

incorporated into the synapse at the mature NMJs, although the synaptic membrane in adult muscle appears macroscopically to be stable (64-66). Therefore, the mechanisms at play during AChR clustering in developing NMJs are also required in mature NMJ where postsynaptic complexes including those with AChR and MuSK dynamically turn over for the maintenance of muscle function. Furthermore, MuSK requires the development and maintenance of synaptic structures at NMJs after birth. The disassembly of NMJ structures *in vivo* can be induced by a reduction of MuSK expression using the RNA interference technology in single muscle fibers of adult rats (67). Postnatal inactivation of MuSK using the loxP/Cre system in mice also causes loss of AChRs and disassembly of the postsynaptic organization (68). The conditional inactivation of MuSK in mice during postnatal development leads to defects in NMJ maintenance and premature death. Studies of these dynamics indicated that MuSK is required for retrograde signals, so far unidentified, to maintain the pre-synaptic structure in mature NMJs (69). Moreover, histopathological studies disclosed the same changes of NMJs in mature animals with MuSK EAMG.

4. Pathogenic roles of anti-MuSK antibodies in EAMG

The establishment of MuSK EAMG, which reproduces the clinical and electrophysiological features of MG, enabled assessment of the pathogenic roles of the relevant autoantibodies in experimental animals (10). Histopathologic examination of

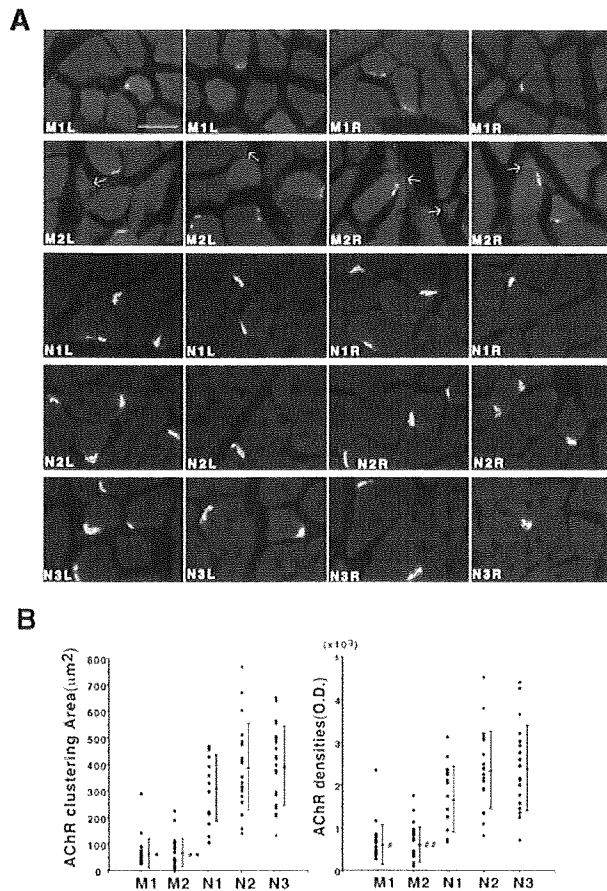


Figure 8. Reduction of the size and density of AChR clusters at the NMJs in paretic rabbits with MuSK EAMG. (A) Cross sections from the soleus muscles of 2 paretic (M1 and M2) and 3 normal rabbits (N1, N2, and N3). (B) Quantification of the area and intensity of AChR clustering. (Reprinted with permission from Shigemoto et al., *J Clin Invest* 116:1016-1024, 2006)

NMJs in rabbits with MuSK EAMG revealed that anti-MuSK antibodies interfere with MuSK functions at play in the maintenance of mature NMJs described in the previous section. Reduced expression of AChRs at the NMJs was observed by using fluorescence microscopy after applying a rhodamine-conjugated AChR agonist, α -BTX (Figure 8A and B) (10). The areas and intensity of AChR fluorescence at NMJs in muscles of paretic rabbits were significantly reduced compared with those in normal rabbits. Anti-MuSK antibodies in rabbits with EAMG blocked agrin-induced clustering of

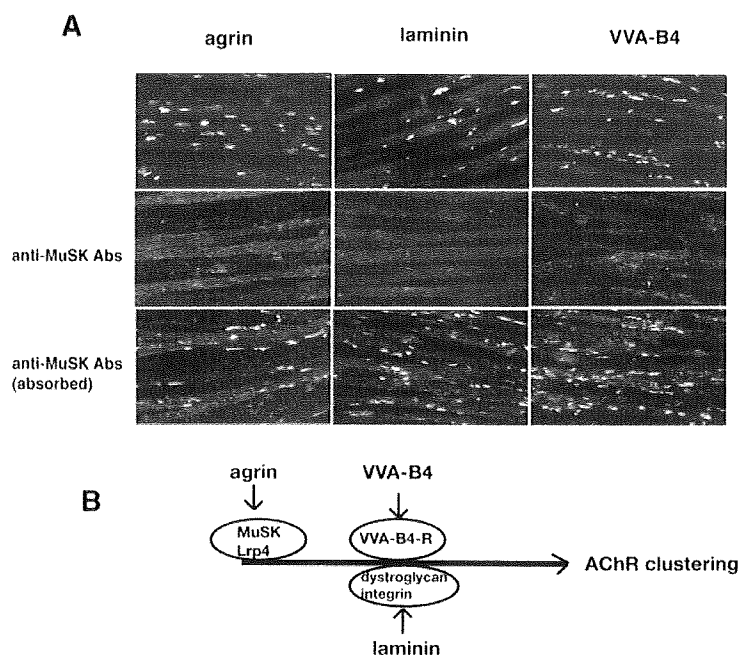


Figure 9. (A) Inhibition of agrin-induced and agrin-independent AChR clustering by MuSK antibodies. This inhibition was blocked by absorption of the MuSK antibodies with MuSK protein before treatment of the cells. (Reprinted with permission from Shigemoto et al., *J Clin Invest* 116:1016-1024, 2006) (B) VVA-B4 binds the unidentified receptors and induces AChR clustering. Laminin induces AChR clustering by binding with dystroglycan and integrin.

AChRs in C2C12 myotubes as well as the human antibodies, whereas absorption of the antibodies with purified MuSK products prevented this blocking effect as illustrated in Figure 9A.

To elucidate the mechanisms of AChR clustering at NMJs, numerous studies have been performed using cultured C2C12 myotubes (44). Agrin induces the clustering of AChRs in C2C12 myotubes following autophosphorylation by MuSK. *In vivo*, this event represents a major cascade of AChR clustering at the NMJs after innervation by motoneurons (42). These results showed that MuSK antibodies effectively inhibited the formation of agrin-induced AChR clustering. The monovalent Fab fragments of MuSK antibodies, from rabbits with MuSK EAMG also inhibited AChR clustering by agrin on C2C12 cells indicating that complement-mediated mechanisms are not necessarily required for such inhibition (unpublished data). In addition, the anti-MuSK antibodies in

rabbits strongly inhibited AChR clustering induced by all known agrin-independent pathways as well as by agrin itself (Figure 9A) (10). Soluble laminin and the *N*-acetylgalactosamine (GalNAc)-specific lectin *Vicia villosa* agglutinin (VVA-B4) also induce AChR clustering on C2C12 myotubes (Figure 9A and B), without activation of MuSK (10, 70-73). Laminin can induce clustering of AChRs via a pathway that involves tyrosine kinases downstream of MuSK by association with dystroglycan and β 1 integrin (Figure 9B) (74, 75). VVA-B4 selectively stains NMJs in skeletal muscles and AChR-rich portions of the C2C12 myotube surface (76, 77); however, the receptor remains to be identified (78). Laminin and VVA-B4 receptors lie downstream from and are common to agrin-mediated AChR clustering pathways (78). These receptors may be associated at postsynaptic membranes and cooperate in the maintenance of AChRs in NMJs. Without question, autoantibodies to MuSK will serve as useful tools for comprehending how MuSK functions at mature NMJs and the pathogenic mechanisms of MuSK MG.

In addition to the loss of AChR during EAMG, we have also noted the disassembly of NMJ structures and a reduction in the size and branching of the motor terminals in the paretic rabbits (Figure 10A). Electron microscopic observations of NMJs in rabbits with MuSK EAMG demonstrated a significant loss of complexity in the convoluted synaptic folds but not their destruction (unpublished data). Similarly, the NMJs of mice with MuSK EAMG incited by active immunization with MuSK (Figure 10B) and passive transfer of IgG from MuSK MG patients presented with the same histopathological picture (11-13). A reduction in the size and branching of the motor terminals contributes to a decreased ACh output, and a loss of AChRs and post-synaptic folding at NMJs increases the threshold for generation of muscle fiber action

potential. These structural abnormalities in NMJs, including both pre- and post-synaptic structures, thus impair neuromuscular transmission in animals with MuSK EAMG (10-13, 69).

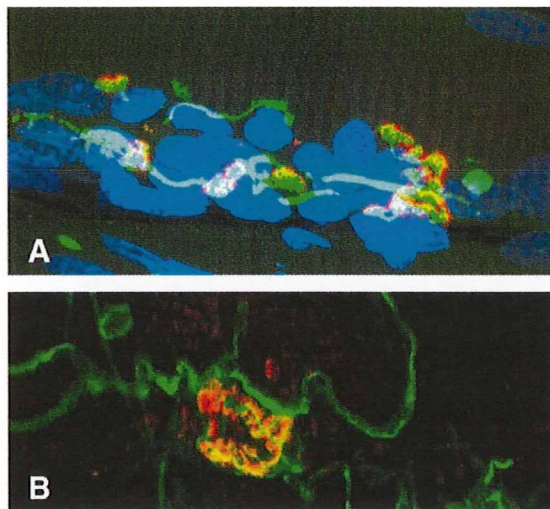


Figure 10. Disassembly of NMJs induced by anti-MuSK antibodies. Motor axon (green), AChR (red), subsynaptic nucleus (blue). (A) NMJs in a rabbit with MuSK EAMG. (B) NMJs in a mouse with MuSK EAMG.

The susceptibility to muscle weakness caused by anti-MuSK antibodies may vary among animal species, since the structures of NMJs differ greatly among them (79). Human NMJs are among the smallest found in the well-studied vertebrates. Therefore, the structural factors affecting the impairment of neuromuscular transmission by anti-MuSK antibodies may also vary among animals (79). However, anti-MuSK antibodies in rabbits and mice with MuSK EAMG inhibit agrin-induced AChR clustering on C2C12 myotubes much like that in MG patients (7), indicating the commonality of anti-MuSK antibodies as a cause of MG beyond the difference of species (10, 11).

The mechanisms used by anti-AChR antibodies to cause MG are well delineated (3, 4, 27), but those mechanisms simply do not apply to MG associated with anti-MuSK antibodies. One route of pathogenicity by anti-AChR antibodies is to damage the

postsynaptic membrane in NMJs, whereas antibodies to MuSK may interfere directly with the functions of MuSK required for maintaining NMJ structures (10, 12). Anti-MuSK antibodies in MG patients have been identified as predominantly IgG4, which does not activate complement (15, 16). Although the IgG1 of anti-MuSK antibodies is present in MG patients, only the titers of IgG4 were significantly associated with disease severity (17). Therefore, IgG4-antibodies binding to MuSK could accelerate the degradation of MuSK molecules (antigenic modulation) and/or inhibit MuSK functions directly (10, 69). In either case, anti-MuSK antibodies inhibit the functions of MuSK in NMJs.

We effectively generated EAMG in complement-deficient mice with severe symptoms by inoculating them with MuSK (unpublished data), whereas the deficient mice were highly resistant to EAMG from AChR immunization (80). Therefore tissue degradation by complement activation is not necessarily required for the manifestation of myasthenia by MuSK antibodies. Some MG patients with anti-MuSK antibodies also have low-affinity antibodies to AChRs found by testing with a highly sensitive immunofluorescence method (21, 81). Currently, the significance of anti-AChR antibodies in patients is not clear, but the clinical features of MuSK-MG are distinct from those for AChR-MG, thus anti-AChR antibodies may not produce visible symptoms in MuSK-MG patients.

Although significant AChR loss and structural changes in NMJs of animals with EAMG are commonly observed, those changes were not reported in the studies of biceps brachii muscles of anti MuSK-positive patients in a previous study (14). However, further histopathological analysis of NMJs in patients with severely weakened muscles would be beneficial for comparison with the animal under study for a similar condition. In

such patients, the weakness and atrophy are not observed uniformly; some anti MuSK-positive patients have more focal weakness in the neck, shoulder and respiratory muscles with prominent cranial and bulbar involvement than patients with anti-AChR antibodies. The limb muscles of individuals with MuSK-MG were less severely affected and inconsistently damaged, whereas patients with generalized AChR-MG patients often suffered from clearcut limb deterioration (82, 83). The mechanisms that favor particular sites of muscle weakness are unknown, and the nature of NMJs among various muscles may not be uniform.

5. The use of animals with EAMG to improve medication

MuSK MG is frequently a severe disease requiring emergent and aggressive therapies. The response to therapy for MuSK MG differs from that for AChR MG (84). Experimentation with animal models of EAMG will contribute to the development of more effective medication as the underlying pharmacological mechanisms become known. Acetylcholinesterase inhibitors are often used as symptomatic treatment and are effective for AChR MG. However, patients with MuSK MG are frequently unresponsive or develop cholinergic crises characterized by increasing muscle weakness that sometimes results in dysphagia and respiratory insufficiency (19, 20, 84, 85). Thus, differentiating a cholinergic crisis from a myasthenic crisis is critical for the decision on medical care of each patient (84, 86). Abnormal sensitivity to ACh after MuSK-MG patients receive acetylcholinesterase inhibitors can sometimes be recorded by electromyography (EMG) as extra discharges occurring after the CMAP with low-

frequency motor nerve stimulation, as shown in Figure 11 (86, 87). Such hypersensitivity to ACh may be caused by the interference of anchoring acetylcholinesterase to the synaptic cleft of postsynaptic membranes by MuSK (41). The same patterns of EMG showing hypersensitivity to ACh can be reproduced in mice with MuSK EAMG (unpublished data). The mechanisms of this hypersensitivity may be elucidated by studies of such animals with MuSK EAMG.

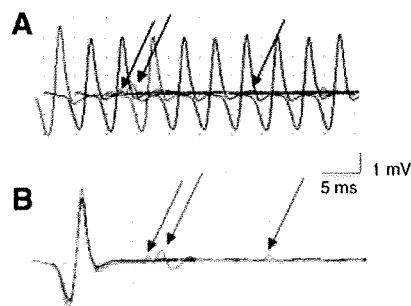


Figure 10. Extra repetitive discharges after the CMAP at low-frequency stimulation were recorded in a MuSK-MG patient with acetylcholinesterase inhibitor treatments. (Reprinted with permission from Punga et al., *Muscle Nerve* 34:111-115.)

Other symptomatic therapy such as 3,4-diaminopyridine, which increases ACh release from the nerve terminal, may be worth trying in MuSK-MG patients. Congenital myasthenic syndrome due to MuSK or Dok-7 mutations has been reported to respond favorably to 3,4-diaminopyridine (88-90). Dok-7 is essential for the formation of NMJs through its interaction with MuSK (91). Patients with MuSK MG apparently do not respond to immunosuppressive therapy as well as those with AChR MG; in fact, some of the former patients who did not respond to the therapy rapidly progressed to life-threatening muscle atrophy (20, 84). However, long-term treatment with higher dose steroids to unrefractory patients may accelerate the distortion of NMJ morphology by anti-MuSK antibodies (92). In view of the clinical variables involved, studying animals

with MuSK EAMG will provide important clues for developing and assessing the appropriate medication for all patients afflicted with MuSK MG.

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