet 2004;13:3229-40.
25. Okada K, Inoue A, Okada M, et al. *The muscle protein Dok-7 is essential for neuromuscular synaptogenesis.* Science 2006;312:1802-5.
26. Beeson D, Higuchi O, Palace J, et al. *Dok-7 mutations underlie a neuromuscular junction synaptopathy.* Science 2006;313:1975-8.
27. Palace J, Lashley D, Newsom-Davis J, et al. *Clinical features of the DOK7 neuromuscular junction synaptopathy.* Brain 2007.
28. Slater CR, Fawcett PR, Walls TJ, et al. *Pre- and post-synaptic abnormalities associated with impaired neuromuscular transmission in a group of patients with "limb-girdle myasthenia".* Brain 2006;129:2061-76.

Myasthenia Gravis Experimentally Induced with Muscle-specific Kinase

KAZUHIRO SHIGEMOTO,[a,*] SACHIHO KUBO,[b] CHEN JIE,[c] NAOHITO HATO,[d] YASUHITO ABE,[e] NORIFUMI UEDA,[e] NAOTO KOBAYASHI,[f] KENJI KAMEDA,[g] KATSUMI MOMINOKI,[h] ATSUO MIYAZAWA,[i] AKIHITO ISHIGAMI,[b] SEIJI MATSUDA,[c] AND NAOKI MARUYAMA[b]

[a]*Department of Preventive Medicine, Ehime University School of Medicine, Ehime, Japan*

[b]*Department of Molecular Pathology, Tokyo Metropolitan Institute for Gerontology, Tokyo, Japan*

[c]*Department of Integrated Basic Medical Science, Ehime University School of Medicine, Ehime, Japan*

[d]*Department of Otolaryngology, Ehime University School of Medicine, Ehime, Japan*

[e]*Department of Molecular Pathology, Ehime University School of Medicine, Ehime, Japan*

[f]*Department of Bioscience, Medical Education Center, Ehime University School of Medicine, Ehime, Japan*

[g]*Department of Bioscience, The Integrated Center for Science, Ehime University, Ehime, Japan*

[h]*Department of Animal Resources, Advanced Science Research Center, Okayama University, Okayama, Japan*

[i]*Membrane Dynamics Project, RIKEN Harima Institute, Hyogo, Japan*

Here we present the first evidence that muscle-specific kinase (MuSK) antigen can cause myasthenia in animals. MuSK is expressed at the postsynaptic membranes of neuromuscular junctions (NMJ) and forms complexes with acetylcholine receptors (AChR) and rapsyn. MuSK is activated by agrin, which is released from motoneurons, and induces AChR clustering and subsequent formation of NMJ in embryos. Notably, autoantibodies against MuSK were found in a proportion of patients with generalized myasthenia gravis (MG) but without the characteristic AChR autoantibodies. However, MuSK autoantibodies had no known pathogenic potential, and animals immunized with purified MuSK proteins did not develop MG in former studies. In contrast, we have now injected rabbits with MuSK ectodomain protein *in vivo* and evoked a MG-like muscle weakness with a reduction of AChR clustering at the NMJ. Our results showed that MuSK is required for maintenance of synapses and that interference with that function by MuSK antibodies causes myasthenic weakness. *In vitro*, AChR clustering in myotubes is induced by agrin and agrin-independent inducers, which do not activate MuSK. Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty, but MuSK autoantibodies in myasthenic animals inhibited both agrin and agrin-independent AChR clustering. MuSK plays multiple roles in pre-patterning of the postsynaptic membrane before innervation and formation of NMJ in embryos. Some of these mechanisms may also participate in the maintenance of mature NMJ. This model system would provide new knowledge about the molecular pathogenesis of MG and MuSK functions in mature NMJ.

Key words: **myasthenia gravis (MG); experimental autoimmune MG (EAMG); muscle-specific kinase (MuSK); acetylcholine receptor (AChR); neuromuscular junction (NMJ); congenital myasthenic syndromes (CMS)**

Introduction

Although autoantibodies against muscle-specific kinase (MuSK) have been found in patients with myasthenia gravis (MG),[1] any pathogenic contribution of MuSK antibodies to the muscle weakness that typifies MG has remained in dispute. That is, until now, MuSK

Present address: Tokyo Metropolitan Institute of Gerontology, Research Team for Molecular Biomarkers, Tokyo, Japan.

Address for correspondence: Kazuhiro Shigemoto, Tokyo Metropolitan Institute of Gerontology, Research Team for Molecular Biomarkers, Sakaecho 35-2, Itabashi-Ku, Tokyo 173-0015, Japan. Voice: +81-3-3964-3241, ext. 3025; fax: +81-3-3579-4776.

kazshige@tmig.or.jp

Ann. N.Y. Acad. Sci. 1132: 93–98 (2008). © 2008 New York Academy of Sciences.
doi: 10.1196/annals.1405.002

antibodies have not produced experimental autoimmune MG (EAMG).[2,3] Here we describe the recent progress toward understanding this phenomenon.

Autoantibodies against MuSK

About 80% of patients with MG have autoantibodies against acetylcholine receptor (AChR). A seminal experiment by Patrick and Lindstrom demonstrated the pathogenicity of autoantibodies to AChR about 30 years ago.[4] Although a number of studies have documented that AChR antibodies cause structural and functional damage to the neuromuscular junction (NMJ), autoantigens, in the nearly 20% of MG patients without such antibodies, remained obscure.[1] Then, in 2001, Hoch *et al.* identified antibodies against MuSK in a proportion of patients with generalized MG.[1] MuSK is required for clustering of AChR during the formation of NMJ and is expressed predominantly at the postsynaptic membrane in mature NMJ.[5,6] In MuSK knockout mice, AChRs fail to cluster opposite to growing motoneuron terminals on the surfaces of myotubes.[7] Additionally, a case of heteroalleric MuSK mutations that caused the reduction of MuSK expression has been associated with congenital myasthenic syndrome (CMS).[8] Further, the reduction of MuSK expression in rat muscles *in vivo* upon RNA interference induced disassembly of synapses.[9] Even though the function of MuSK in mature NMJ is still uncertain, a causal relationship between MuSK autoantibodies and MG has been proposed.[1,10,11]

Recent studies by Vincent and others showed that the frequency of MuSK antibodies in MG patients who were AChR seronegative (lacked autoantibodies to AChR) varied from 4 to 50%.[11-17] We detected MuSK antibodies in 29% of seronegative MG patients but not in any MG patients with AChR antibodies (seropositive MG) or with other autoimmune diseases.[16] Previously, we identified antibodies against a recombinant MuSK fusion protein with human alkaline phosphatase (AP) in seropositive MG patients[15] and later revealed that 8.8% of seropositive MG patients had autoantibodies to AP but not to MuSK.[16] We are currently studying the clinical significance of the autoantibodies to AP in seropositive MG.

Clinical features of patients with MG and MuSK antibodies are distinctive. Such patients often have severe bulbar dysfunctions that can be difficult to treat effectively with immunosuppressive and immunomodulatory strategies, and atrophy of facial and tongue muscles is common.[12,13,18,19] After the identification of MuSK antibodies in MG patients, laboratory quantification of these antibodies is now required to confirm the diagnosis of MG, the appropriate clinical treatment, as well as the presence of AChR antibodies.[18,20,21]

Experimental Autoimmune MG

Although MuSK antibodies are present in some seronegative MG patients and the clinical features are distinctive, proving the pathogenicity of MuSK antibodies has been difficult because these antibodies did not induce myasthenia in experimental animals. Formerly, the pathogenicity of AChR antibodies was shown when rabbits injected with AChR protein purified from electric eels developed muscle weakness and paralysis.[4] Injection of eel AChR protein stimulates the production of antibodies that cross-react with rabbit AChR at the NMJ. Electrophysiological studies confirmed that the flaccid paralysis in this animal model resembled that in MG patients. Similarly, EAMG appeared in other species after immunization with purified AChR protein. In addition, the antibodies to AChR in human MG patients could passively transfer disease to mice.[22] Therefore, creating an EAMG model induced by MuSK antibodies was indispensible for proving the pathogenicity of MuSK antibodies and investigating their pathogenic mechanisms in MG.[10,20,21]

To pursue this objective, we recently immunized rabbits with MuSK ectodomain, which caused myasthenic weakness and produced electromyographic findings that were compatible with a diagnosis of MG, as shown by Patrick and Lindstrom.[23] The extracellular segment of MuSK comprises five distinct domains, i.e., four immunoglobulin-like domains and one cysteine-rich region.[5,6] The fusion protein expression constructs we generated consisted of mouse MuSK ectodomain with the Fc region of human IgG1 or His-tag and were used to transfect COS-7 cells.[23] The recombinant MuSK-Fc and MuSK-His proteins secreted were purified by using protein-A Sepharose and histidine affinity columns, respectively (FIG. 1). New Zealand white rabbits were then immunized with 100–400 mg of this purified MuSK recombinant protein. After three to four injections of MuSK protein, all six treated rabbits manifested flaccid paralysis (FIG. 2). Sera from the paretic rabbits contained high titers of MuSK antibodies that reacted specifically with MuSK molecules as observed by testing sera from MG patients with MuSK antibodies.[1,24] The paretic rabbits developed severe muscular exhaustion revealed by histological studies showing alterations in muscle fibers

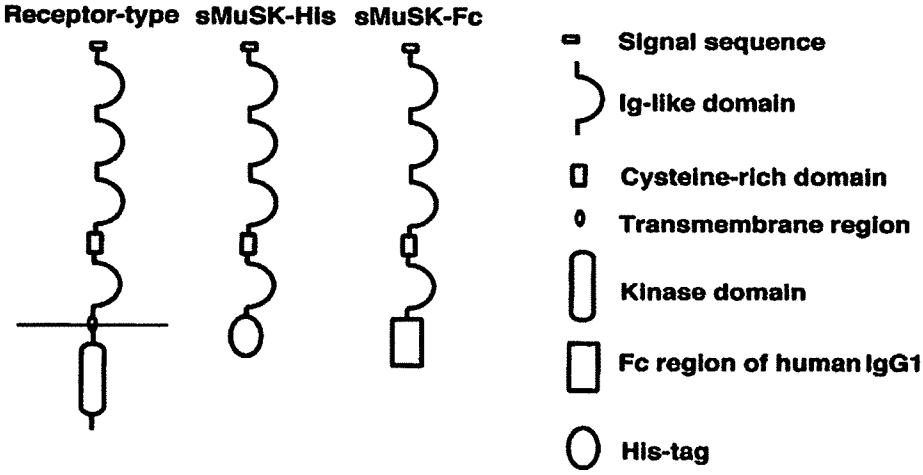

FIGURE 1. Schematic representation of the muscle-specific kinase (MuSK) domain structure and expression of secretory MuSK proteins in COS-7 cells. The domain structures of recombinant secretory MuSK protein (MuSK-His and MuSK-Fc) and receptor-type MuSK are shown. The whole coding region of the MuSK extracellular domain was fused with the His-tag or Fc region of human IgG1 as shown.

ranging from subtle to angular atrophy intermingled with normal muscle. Atrophic changes of this type can result from MG, reduced mechanical activity of muscles, or cachexia. Repetitive electromyograms of a paretic rabbit were then done to measure the result of stimulating the retroauricular branch at 20 Hz and recording responses from the retroauricular muscle. The compound muscle action potential (CMAP) showed a decremental pattern, consistent with MG.[23] However, the injection of ACh esterase inhibitor did not significantly offset the CMAP decrement or decrease the symptoms. Importantly, the induction of EAMG by MuSK antibodies is not limited to rabbits, i.e., we and others have also produced EAMG in mice by injecting MuSK protein (FIG. 2).[25]

AChR Clustering and Structure of NMJ in Rabbits with EAMG and MuSK Antibodies

The clustering of AChR necessary for NMJ formation is completely abolished in MuSK knockout mice,[7] and AChR clustering at the NMJ is reduced in subjects with CMS and MuSK mutations.[8] In a previous RNA interference experiment, injection of double-stranded RNA (dsRNA) targeting MuSK diminishes the expression of MuSK protein and AChR clusters in rat muscle fibers *in vivo*, whereas dsRNA targeting nonessential proteins does not have any effect (RNA-interference experiment).[9] Therefore, we examined the expression of AChR at NMJ in soleus muscles of paretic and normal rabbits by fluorescence microscopy after applying a rhodamine-conjugated AChR agonist, a-bungarotoxin. Images of AChR clustering were then recorded by using a digital camera.[23] The sizes and optical densities were measured using National Institute of Health (NIH) image analysis software with unprocessed digitized NIH images (version 1-62; http://rsb.info.nih.gov/nih-image). The results unequivocally pictured a significantly reduced area and intensity of AChR fluorescence in the paretic rabbits compared with their normal counterparts. In addition, a structural examination showed that the size and branching of the NMJ were significantly diminished in paretic rabbits. Similar changes of NMJ structure were observed in rats with reduced expression of MuSK evident by RNA interference,[9] in a patient with CMS and MuSK mutations, and in mice expressing the missense mutation by electroporation experiments.[8] Our results demonstrated that MuSK antibodies also elicited synaptic changes in EAMG, including the reduced expression of surface AChR at postsynaptic membranes of NMJ. Further examination of MuSK knockout mice disclosed presynaptic defects in addition to postsynaptic ones,[7] indicating that MuSK is also required for presently unidentified retrograde signals to maintain the presynaptic structure in mature NMJ.

Pathogenic Mechanisms of MuSK Antibodies in AChR Clustering at NMJ

MuSK plays multiple roles in clustering AChR during development of the postsynaptic membrane of NMJ. Contact of the motor-nerve growth cone with

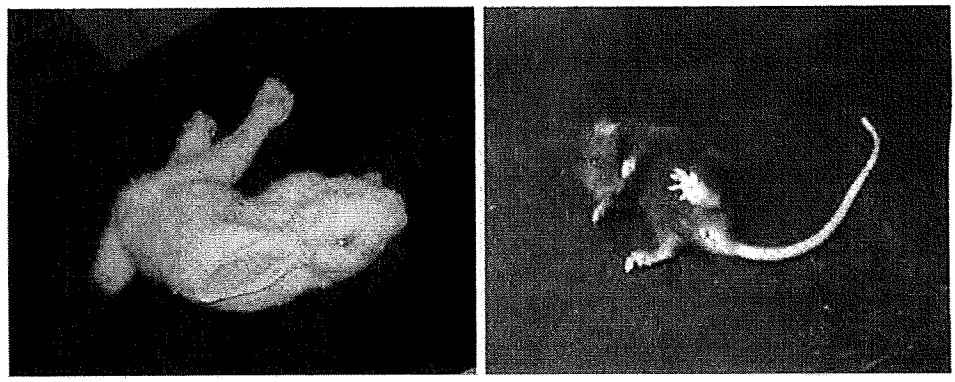

FIGURE 2. Manifestation of muscle weakness after injection of purified MuSK proteins in experimental animals (*left*, a paretic rabbit; *right*, a paretic mouse).

the muscle induces a narrow, distinct, endplate zone in the mid-muscle that is marked by a high density of AChR clustering.[26–29] In this step, agrin released from motoneurons activates MuSK and redistributes AChR clusters to synaptic sites. However, a direct physical interaction between MuSK and agrin has so far not been demonstrated, despite many efforts to do so.[27] Thus, the mechanisms of MuSK activation and the following events remain obscure, although a co-receptor of MuSK, co-ligand of agrin or either post-translational modification of agrin or MuSK have been postulated. Intriguingly, MuSK is also required for organizing a primary synaptic scaffold to establish the post-synaptic membrane.[30,31] Preceding muscle innervations, AChR clusters form at the central regions of muscle fibers, creating an endplate zone that is somewhat broader than that in innervated muscle. Thus, MuSK is required for pre-patterning of AChR clustering in the absence of motor innervation. The scenario of MuSK's roles in the process is somewhat complicated; possibly an element other than agrin achieves activation of MuSK and triggers postsynaptic specialization at the NMJ, and/or MuSK acts as a primary scaffold molecule without activation. The listed pleiotropic roles of MuSK in AChR clustering at NMJ development could also require the maintenance of mature NMJ. Studies performed *in vivo* have shown that synaptic AChRs intermingle completely over a period of approximately 4 days and that many extrasynaptic AChRs are incorporated into the synapse at the mature NMJ, although the synaptic membrane in adult muscle appears to be macroscopically stable.[32,33] Therefore, the mechanisms at play in AChR clustering during NMJ development are also required in mature NMJ when postsynaptic complexes, including AChR and MuSK, are dynamically turning over for maintenance.

To elucidate the mechanisms of AChR clustering at NMJ, a number of studies were performed using cultured C2C12 myotubes. Agrin induces clustering of AChR in C2C12 myotubes following MuSK autophosphorylation.[26,27,29] This event *in vitro* represents a major cascade of AChR clustering at the NMJ after innervation by motoneurons.[27,34–36] Laminin-1 and the *N*-acetylgalactosamine (GalNAc)-specific lectin *Vicia villosa* agglutinin (VVA-B4) can induce AChR clustering in C2C12 myotubes without activation of MuSK.[34,37–40] Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty. However, these mechanisms may also play important roles in the maintenance of NMJs via agrin-independent pathways and in their formation, as shown by genetic studies.[30,31]

In their previous study, Hoch *et al.* observed that the MuSK antibodies of MG patients inhibited agrin-induced AChR clustering in C2C12 myotubes.[1] We also found that agrin-induced clustering of AChR was strongly blocked in the presence of MuSK antibodies, whereas absorption of the antibodies with purified MuSK products prevented this blocking effect.[23] Thus, MuSK antibodies were responsible for inhibiting the formation of agrin-induced AChR clustering. We also perceived that MuSK-specific antibodies strongly inhibited AChR clustering induced by all known agrin-independent pathways as well as by agrin itself.

Conclusions

In our experimental model of myasthenia, MuSK antibodies routinely mediated pathogenesis in rabbits and mice.[23,25] Consequently, we now believe that MuSK antibodies cause MG in patients. However,

the pathogenic mechanisms of these antibodies entail multiple events in which MuSK acts as a multifunctional platform from which to regulate synapse formation and maintenance. These are reflected in a diversity of clinical features ranging from typical MG to a multitude of variants.[12,13,18,19]

AChR antibodies have been shown to affect neuromuscular transmission by three main mechanisms: (a) binding and activation of complement at the NMJ; (b) accelerated degradation of AChR molecules cross-linked by antibodies (antigenic modulation); and (c) functional AChR block.[20,21] Intriguingly, MuSK antibodies in MG patients are mainly of the IgG4 subclass, which does not activate complement.[41] Electron microscopic observations of NMJ in the EAMG rabbits demonstrated a significant reduction of synaptic folds but no destruction, thus our EAMG model resembles the phenotypes of MG with MuSK antibodies. MuSK antibodies against compound antigenic determinants on the extracellular domain may elicit pathogenic effects through antigenic modulation and/or restraint of MuSK functions,[41] and the consequences of these effects range from a partial to entire loss of MuSK functions.

Recently, a new MuSK-interacting cytoplasmic protein, called Dok-7, has been discovered.[42] Dok-7 knockout mice underwent a marked disruption of neuromuscular synaptogenesis that was indistinguishable from the features found in MuSK-deficient mice. Mutations in Dok-7 caused a genetic form of limb-girdle myasthenia (CMS).[43,44] Some clinical features of these patients resemble the severe type of MG with MuSK antibodies[44]; therefore, the EAMG model with MuSK antibodies presented here promises to facilitate resolution of the pathogenic basis of MG and CMS at the molecular level and identification of beneficial treatment strategies against them.

Acknowledgments

We thank Ms. P. Minick for excellent editorial assistance. This study was supported by a Health Science Research Grant, Research on Psychiatric and Neurological Diseases and Mental Health, from the Ministry of Health, Labor, and Welfare, Japan; and by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan; and by a grant from the Kato Memorial Trust for Nambyo Research, Japan; and by a grants from Kurozumi Medical Foundation, Japan. We are also grateful to the staff of the Integrated Center for Science of Ehime University for assistance with animal care and sequence analysis.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Hoch, W., J. McConville, S. Helms, *et al.* 2001. Autoantibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. Nat. Med. **7:** 365–368.
2. Lindstrom, J. 2004. Is "seronegative" MG explained by autoantibodies to MuSK? Neurology **62:** 1920–1921.
3. Selcen, D., T. Fukuda, X.M. Shen & A.G. Engel. 2004. Are MuSK antibodies the primary cause of myasthenic symptoms? Neurology **62:** 1945–1950.
4. Patrick, J. & J. Lindstrom. 1973. Autoimmune response to acetylcholine receptor. Science **180:** 871–872.
5. Ganju, P., E. Walls, J. Brennan & A.D. Reith. 1995. Cloning and developmental expression of Nsk2, a novel receptor tyrosine kinase implicated in skeletal myogenesis. Oncogene **11:** 281–290.
6. Valenzuela, D.M., T.N. Stitt, P.S. DiStefano, *et al.* 1995. Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. Neuron **15:** 573–584.
7. DeChiara, T.M., D.C. Bowen, D.M. Valenzuela, *et al.* 1996. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell **85:** 501–512.
8. Chevessier, F., B. Faraut, A. Ravel-Chapuis, *et al.* 2004. MUSK, a new target for mutations causing congenital myasthenic syndrome. Hum. Mol. Genet. **13:** 3229–3240.
9. Kong, X.C., P. Barzaghi & M.A. Ruegg. 2004. Inhibition of synapse assembly in mammalian muscle in vivo by RNA interference. EMBO Rep. **5:** 183–188.
10. Vincent, A. 2002. Unravelling the pathogenesis of myasthenia gravis. Nat. Rev. Immunol. **2:** 797–804.
11. Vincent, A., J. Bowen, J. Newsom-Davis & J. McConville. 2003. Seronegative generalised myasthenia gravis: clinical features, antibodies, and their targets. Lancet Neurol. **2:** 99–106.
12. Evoli, A., P.A. Tonali, L. Padua, *et al.* 2003. Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis. Brain **126:** 2304–2311.
13. Sanders, D.B., K. El-Salem, J.M. Massey, *et al.* 2003. Clinical aspects of MuSK antibody positive seronegative MG. Neurology **60:** 1978–1980.
14. Yeh, J.H., W.H. Chen, H.C. Chiu & A. Vincent. 2004. Low frequency of MuSK antibody in generalized seronegative myasthenia gravis among Chinese. Neurology **62:** 2131–2132.
15. Ohta, K., K. Shigemoto, S. Kubo, *et al.* 2004. MuSK antibodies in AChR Ab-seropositive MG vs AChR Ab-seronegative MG. Neurology **62:** 2132–2133.
16. Ohta, K., K. Shigemoto, S. Kubo, *et al.* 2005. MuSK Ab described in seropositive MG sera found to be Ab to alkaline phosphatase. Neurology **65:** 1988.
17. Vincent, A., J. McConville, M.E. Farrugia, *et al.* 2003. Antibodies in myasthenia gravis and related disorders. Ann. N.Y. Acad. Sci. **998:** 324–335.

18. Bartoccioni, E., F. Scuderi, G.M. Minicuci, *et al.* 2006. Anti-MuSK antibodies: correlation with myasthenia gravis severity. Neurology **67:** 505–507.
19. Deymeer, F., O. Gungor-Tuncer, V. Yilmaz, *et al.* 2007. Clinical comparison of anti-MuSK- vs anti-AChR-positive and seronegative myasthenia gravis. Neurology **68:** 609–611.
20. Conti-Fine, B.M., M. Milani & H.J. Kaminski. 2006. Myasthenia gravis: past, present, and future. J. Clin. Invest. **116:** 2843–2854.
21. Vincent, A., B. Lang & K.A. Kleopa. 2006. Autoimmune channelopathies and related neurological disorders. Neuron **52:** 123–138.
22. Toyka, K.V., D.B. Drachman, D.E. Griffin, *et al.* 1977. Myasthenia gravis. Study of humoral immune mechanisms by passive transfer to mice. N. Engl. J. Med. **296:** 125–131.
23. Shigemoto, K., S. Kubo, N. Maruyama, *et al.* 2006. Induction of myasthenia by immunization against muscle-specific kinase. J. Clin. Invest. **116:** 1016–1024.
24. Scuderi, F., M. Marino, L. Colonna, *et al.* 2002. Anti-p110 autoantibodies identify a subtype of "seronegative" myasthenia gravis with prominent oculobulbar involvement. Lab. Invest. **82:** 1139–1146.
25. Jha, S., K. Xu, T. Maruta, M. Oshima, *et al.* 2006. Myasthenia gravis induced in mice by immunization with the recombinant extracellular domain of rat muscle-specific kinase (MuSK). J. Neuroimmunol. **175:** 107–117.
26. Ruegg, M.A., K.W. Tsim, S.E. Horton, *et al.* 1992. The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. Neuron **8:** 691–699.
27. Glass, D.J., D.C. Bowen, T.N. Stitt, *et al.* 1996. Agrin acts via a MuSK receptor complex. Cell **85:** 513–523.
28. Cohen, I., M. Rimer, T. Lomo & U.J. McMahan. 1997. Agrin-induced postsynaptic-like apparatus in skeletal muscle fibers in vivo. Mol. Cell Neurosci. **9:** 237–253.
29. Ferns, M., M. Deiner & Z. Hall. 1996. Agrin-induced acetylcholine receptor clustering in mammalian muscle requires tyrosine phosphorylation. J. Cell. Biol. **132:** 937–944.
30. Lin, W., R.W. Burgess, B. Dominguez, *et al.* 2001. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature **410:** 1057–1064.
31. Yang, X., S. Arber, C. William, *et al.* 2001. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron **30:** 399–410.
32. Akaaboune, M., S.M. Culican, S.G. Turney & J.W. Lichtman. 1999. Rapid and reversible effects of activity on acetylcholine receptor density at the neuromuscular junction in vivo. Science **286:** 503–507.
33. Akaaboune, M., R.M. Grady, S. Turney, *et al.* 2002. Neurotransmitter receptor dynamics studied in vivo by reversible photo-unbinding of fluorescent ligands. Neuron **34:** 865–876.
34. Gautam, M., T.M. DeChiara, D.J. Glass, *et al.* 1999. Distinct phenotypes of mutant mice lacking agrin, MuSK, or rapsyn. Brain Res. Dev. Brain Res. **114:** 171–178.
35. Burgess, R.W., Q.T. Nguyen, Y.J. Son, *et al.* 1999. Alternatively spliced isoforms of nerve- and muscle-derived agrin: their roles at the neuromuscular junction. Neuron **23:** 33–44.
36. Gautam, M., P.G. Noakes, L. Moscoso, *et al.* 1996. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell **85:** 525–535.
37. Sanes, J.R. & J.M. Cheney. 1982. Lectin binding reveals a synapse-specific carbohydrate in skeletal muscle. Nature **300:** 646–647.
38. Sugiyama, J.E., D.J. Glass, G.D. Yancopoulos & Z.W. Hall. 1997. Laminin-induced acetylcholine receptor clustering: an alternative pathway. J. Cell. Biol. **139:** 181–191.
39. Martin, P.T. & J.R. Sanes. 1995. Role for a synapse-specific carbohydrate in agrin-induced clustering of acetylcholine receptors. Neuron **14:** 743–754.
40. Marangi, P.A., S.T. Wieland & C. Fuhrer. 2002. Laminin-1 redistributes postsynaptic proteins and requires rapsyn, tyrosine phosphorylation, and Src and Fyn to stably cluster acetylcholine receptors. J. Cell. Biol. **157:** 883–895.
41. McConville, J., M.E. Farrugia, D. Beeson, *et al.* 2004. Detection and characterization of MuSK antibodies in seronegative myasthenia gravis. Ann. Neurol. **55:** 580–584.
42. Okada, K., A. Inoue, M. Okada, *et al.* 2006. The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science **312:** 1802–1805.
43. Beeson, D., O. Higuchi, J. Palace, *et al.* 2006. Dok-7 mutations underlie a neuromuscular junction synaptopathy. Science **313:** 1975–1978.
44. Palace, J., D. Lashley, J. Newsom-Davis, *et al.* 2007. Clinical features of the DOK7 neuromuscular junction synaptopathy. Brain **130:** 1507–1515.

Muscle Weakness and Neuromuscular Junctions in Aging and Disease

Kazuhiro Shigemoto[1], Sachiho Kubo[1], Syuuichi Mori[1], Shigeru Yamada[1], Takuyu Akiyoshi[1,2] and Tsuyoshi Miyazaki[1]

[1]Tokyo Metropolitan Institute of Gerontology, Research Team for Molecular Biomarkers
Sakaecho 35-2, Itabashi, Tokyo 173-0015 Japan

[2]Department of Geriatric Medicine, Tokyo University School of Medicine,

To whom correspondence should be addressed:

Kazuhiro Shigemoto M.D., Ph.D
Tokyo Metropolitan Institute of Gerontology
Research Team for Molecular Biomarkers

Sakaecho 35-2, Itabashi-ku
Tokyo 173-0015 Japan
kazshige@tmig.or.jp
tel: +81-3-3964-3241 (ext.3025)
fax: +81-3-3579-4776

Key words: sarcopenia, neuromuscular junction, myasthenia gravis, muscle-specific kinase

Introduction

A critical issue in today's super-aging society is the need to reduce the burden of family care while continuing to make our medical institutions supportive. A rapidly emerging, major health concern is the debilitating effect of muscle weakness and atrophy from aging, termed *sarcopenia;*, however, the molecular basis of this condition is not well understood. Our research aim is to elucidate the molecular mechanisms of age-related muscle atrophy and to devise new measures for preventing and treating this disability. A promising treatment for muscle atrophy is the promotion of muscle regeneration by recruiting stem cells into the targeted region. The first requirement is to understand how the motor system, which consists of muscles and motoneurons, is maintained to accomplish that goal. Recent studies in the field of neuroscience have focused on neuromuscular junctions (NMJs), which play important roles in the maintenance of both motor nerves and muscle fibers. Signaling between muscles and motoneurons at NMJs supports interactions within the motor system. To understand the mechanisms involved, we focus our research on the pathogenic processes underlying neuromuscular diseases. The well-known autoimmune disease, myasthenia gravis (MG), serves as a model not only for tracking the pathogenesis and treatment outcomes of all autoimmune diseases, but also for understanding synaptic functions in maintaining the motor system. Here we describe recent insights into the molecular mechanisms required for the maintenance of NMJs and the related causes of muscle atrophy.

Neuromuscular Junctions

NMJs, which are structures located between motor terminals and muscles, are the sites of synapses between motor nerves and muscle fibers. At the anterior horn of the spinal cord and brainstem, skeletal muscle fibers are innervated by large motor neurons. The terminal arborization of each α-motor neuron is situated in **a** shallow depression of the muscle cell membrane, which is invaginated further into deep and regular folds, termed postjunctional folds (Figure 1). The motor nerve terminal is specialized for neurotransmitter (acetylcholine:ACh) release. Synaptic vesicles containing ACh cluster adjacent to specialized structures of the presynaptic membrane, called active zones. The active zones are aligned precisely with mouths of the postjunctional folds. ACh receptors (AChRs) are highly concentrated, with a density of about 12,000 receptors per μm^2, at the post-junctional membrane nearest the fold's peak (Figure 1). When the

nerve action potential reaches the terminal, depolarization opens voltage-gated Ca^+ channels on the presynaptic membrane. This allows a Ca^+ influx that triggers the fusion of synaptic vesicles with the presynaptic membrane and the release of ACh. The postsynaptic membrane responds rapidly and dependably to ACh released from the overlying active zones in the nerve terminal. AChRs, by binding ACh, become transiently permeable to both Na^+ and K^+, then opening the associated voltage-gated ion channels, which contribute to the action potential and muscle contraction. The synaptic cleft between nerve terminals and postsynaptic membrane is about 50 nm wide. A layer of connective tissue called basal lamina (basement membrane) sheaths each muscle fiber, passes through the synaptic clef and extends into the junctional folds. Both the presynaptic terminal and the muscle fiber secrete molecules including collagen IV, laminin, ectactin and heparan sulfate proteoglycans to the basal lamina. However, synaptic portions of the basal lamina contain their distinctive isoform composition separate from that of the extrasynaptic portions. Synaptic basal lamina also contain the enzyme acetylcholinesterase, which quickly inactivates the ACh released from the presynaptic terminal by hydrolyzing it to acetate and choline. Concentrations of released ACh in the synaptic cleft decrease rapidly by diffusion and interaction with acetylcholinesterase, upon which the neuromuscular transmission terminates.

Myasthenia gravis and autoantibodies to AChR

MG is a rare neuromuscular disease, but a well-recognized disorder because of such characteristic clinical features as ptosis with fluctuating general fatigue and muscle weakness that worsens with repeated activity (1, 2) but tends to improve with rest. Ptosis and diplogia occur early in the majority of these patients. With passing time, when the bulbar and respiratory muscles deteriorate, the disease becomes life-threatening so that intubation with mechanical ventilation is required. About 80% of patients with MG have autoantibodies against AChR (1, 2). In 1973, Patrick and Lindstrom provided the first evidence indicating the pathogenicity of AChR antibodies in a model of experimentally induced MG (3). Thereafter, a number of studies showed the pathogenic roles of AChR antibodies in causing structural and functional damage of the NMJ, but no such autoantigens could be identified in ~20% of these MG patients (4). However, even patients who did not have AChR antibodies responded to immunotherapies, and their serum antibodies transferred a defect in neuromuscular

transmission to mice, indicating that autoantibodies against NMJ can induce the muscle weakness.

Previously, studies on the mechanism(s) of synaptic transmission at the NMJ had facilitated understanding of how antibodies to AChR induce the pathogenicity typical of MG (1, 2). Effective neuromuscular transmission depends on numerous interactions between ACh and its receptor, AChR, and the failure of neuromuscular transmission results in myasthenic weakness and fatigue. To evoke action potential for the contraction of muscle fibers, a large enough number of AChRs must be present at postsynaptic membranes. In 1973, Fambrough and colleagues found an abnormal decrease in the number of AChR at postsynaptic membranes of the NMJ of patients with MG (5). Others showed that AChR antibodies affect neuromuscular transmission by three main mechanisms: (a) Complement-mediated lysis of post-synaptic membrane by binding and activation of complement at the NMJ; (b) accelerated degradation of AChR molecules cross-linked by antibodies (antigenic modulation); (c) functional AChR block by antibodies. The predominant pathogenicity is caused by the complement-mediated mechanisms (6), but all three mechanisms tend to reduce the number of available AChRs and, thereby, decrease neuromuscular transmission between motor nerve endings and postsynaptic membranes. Therefore, an individual nerve impulse cannot generate enough postsynaptic depolarization to achieve the crucial firing threshold required for opening of sufficient voltage-gated sodium channels to initiate an action potential in the muscle fiber (7).

Antibodies to muscle-specific kinase in MG patients

For the last three decades, causative autoantibodies other than those to AChR have been sought in MG patients but have eluded identification in spite of extensive research efforts (1, 2). In 2001, Hoch et al. found autoantibodies against muscle-specific kinase (MuSK) in a proportion of patients with generalized MG (4). MuSK is essential during the development of NMJ, when it organizes fetal AChR clustering at the postsynaptic membrane. Subsequently, in mature NMJ, MuSK is expressed predominantly at the postsynaptic membrane. Studies by Vincent and others showed that the frequency of MuSK antibodies in "seronegative MG patients," i.e., those who lack autoantibodies to AChR, varied from 4 to 50% (4, 8-11). Ohta et al. detected MuSK antibodies in about 30% of seronegative MG patients but not in any MG patients with AChR antibodies

(seropositive MG) or other autoimmune diseases (12-14). The clinical features of patients with MG and MuSK antibodies are distinctive. These individuals often suffer from a severe bulbar dysfunction that is difficult to resolve with immunosuppressive and immunomodulatory treatments, and muscular atrophy of facial and tongue muscles is common (14, 15). The response to acetylcholine esterase inhibitors is generally unsatisfactory with risk of worsening symptoms, especially when starting treatment in patients with bulbar symptoms or an impending respiratory crisis (16). Thymectomy does not alleviate the symptoms (14). In short-term therapy, patients with MuSK-positive MG respond as well to plasma exchange and intravenous immunoglobulin as those with AChR seropositive MG (14). Even so, those patients whose neck and shoulder muscles are affected often experience respiratory weakness (15). MG in which weakness is limited to the ocular muscle is not frequent but does occur (15).

A number of clinical studies showed that MuSK MG constitutes a distinct subclass of the disease (8-10, 15). The reason is that many patients with MuSK antibodies develop severe muscle weakness and eventual atrophy, which is less common in patients with AChR seropositive MG, and the former respond differently to therapy than persons in the latter group. After the identification of MuSK antibodies in an MG patient, laboratory testing is now required to confirm the diagnosis of MG, to seek AChR antibodies and to formulate the clinical treatment.

MuSK functions in NMJ

MuSK plays multiple roles in clustering AChR during development of the postsynaptic membranes of NMJ (17, 18). Contact of the motor-nerve growth cone with the muscle induces a narrow, distinct endplate zone in the mid-muscle that is marked by a high density of AChR clustering. In this step, agrin released from motoneurons activates MuSK and redistributes AChR clusters to synaptic sites (18, 19). However, agrin does not bind MuSK, and additional components are required to activate MuSK (17, 19). Recent studies demonstrated that Lrp4, a member of the LDLR family, is a receptor of agrin, forms a complex with MuSK, and mediates MuSK activation by agrin (20, 21). Intriguingly, MuSK is also required for organizing a primary synaptic scaffold to create the post-synaptic membrane (18). Prior to muscle innervation, AChR clusters form at the central regions of muscle fibers, creating an endplate zone that is somewhat broader than that in innervated muscle. Thus, MuSK is required for pre-patterning of

AChR clustering in the absence of motor innervation. However, establishing a scenario for MuSK's participation in the process is somewhat complicated. For example, an element other than agrin may activate MuSK and trigger the postsynaptic specialization at NMJs. Simultaneously or alternatively, MuSK could act as a primary scaffold molecule without activation. The listed pleiotropic roles of MuSK in AChR clustering at developmental NMJs could also be required for the maintenance of mature NMJs (22, 23). Studies performed *in vivo* have shown that synaptic AChRs intermingle among themselves completely over a period of ~four days and that many extra-synaptic AChRs are incorporated into the synapse at the mature NMJ, although the synaptic membrane in adult muscle appears macroscopically to be stable (24). Therefore, the mechanisms at play during AChR clustering in developing NMJs are also required in mature NMJs where postsynaptic complexes including those with AChR and MuSK are dynamically turning over for the maintenance of muscle function.

Do MuSK antibodies cause MG?

In contrast to the well-accepted mechanisms by which AChR antibodies function in MG, the pathogenic role of MuSK antibodies has been unclear (25). First, no significant loss of AChR at NMJs was observed in biopsies from biceps brachii muscles of MuSK-positive patients with MG (26). Second, MuSK antibodies are mainly in the IgG4 subclass, which does not activate complement (14), and complement-mediated damage to postsynaptic membranes is considered a major source of pathogenicity in MG patients with AChR antibodies. Third, no research results have shown that passive transfer of MuSK serum from MG patients generates the equivalent disease in mice. Fourth, no experimental animal model of myasthenia gravis (EAMG) induced by immunization of MuSK protein has been developed. However MuSK antibodies from MG patients can inhibit MuSK functions *in vitro* (4).

The pathogenicity of AChR antibodies was simulated experimentally by the induction of muscle weakness and development of paralysis in rabbits immunized with AChR protein purified from the electric eel (3). This AChR protein induced the production of antibodies that cross-reacted with rabbit AChR at the NMJ. The flaccid paralysis that followed and electrophysiological studies of these animals provided a model that resembled the MG of humans. Therefore, the demonstration of experimental autoimmune MG in animals induced by MuSK antibodies was essential for proving

their pathogenicity and investigating their mechanisms of eliciting MG.

In 2006, we have found that immunization of rabbits with MuSK ectodomain caused myasthenic weakness and produced electromyographic findings that were compatible with a diagnosis of MG (23), as shown earlier by Patrick and Lindstrom (3). The extracellular segment of MuSK comprised five distinct domains, i.e., four immunoglobulin-like domains and one cysteine-rich region (Figure 2). The fusion protein expression constructs, which consisted of mouse MuSK ectodomain with the Fc region of human IgG1 or His-tag, were generated and transfected into COS-7 cells. The secreted recombinant MuSK-Fc and MuSK-His proteins were purified by using protein-A Sepharose and histidine affinity columns, respectively. New Zealand White rabbits were then immunized with 100 to 400 μg of purified MuSK recombinant protein. After three to four injections of MuSK protein, all of six rabbits manifested flaccid paralysis (Figure 3A). Sera from the paretic rabbits contained a high titer of MuSK antibodies that reacted specifically with MuSK molecules on the surfaces of C2C12 myotubes as observed in sera from MG patients who were positive for MuSK antibodies (23). Histological studies of the muscle tissues from the paretic rabbits, which had manifested severe exhaustion, revealed alterations in muscle fibers ranging from subtle to angular atrophy intermingled with normal muscle tissue (Figure 3B). The histological changes typical of atrophied muscle fibers can result from MG, reduced mechanical ability or cachexia. In repetitive electromyograms from one of these paretic rabbits, the retroauricular branch of facial nerve was stimulated at 20 Hz, and recordings were taken from adjacent retroauricular muscle (Figure 3C). The compound muscle action potential (CMAP) showed a decremental pattern, consistent with MG. However, injections of acetylcholine esterase inhibitor did not significantly reverse either the CMAP defect or the paralytic symptoms. Importantly, induction of myasthenia by MuSK antibodies is not confined to the rabbit, since we and others also produced myasthenia in mice by injection of MuSK protein (Figure 4) (27, 28).

How do antibodies to MuSK cause MG?

We have provided the first piece of evidence that active immunization with MuSK protein reproduces the MG-like disease in animals (23, 28). Next, we focused on how MuSK antibodies cause MG. The pathogenic role of MuSK antibodies in MG has been questioned, since the number of AChRs is not reduced and complement is not deposited

at the NMJs of biceps brachii muscles from MuSK-positive patients with MG (26). The mechanisms used by AChR antibodies to cause MG are well delineated (1, 2), but those mechanisms simply do not apply to MG associated with MuSK antibodies. MuSK antibodies have been identified as predominantly IgG4, which does not activate complement. However, antibodies binding to MuSK could accelerate the degradation of MuSK molecules (antigenic modulation) and/or inhibit MuSK functions directly. MuSK is essential for AChR clustering at the developing NMJs, and its deficiency may lead to the complete loss of junctional ultrastructure (22, 29). Further, MuSK may also play important roles in the maintenance of AChR clustering and the structure of mature NMJs. To reveal precisely how MuSK antibodies participate in MG, unraveling the way in which MuSK acts at mature NMJ is necessary.

To elucidate the mechanisms of AChR clustering at NMJs, numerous studies were performed using cultured C2C12 myotubes (Figure 5). Agrin induces clustering of AChR in C2C12 myotubes following autophosphorylation by MuSK. *In vivo*, this event represents a major cascade of AChR clustering at the NMJ after innervation by motoneurons. Laminin-1 and the *N*-acetylgalactosamine (GalNAc)-specific lectin *Vicia villosa* agglutinin (VVA-B4) also induce AChR clustering on C2C12 myotubes, without activation of MuSK. Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty. However, these mechanisms may also be important for the formation and maintenance of NMJs, the latter via agrin-independent pathways as shown by genetic studies (23).

In a previous study, Hoch et al. observed that the MuSK antibodies of MG patients inhibited agrin-induced AChR clustering in C2C12 myotubes (4). We also found that agrin-induced clustering of AChR was strongly blocked in the presence of MuSK antibodies, whereas absorption of the antibodies with purified MuSK products prevented this blocking effect as illustrated in Figure 5 (23). These results showed that MuSK antibodies effectively inhibited the formation of agrin-induced AChR clustering. Intriguingly, the monovalent Fab fragments of MuSK antibodies from rabbits with experimental autoimmune MG also inhibited AChR clustering by agrin on C2C12 cells, indicating that complement-mediated mechanisms are not necessarily required for such inhibition (unpublished data). We also noted that MuSK-specific antibodies strongly inhibited AChR clustering induced by all known agrin-independent pathways as well as by agrin itself (Figure 5) (23).

We then examined the reduced expression of AChR at NMJs in soleus muscles of paretic and normal rabbits by using fluorescence microscopy after applying a rhodamine-conjugated AChR agonist, α-BTX (Figure 6). The use of a digital camera and staining with rhodamine-conjugated α-BTX enabled us to record the size and optical densities of AChR clusters. The resulting images were measured by using NIH image analysis software (23). The areas and intensity of AChR fluorescence in muscles of these paretic rabbits were significantly reduced compared with those in normal rabbits. In addition, the structure of NMJs in our paretic rabbits as well as the size and branching of the motor terminals were significantly reduced. Electron microscopic observations of NMJs in rabbits with EAMG induced by injection of MuSK protein demonstrated a significant loss of complexity in the convoluted synaptic folds but no destruction. A particularly important observation was that the EAMG model cited here resembles the phenotype of humans with MG and MuSK antibodies (Figure 7). In the intricate and convoluted synaptic folds, the high density of voltage gated sodium channels in the membranes' depths amplify the end-plate current, thus enhancing neuromuscular transmission and muscle contraction (30). A reduction in the size and branching of the motor terminals contributes to the reduced ACh output, and reduced post-synaptic folding increases the threshold for generation of muscle fiber action potential. These structural abnormalities in NMJ, including both pre- and post-synaptic structures, thus impair neuromuscular transmission in rabbits with EAMG (28).

Intriguingly, similar abnormalities of NMJ structure were also observed in rats with reduced expression of MuSK as noted by RNA interference (22), in a patient with congenital myasthenic syndromes (CMS) caused by MuSK mutations and also in mice expressing the MuSK missense mutation seen by electroporation experiments (31). MuSK knock-out mice also displayed presynaptic defects in addition to postsynaptic ones, indicating that MuSK is required for retrograde signals, so far unidentified, to maintain the pre-synaptic structure in mature NMJ.

Dok-7 is required for the maintenance of NMJs

In 2006, a MuSK-interacting protein called Dok-7 was discovered (32) and identified as a member of the Dok family of cytoplasmic proteins. Dok-7 is postulated to have three main functional domains: a pleckstrin homology (PH) domain, essential for membrane association, a phosphotyrosine-binding (PTB) domain involved in the Dok-7

induced activation of MuSK and a large C-terminal domain containing multiple tyrosine residues. Dok-7 knockout mice showed a marked disruption of neuromuscular synaptogenesis that was indistinguishable from the features found in MuSK-deficient mice. Thus, Dok-7 is essential for neuromuscular synaptogenesis through its interaction with MuSK.

Mutations in the Dok-7 protein cause the genetic form of limb-girdle myasthenia called CMS (33). Some clinical features of these patients resemble those in the severe type of MG accompanied by MuSK antibodies (34). Proximal muscles are usually more affected than those in distal regions, as evident in MuSK MG patients, and ptosis is often present. However, limb-muscle weakness is comparatively less severe. Previous studies showed no reduction of AChR clustering with significant changes in NMJ of MuSK MG patients (26), but further structural analysis of NMJ is required in muscles where severe weakness occurs commonly. The weakness and atrophy are not observed uniformly in muscles of these patients, although both MuSK and Dok-7 are essential for the formation of NMJs during the embryonic stage (32). Notably, one of the major distinctions between acquired MuSK MG and CMS with the Dok-7 mutation is the timing when weakness begins. The CMS patients typically have difficulty in walking after reaching that normal motor milestone during early childhood, whereas the onset of weakness of MG patients, in most instances, occurs in adulthood. Interestingly, AChR clustering and post-synaptic folds are reduced and have small motor terminals as observed at NMJs in patients with CMS and Dok-7 mutations (35). The effect of Dok-7 mutations on post-synaptic structures may also be an alteration of retrograde signaling to the pre-synaptic nerve terminals resulting in a reduced NMJ size in these patients (Figure 7). Dok-7, along with MuSK, is also required for the maintenance of NMJ, not only for synaptogenesis.

MuSK plays important roles in the maintenance of NMJs

We have shown that MuSK is required for the maintenance as well as the generation of NMJs (23, 28). Disruption of those mechanisms by MuSK antibodies causes MG in humans. Use of an experimental model for MG revealed that MuSK antibodies mediate the pathogenesis of this syndrome in rabbits and mice (23, 27, 28). In most cases, the symptoms take more than three months to manifest themselves in rabbits and more than four weeks in mice. Moreover, the symptoms were also induced experimentally by