IV. 研究成果の刊行物・別刷

Induction of myasthenia by immunization against muscle-specific kinase

Kazuhiro Shigemoto,[1,2] Sachiho Kubo,[2] Naoki Maruyama,[2] Naohito Hato,[3] Hiroyuki Yamada,[3] Chen Jie,[4] Naoto Kobayashi,[4] Katsumi Mominoki,[5] Yasuhito Abe,[6] Norifumi Ueda,[6] and Seiji Matsuda[4]

[1]Department of Preventive Medicine, Ehime University School of Medicine, Ehime, Japan. [2]Department of Molecular Pathology, Tokyo Metropolitan Institute for Gerontology, Tokyo, Japan. [3]Department of Otolaryngology and [4]Department of Integrated Basic Medical Science, Ehime University School of Medicine, Ehime, Japan. [5]Department of Biological Resources, The Integrated Center for Science, Ehime University, Ehime, Japan. [6]Department of Molecular Pathology, Ehime University School of Medicine, Ehime, Japan.

Muscle-specific kinase (MuSK) is critical for the synaptic clustering of nicotinic acetylcholine receptors (AChRs) and plays multiple roles in the organization and maintenance of neuromuscular junctions (NMJs). MuSK is activated by agrin, which is released from motoneurons, and induces AChR clustering at the postsynaptic membrane. Although autoantibodies against the ectodomain of MuSK have been found in a proportion of patients with generalized myasthenia gravis (MG), it is unclear whether MuSK autoantibodies are the causative agent of generalized MG. In the present study, rabbits immunized with MuSK ectodomain protein manifested MG-like muscle weakness with a reduction of AChR clustering at the NMJs. The autoantibodies activated MuSK and blocked AChR clustering induced by agrin or by mediators that do not activate MuSK. Thus MuSK autoantibodies rigorously inhibit AChR clustering mediated by multiple pathways, an outcome that broadens our general comprehension of the pathogenesis of MG.

Introduction

Myasthenia gravis (MG) is an antibody-mediated autoimmune disease in which the nicotinic acetylcholine receptor (AChR) at neuromuscular junctions (NMJs) is the major autoantigen (1). AChR-specific antibodies are detected in 90% of nonimmunosuppressed patients with generalized MG. However, Hoch et al. found antibodies to a novel antigen, muscle-specific kinase (MuSK), in approximately 66% of patients with generalized MG that were lacking detectable AChR autoantibodies (seronegative MG) (2). Subsequent studies have reported MuSK antibody frequencies of 4–47.4% in MG patients seronegative for AChR antibodies (3–9). MG patients with MuSK antibodies tend to develop severe facial weakness and bulbar symptoms, including dysphagia, dysarthria, and respiratory cirsis with some atrophy of facial muscles, that are often difficult to treat effectively with immunosuppressive therapies (3, 7). The pathogenic mechanisms of MG caused by AChR antibodies are well delineated, but pathogenicity has not been demonstrated for MuSK antibodies (10). Furthermore, no reports have described the induction of MG by immunization of animals with purified MuSK protein. The present study was undertaken to explore this issue. Here we describe the development of myasthenia and reduction of AChR density in rabbits immunized with the ectodomain of MuSK. The molecular pathogenesis of MG was further investigated using an in vitro assay of AChR clustering on myotubes that was mediated by MuSK antibodies.

MuSK is an AChR-associated transmembrane protein. During development of skeletal muscle, MuSK is initially required for organizing a primary synaptic scaffold to establish the postsynaptic membrane (11, 12). 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 (13, 14). MuSK and rapsyn, which is a 43-kDa, membrane-associated cytoplasmic protein, must be expressed before the endplate zone forms (11, 15–17). Subsequent contact of the motor-neuron growth cone with the muscle extinguishes extrasynaptic AChR clusters, resulting in a narrow, distinct endplate zone in the midmuscle that is marked by a high density of AChR clustering (13, 14). In this step, agrin released from motoneurons activates MuSK and redistributes AChR clusters to synaptic sites (13, 14, 17–20). Therefore the formation of NMJs either in the absence or presence of agrin requires the expression of MuSK at the endplate membrane.

The extracellular segment of MuSK comprises 5 distinct domains, i.e., 4 immunoglobulin-like domains and 1 cysteine-rich region (21–25). All 5 domains are conserved in *Torpedo spp*, mice, humans, rats, *Xenopus*, and chickens. MuSK exerts its multiple effects through interaction of the extracellular domains with other molecules. Transfection of MuSK$^{-/-}$ myotubes with a series of mutant MuSK constructs demonstrated that the amino-terminal immunoglobulin-like domain is required for activation by agrin (26). The extracellular domains of MuSK interact with rapsyn-AChR complexes (27, 28). Although the synaptic membrane in adult muscle appears to be macroscopically stable, in vivo studies 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 NMJs (29, 30). In addition, RNA interference targeting MuSK in adult mammalian muscle in vivo induced the disassembly of postsynaptic AChR clusters, with effects ranging from fragmentation to disappearance (31). Hence the mechanisms of NMJ formation by MuSK are also likely to be important for the maintenance of AChR clusters at the NMJs.

Nonstandard abbreviations used: BTX, α-bungarotoxin; GalNAc, *N*-acetylgalactosamine; MG, myasthenia gravis; MuSK, muscle-specific kinase; MuSK-AP, chimeric protein of the MuSK ectodomain and human placental alkaline phosphatase; MuSK-Fc, chimeric protein of the MuSK ectodomain and the Fc region of human IgG1; NMJ, neuromuscular junction; VVA-B4, *Vicia villosa* agglutinin.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **116**:1016–1024 (2006). doi:10.1172/JCI21545.

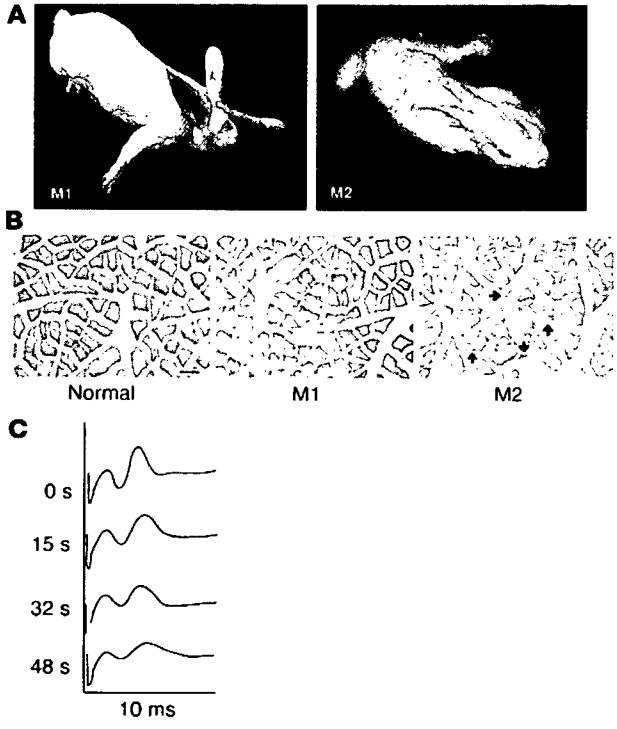

Figure 1
Rabbits manifest MG-like paresis after immunization with MuSK protein. (**A**) Two rabbits, representative of the 4 animals that showed myasthenia, manifested myasthenic weakness after immunization with the recombinant MuSK protein. After 3 injections of MuSK protein, M1 and M2 rabbits manifested flaccid weakness within 3 and 9 weeks, respectively. The M2 rabbit developed severe exhaustion with muscle weakness. (**B**) Cross sections from the soleus muscles of 2 paretic (M1 and M2) and 1 normal rabbit were stained with H&E. Muscle fibers in the M1 paretic rabbit showed only subtle changes in shape and smallness, whereas an atrophy of muscle fibers in the M2 paretic rabbit was observed as small angular fibers (arrows). Scale bar: 50 μm. (**C**) Electromyograms recorded from the M1 paretic rabbit. The retroauricular branch of the facial nerve was continuously stimulated by a constant-current stimulator, which delivered square-wave pulses of 0.1 ms at 20 Hz, and the compound muscle action potential (the second peak observed on the oscilloscope screen was recorded at the indicated time points during stimulation) showed a decremental pattern, consistent with MG.

In cultured myotubes, AChR clustering is induced by laminin-1 and the *N*-acetylgalactosamine-specific (GalNAc-specific) lectin *Vicia villosa* agglutinin (VVA-B4) without activation of MuSK (32–36). Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty. Even so, 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 (13, 14). The data we present herein demonstrate that MuSK autoantibodies inhibit AChR clustering by agrin itself and also by all known agrin-independent pathways.

Results

Immunization with purified MuSK protein causes flaccid weakness in rabbits. Rabbit antibodies were raised against a purified chimeric protein composed of the MuSK ectodomain and the Fc region of human IgG1 (MuSK-Fc). All of 4 recipient rabbits manifested flaccid weakness after 3 or 4 repeated injections with MuSK-Fc. Three of these rabbits developed flaccid weakness within 3 weeks after the last injection of MuSK protein, and the fourth rabbit manifested flaccid weakness 9 weeks after the third injection. Two rabbits that manifested flaccid weakness (M1 and M2 paretic rabbits) are shown in Figure 1A and Supplemental Movies 1 and 2 (supplemental material available online with this article; doi:10.1172/JCI21545DS1). Two of 4 paretic rabbits developed severe exhaustion (Figure 1A and Supplemental Movie 2; M2 paretic rabbit). Histological studies of the muscle tissues in the paretic rabbits revealed that the angular atrophic muscle fibers in the M2 paretic rabbit were intermingled with normal fibers, whereas the M1 rabbit had only subtle changes in the muscles (Figure 1B). No muscle regeneration was observed in M1 and M2 paretic rabbits (Figure 1B). The histological changes of the atrophic muscle fibers observed in the M2 paretic rabbit can result from MG, reduced mechanical activity of muscles, or cachexia (37).

These results suggest that the muscle weakness was caused by a disturbance of neuromuscular transmission due to the inhibition of MuSK functions in mature NMJs. This possibility was investigated first by an electromyographic study (38). Repetitive nerve stimulation (RNS) at a rate of 20/s in a paretic animal elicited a decremental response in compound muscle action potential (CMAP), characteristic of MG (Figure 1C). However, the CMAP in a normal rabbit did not change significantly during RNS at the same rate (data not shown). This result is consistent with the M1 rabbit having a disorder of the postsynaptic membrane as the cause of its weakness following immunization with a fragment of MuSK protein.

Reduction of AChR clustering at NMJs in paretic rabbits. Our second objective was to examine the NMJs of soleus muscles in 2 paretic (M1 and M2) and 3 normal rabbits (N1, N2, and N3) by fluorescence microscopy after applying a rhodamine-conjugated AChR antagonist, α-bungarotoxin (BTX). For semiquantitative measurement of the size and density of AChR clustering at NMJs, we determined an appropriate concentration of rhodamine-conjugated BTX by using serial dilutions of this reagent for application to frozen sections of soleus muscles (Figure 2A). A digital camera recorded images of the AChR clustering. Ten AChR clusters of NMJs were randomly chosen from the left and 10 from the right soleus muscles, after which the sizes and ODs were measured using NIH Image analysis software with the unprocessed digitized images (Supplemental Figure 1). In agreement with the electromyographic studies described above, the area and intensity of AChR fluorescence at the NMJs in both M1 and M2 paretic rabbits were significantly reduced compared with normal rabbits (area: M1, 67.9 ± 58.2 μm^2; M2, 75.0 ± 55.9 μm^2; compared with N1, 303.9 ± 125.9 μm^2; N2, 387.7 ± 163.0 μm^2; N3, 395.4 ± 150.0 μm^2; density: M1, 6219 ± 4659 OD; M2, 6889 ± 4259 OD; compared with N1, 17,770 ± 7,693 OD; N2, 23,259 ± 9,046 OD; N3, 23,870 ± 9,997 OD; Figure 2B).

Specific binding of antibodies to MuSK. As we found that muscle weakness caused by a disorder in the postsynaptic membrane was induced in rabbits via immunization with a purified MuSK protein, we next examined the ability of immune serum from paretic rabbits to react specifically with MuSK. To test whether MuSK

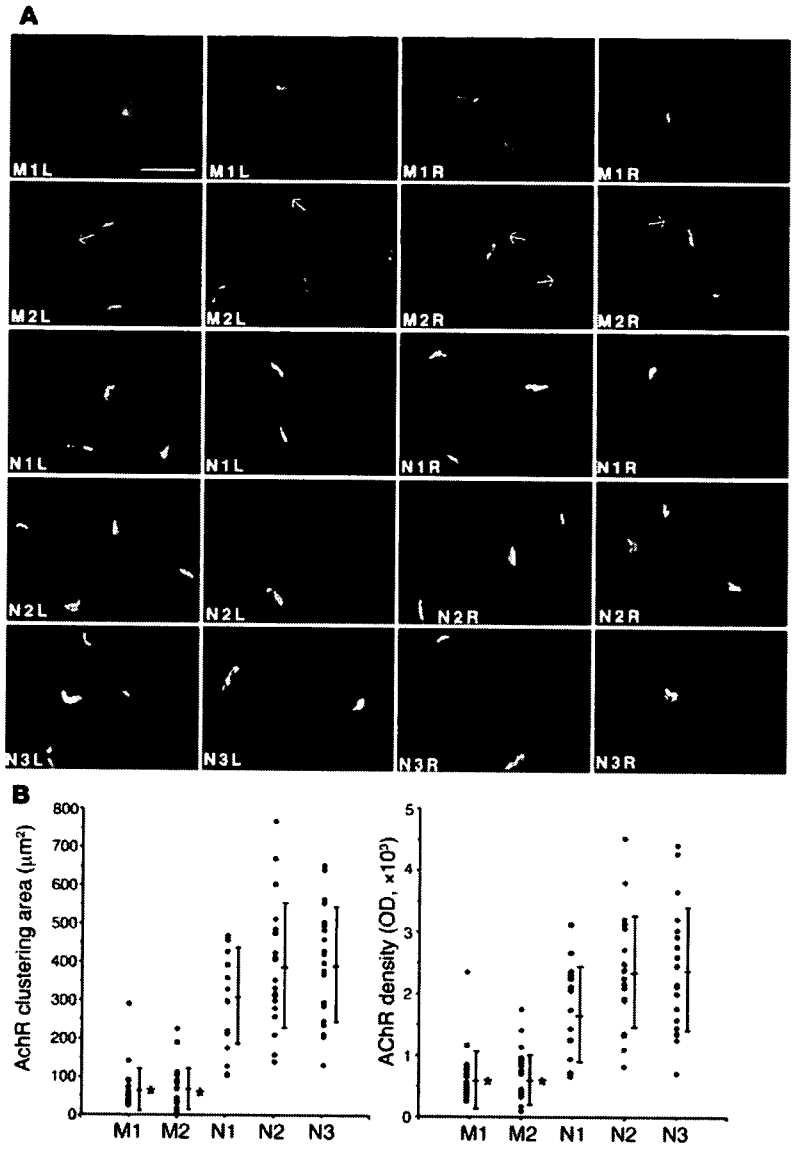

Figure 2
Reduction of the size and density of AChR clusters at the NMJs in paretic rabbits. (**A**) Cross sections from the soleus muscles of 2 paretic (M1 and M2) and 3 normal rabbits (N1, N2, and N3) were stained with 10 nM rhodamine-conjugated BTX. Bright crescents of bound BTX, indicative of endplate AChR, were smaller and less intense in the paretic rabbits' muscle fibers than in those of the normal rabbits. Arrows indicate the small angular fibers in M2 soleus muscles. L, left; R, right. Scale bar: 50 μm. (**B**) Images of 10 AChR clusters at NMJs in the right and 10 in the left soleus muscles of the paretic and normal rabbits were randomly recorded by a digital imaging camera. Quantification of the area and intensity of AChR clustering in the unprocessed images were measured using NIH Image software. Bars indicate mean ± SD. $^*P < 0.01$ versus normal rabbits.

antibodies could be used to detect MuSK in Western blots, a chimeric protein that combined the MuSK ectodomain with human placental alkaline phosphatase (MuSK-AP) was transfected into COS-7 cells. As expected, proteins representing the chimeric products (~130 kDa) were secreted into the culture medium (Figure 3A). The immune serum did not react with any of the AChR subunits precipitated from a lysate of C2C12 myotubes, as determined using biotinylated BTX and streptavidin-agarose. In contrast, the AChR β subunit was detected in the same precipitate when using a monoclonal antibody (data not shown). In addition, immunohistochemical analysis showed that MuSK antibodies reacted with native MuSK molecules and colocalized with agrin-induced AChR clusters on the surfaces of differentiated C2C12 myotubes (Figure 3B). Therefore a subset of MuSK antibodies reacts specifically with MuSK molecules on the cell surface.

Activation of MuSK by autoantibodies against MuSK. Hopf and Hoch (39) reported that rabbit polyclonal antibodies against the MuSK N-terminal sequence induced the activation of MuSK and the clustering of AChR on the surfaces of C2C12 myotubes in the absence of agrin. Therefore, the effect of MuSK antibodies on the tyrosine phosphorylation of MuSK was assessed here in C2C12 cell cultures. After a 30-minute incubation with MuSK antibodies, MuSK protein was immunoprecipitated from detergent extracts of the cells by using an antiserum against its intracellular domain. Tyrosine-phosphorylated MuSK was detected in Western blots by using phosphotyrosine-specific antibodies (Figure 4A). This antibody-induced autophosphorylation was completely inhibited by absorption with an excess of MuSK-AP before the addition of MuSK antibodies to the C2C12 myotube cultures. Thus the MuSK antibodies specifically activated autophosphorylation by inducing dimerization of the MuSK molecules in the absence of agrin, as shown previously using N-terminal-specific MuSK antibodies.

The downstream cascade of MuSK also leads to tyrosine phosphorylation of the AChR β subunit (19, 40, 41). Therefore we next sought to determine whether AChRs are tyrosine phosphorylated in response to MuSK activation by the MuSK antibodies. C2C12 myotubes were exposed to biotinylated BTX to identify AChRs in phosphotyrosine immunoblots. After exposure of the myotubes to MuSK antibodies for 30 minutes, tyrosine phosphorylation of the AChR β subunit was detected at a level similar to that induced by agrin (Figure 4B). AChR phosphorylation was inhibited when the antibodies were absorbed with an excess of MuSK-AP prior to addition to cultured C2C12 myotubes, which demonstrates that the MuSK antibodies specifically induce AChR β subunit phosphorylation in the downstream cascade of MuSK. Antibodies against the intracellular domain of the MuSK protein did not phosphorylate either MuSK or the AChR β subunit (data not shown).

Inhibition of spontaneous AChR clustering by antibodies against the MuSK ectodomain. MuSK antibodies induced tyrosine phosphorylation of both MuSK and the AChR β subunit, whereas AChR clustering at the NMJs of paretic rabbits was reduced. Therefore, we next tested the ability of MuSK antibodies to stimulate AChR

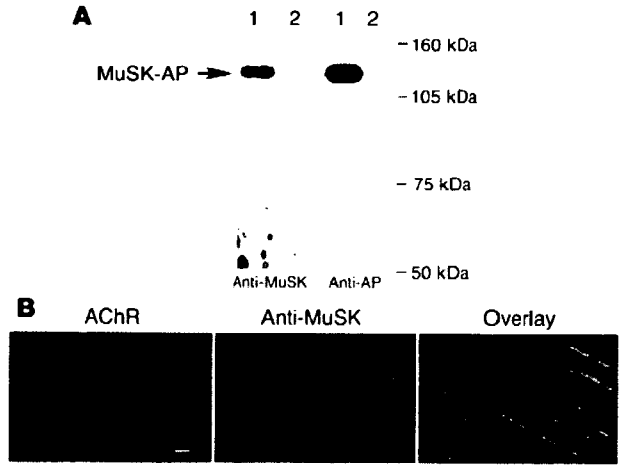

Figure 3
MuSK autoantibodies specifically bind MuSK. (**A**) Detection of secreted MuSK-AP from COS-7 cells by Western blot analysis with MuSK autoantibodies. The culture supernatants from COS-7 cells transfected with MuSK-AP (lane 1) or vector alone (lane 2) were immunoblotted with the MuSK antibodies, and the same immmunoblots were reprobed with an anti–alkaline phosphatase (anti-AP) antibody. (**B**) Specific binding of MuSK autoantibodies to MuSK in C2C12 myotubes. Myotubes were treated with agrin (1 nM for 18 h) and double labeled, first with rhodamine-conjugated BTX (left), and then with MuSK antibodies followed by fluorescein-conjugated secondary antibodies (middle). AChRs and MuSK colocalized, as shown by the overlay of red and green fluorescence (right). Green blotches are artifact. Scale bar: 20 μm.

clustering in the absence of agrin following MuSK autophosphorylation. Overnight incubation of C2C12 myotubes with MuSK antibodies in the absence of soluble agrin and labeling of AChR with rhodamine-conjugated BTX revealed that the MuSK antibodies inhibited spontaneous AChR clustering; that is, only a small number of AChR clusters formed in the myotubes in the absence of additional stimulation (Figure 5, A and B). However, AChR clustering was not blocked by antibodies against the MuSK intracellular domain (data not shown). Taken together, these results suggest that MuSK antibodies do not induce AChR clustering. Instead, they interfere with spontaneous AChR clustering by interacting with MuSK molecules in C2C12 myotubes, despite the activation of MuSK autophosphorylation.

Inhibition of agrin-induced AChR clustering by MuSK antibodies. Agrin induces clustering of AChR in C2C12 myotubes following MuSK autophosphorylation (17, 19, 20). This cascade of events is a major feature of AChR clustering at the NMJs after innervation by motoneurons (17, 36, 42, 43). In a previous study, Hoch et al. observed that the MuSK antibodies of MG patients inhibited agrin-induced AChR clustering (2), whereas we found that MuSK antibodies from paretic rabbits activated MuSK. It was therefore of interest to determine whether MuSK antibodies could interfere with agrin-induced AChR clustering in C2C12 cells as they did in spontaneous clustering. Indeed, agrin-induced clustering of AChR was strongly blocked in the presence of MuSK antibodies, whereas absorption of the antibodies with soluble MuSK-AP fusion products prevented this blocking effect (Figure 6, A and B). This result clearly demonstrates that the MuSK antibodies are responsible for inhibiting the formation of agrin-induced AChR clustering, in contrast to the N-terminal–specific MuSK antibodies, which induce AChR cluster formation in C2C2 myotubes (39).

Inhibition of agrin-independent AChR clustering by antibody against the MuSK ectodomain. Since MuSK is necessary for the signaling and effector mechanisms of agrin-independent AChR clustering at the NMJs in vivo (13, 14), we examined the ability of MuSK antibodies to inhibit AChR clustering induced by agrin-independent stimuli such as laminin-1 and VVA-B4. Incubation of laminin-1 with MuSK antibodies strongly blocked AChR clustering as well as spontaneous and agrin-induced AChR clustering (Figure 6, A and B) on C2C12 cells. After absorption of the antibodies with soluble MuSK-AP fusion products, AChR clustering in response to laminin-1 was restored (Figure 6, A and B). Thus the blocking effect was caused by these antibodies specific for the MuSK ectodomain, as was observed for agrin-induced clustering. In addition, treatment of C2C12 cells with VVA-B4 and MuSK antibodies totally blocked AChR clustering, whereas absorption of the antibodies with soluble MuSK-AP protein prevented this blocking effect (Figure 6, A and B). Therefore blocking by the antibodies was also specific for the MuSK ectodomain. These results indicate that the interaction of MuSK antibodies with MuSK molecules in myotubes leads to a reduction of AChR clustering via an agrin-independent pathway.

Blocking of neuraminidase-stimulated AChR clustering by MuSK antibodies. Treatment of C2C12 cells with neuraminidase increases both the intensity of VVA-B4 staining and the number of AChR clusters compared with spontaneous clustering (34, 43). Since AChR clustering caused by neuraminidase treatment may involve the same signaling mechanism as that induced by VVA-B4, the ability of MuSK antibodies to inhibit this response was examined. We con-

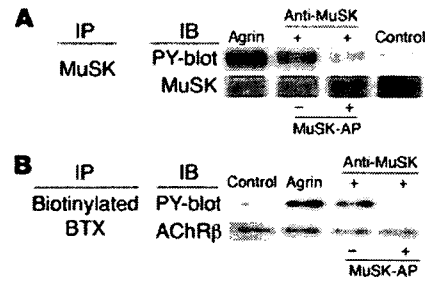

Figure 4
MuSK antibodies specifically activate MuSK and the downstream cascade. Specific phosphorylation of MuSK and AChR β subunit (AChRβ) by MuSK antibodies. C2C12 myotubes were treated with agrin or MuSK antibodies for 30 minutes and then immunoprecipitated with (**A**) MuSK antibodies or (**B**) biotinylated BTX using streptavidin-Sepharose. Immunoblots of immunoprecipitates were probed with antibodies to MuSK (**A**), AChRβ (**B**), or phosphotyrosine (PY, **A** and **B**). Both MuSK and AChRβ were phosphorylated after the treatment of agrin or MuSK antibodies. Phosphorylation of MuSK and AChRβ was inhibited by the absorption of MuSK antibodies with MuSK-AP protein.

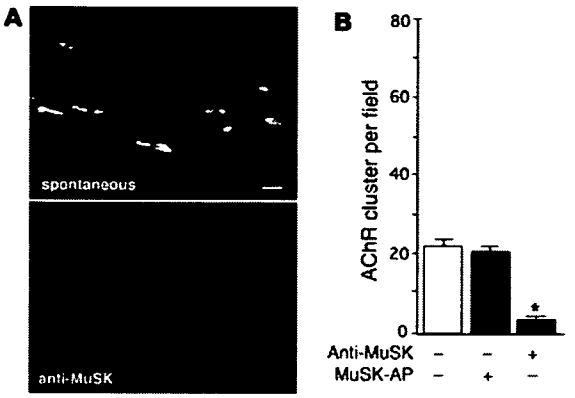

Figure 5
Inhibition of spontaneous AChR clustering by MuSK antibodies. (**A**) AChR clusters on C2C12 myotubes were stained with rhodamine-conjugated BTX with or without MuSK antibodies. Scale bar: 20 μm. (**B**) Quantification of the inhibitory activity of the MuSK antibodies showed that these antibodies provided significant inhibition of spontaneous AChR clustering (*$P < 0.01$, ANOVA). Values represent mean ± SEM of 10–15 fields for each of the 2 experiments per treatment.

firmed that neuraminidase treatment stimulated AChR clustering in C2C12 myotubes. That is, overnight incubation of C2C12 cells with 0.1 U/ml neuraminidase resulted in a 3-fold increase in the number of AChR clusters per field (Figure 7, A and B). When the C2C12 cells were incubated with MuSK antibodies and neuraminidase for 16 hours, AChR clustering was blocked efficiently, as was the clustering induced by VVA-B4. Again, absorption with MuSK-AP prevented blocking (Figure 7, A and B), which demonstrates that the inhibition of AChR clustering is caused by a direct effect of the MuSK antibodies. In summary, MuSK antibodies from paretic rabbits inhibit the AChR clustering in myotubes induced by all known agrin-independent stimuli as well as by agrin itself.

Discussion

MuSK autoantibodies are found in 4–70% of patients with generalized MG who lack detectable AChR autoantibodies (seronegative MG) (2–9); therefore, we investigated the pathogenicity of MuSK autoantibodies, as reported here. Our results demonstrate that immunization of rabbits with MuSK ectodomain protein caused myasthenic weakness and produced electromyographic findings that were compatible with a diagnosis of MG and consistent with a significant reduction of AChR clustering at NMJs. Additionally, MuSK antibodies specifically inhibited in vitro AChR clustering responses to all known stimuli, including those that act via agrin-independent pathways. Surprisingly, when applied to C2C12 muscle cells, MuSK antibodies initiated the phosphorylation of MuSK receptors and the subsequent phosphorylation of AChR in the absence of agrin, despite strongly inhibiting spontaneous AChR clustering. Furthermore, the MuSK antibodies inhibited AChR clustering induced by agrin. The possibility that MuSK antibodies contain a component that reacts with a receptor that stimulates inhibitory signals for AChR clustering seems unlikely,

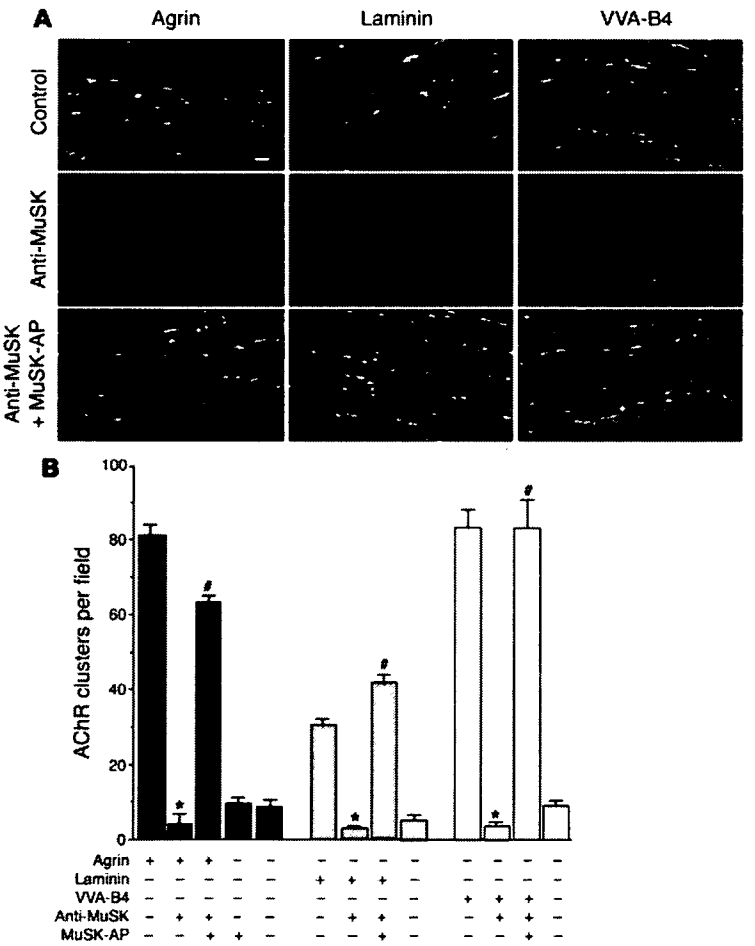

Figure 6
Inhibition of agrin-induced and agrin-independent AChR clustering by MuSK antibodies. (**A**) C2C12 cells were treated with agrin, laminin-1, or VVA-B4. AChR clusters were stained with rhodamine-conjugated BTX. AChR clustering induced by agrin, laminin-1, and VVA-B4 was inhibited in the presence of MuSK antibodies. This inhibition was blocked by absorption of the MuSK antibodies with MuSK-AP before treatment of the cells. Scale bar: 20 μm. (**B**) Quantification of the inhibitory activity of the MuSK antibodies confirmed that they significantly inhibited agrin-, laminin-1–, and VVA-B4–induced AChR clustering. Preabsorption of the MuSK antibodies with MuSK-AP significantly blocked inhibition. Values represent mean ± SEM of 10–15 fields for each of the 2 experiments per treatment. *$P < 0.01$ versus similar treatment without MuSK antibodies; #$P < 0.01$ versus similar treatment without preabsorption; ANOVA.

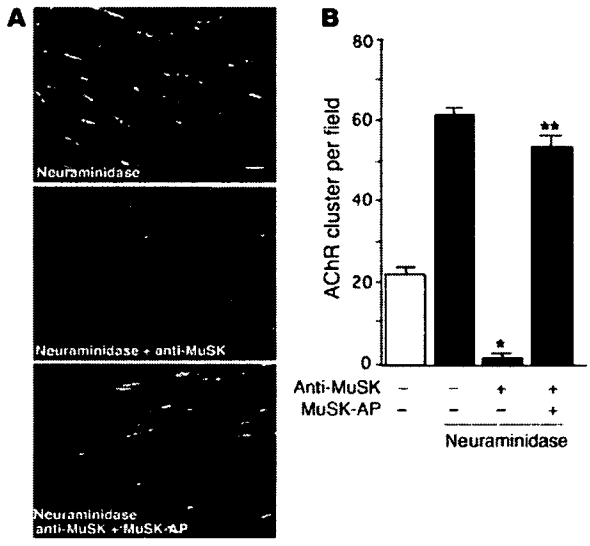

Figure 7
Inhibition of neuraminidase-induced AChR clustering by MuSK antibodies. (**A**) C2C12 cells were treated with *Clostridium perfringens* neuraminidase. AChR clusters were stained with rhodamine-conjugated BTX. AChR clustering induced by neuraminidase was inhibited in the presence of the MuSK antibodies. This inhibition was blocked by absorption of the MuSK antibodies with MuSK-AP before treatment of the cells. Scale bar: 20 μm. (**B**) Quantification of the significant inhibition of neuraminidase-induced AChR clustering by MuSK antibodies. Inhibition was significantly blocked by preabsorption of the MuSK antibodies with 6.5 nM MuSK-AP. Values represent mean ± SEM of 10–15 fields for each of the 2 experiments per treatment. $^{*}P < 0.01$ versus treatment without MuSK antibodies; $^{**}P < 0.01$ versus treatment without preabsorption; ANOVA.

since the ability of these antibodies to inhibit AChR clustering was abolished by absorption with a purified chimeric recombinant protein of MuSK. Hoch et al. have shown that plasma containing MuSK antibodies derived from patients with generalized MG who lack detectable AChR autoantibodies causes modest AChR clustering, whereas the same plasma inhibits agrin-induced AChR clustering (2). Thus, it is conceivable that a subset of MuSK antibodies expresses a strong inhibitory activity that blocks spontaneous AChR clustering, whereas other subsets of MuSK antibodies, such as those directed against the N terminus of the ectodomain, have a modest capacity to induce AChR clustering (39). An alternative possibility is that the longer incubation time with the MuSK antibodies used in the study of Hoch et al. caused the reverse effect by downregulating scaffold molecules required for AChR clustering. The effects on spontaneous clustering of MuSK antibodies reported by Hoch et al. were observed after 5 hours of incubation, whereas our analysis was carried out after a 16-hour incubation with the antibodies (2).

Neither the receptor responsible for nor the activation mechanism of AChR clustering induced on myotubes by laminin-1 is known. However, the signaling mechanisms of agrin and laminin-1 converge at a site downstream from MuSK (19, 35). Another agrin-independent stimulus, VVA-B4, recognizes terminal β-linked GalNAc residues and binds at the NMJ (33). Moreover, even in the absence of rapsyn-AChR complexes, agrin induces MuSK-based scaffolding, including the provision of binding sites for VVA-B4 on myotubes (12). Thus VVA-B4 receptors acting in concert with MuSK appear to play a key role in AChR clustering (12, 44). However, MuSK is not a receptor for VVA-B4, as demonstrated by the absence of O-linked carbohydrates providing O-linked GalNAc residues in MuSK molecules (45). Neuraminidase treatment also induces AChR clustering on myotubes by its action on terminal β-linked GalNAc residues (34). By eliminating sialic acid from glycoconjugates in the membranes, neuraminidase increases the exposure of terminal β-linked GalNAc residues that bind to the GalNAc-specific lectin VVA. We believe our observation that MuSK-specific antibodies strongly inhibited AChR clustering induced by all agrin-independent pathways as well as by agrin to be unprecedented.

Current knowledge offers several possible mechanisms for the global inhibition of this signaling pathway. Prior to innervation, MuSK is essential for organizing a primary synaptic scaffold and initiating postsynaptic membrane maturation events (11, 12, 16). These mechanisms may also be necessary for the maintenance of AChR clusters at mature NMJs, where AChR clusters undergo rapid remodeling (29, 30). Therefore, antibody to MuSK could directly prevent scaffold formation, which may be requisite for AChR clustering taking place in mature NMJs, as shown by RNA interference experiments (31). For example, the interaction of MuSK and rapsyn-AChR complexes is required for AChR clustering by agrin (11, 46). A second possible mechanism governing the inhibition of AChR clustering is an antibody-induced decrease of MuSK expression at the cell surface, which might also prevent scaffolding. In addition, the antibody could act to internalize other associated components required for agrin-dependent or agrin-independent AChR clustering. Through either mechanism, a subset of MuSK antibodies could interfere with AChR clustering even when stimulated by a different subset of MuSK antibodies and subsequently cause the extrajunctional spread of AChRs at mature NMJs.

Finally, the ability to block AChR clustering is not the sole function of MuSK antibodies, since sera containing MuSK antibodies obtained from patients with MG also inhibited agrin-dependent and agrin-independent AChR clustering on myotubes (our unpublished results). The experimental model developed here (Figure 8) is based on the use of MuSK antibodies derived from an animal whose autoimmune response impaired neuromuscular transmission. This model will facilitate further progress in resolving the pathogenic basis of MG at the molecular level and identifying other aspects of pathogenicity available to these autoantibodies in vivo.

Methods

Isolation of MuSK cDNA clones. Mouse MuSK cDNA clones were isolated by constructing a mouse C2C12 myotube cDNA library in the λ ZAPII vector (Stratagene) (47). The library was screened with a human MuSK cDNA probe amplified by reverse transcription PCR, together with a set of primers and total RNA from human muscle (Stratagene). The following primers were designed based on the sequence of the human MuSK cDNA:

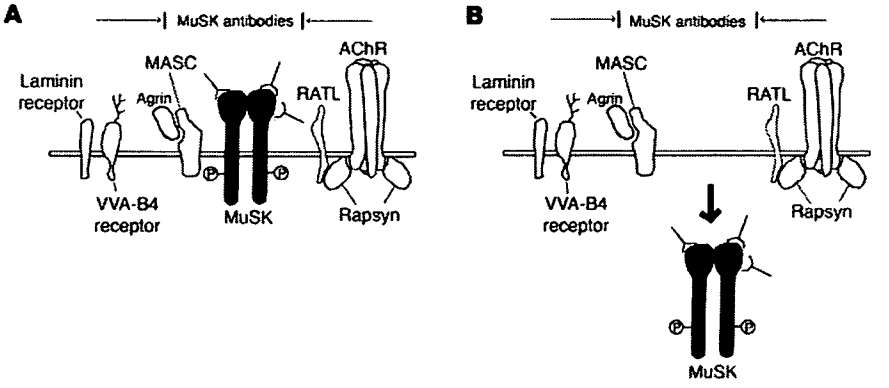

Figure 8

Models of MuSK antibody inhibition of AChR clustering on myotubes. (**A**) MuSK antibodies cover the ectodomain of MuSK and inhibit interactions with other scaffolding molecules required for AChR clustering, including myotube-associated component (MASC), rapsyn-associated transmembrane linker (RATL), and receptors for VVA-B4 and laminin. MASC is a hypothetical molecule that is required for agrin binding and the activation of MuSK. RATL, also a hypothetical molecule, mediates the association of rapsyn with the extracellular domain of MuSK. (**B**) MuSK antibodies induce the internalization of MuSK in the presence or absence of associated molecules. The lack of MuSK on the surfaces of myotubes inhibits the effects of scaffolding and AChR clustering.

5′-GTTCTCCAGAAGGAACTTCGTCCTGC-3′ and 5′-CCGTGCAGCGCAGTAAATGCCATCATC-3′ (25). Sequences were identified using the ABI 310 DNA sequencer and BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequence analysis was carried out using the GCG package of the Human Genome Center, Institute of Medical Science of the University of Tokyo.

Recombinant proteins. A MuSK-AP fusion protein was produced by amplifying the MuSK cDNA from nucleotide 2 in the 5′ untranslated region to nucleotide 1337 (GenBank/EMBL accession number AY360453) using PCR, adding terminal *Bsp*EI sites, and inserting the construct into the *Bsp*EI site of the APtag-2 vector (48). The fusion protein expression construct, which consisted of the MuSK ectodomain and the Fc region of human IgG1, was generated with the same strategy used for the alkaline phosphatase construct. The MuSK cDNA from nucleotides 2 to 1337 was amplified by PCR; an artificial splicing donor signal (GTGAGT) was added at the 3′ terminus of the cDNA, and the construct was inserted between the *Sal*I and *Spe*I sites of the vector pEF-Fc (gift from Y. Yoshihara, RIKEN Brain Science Institute, Wako, Saitama, Japan). We generated expression vectors carrying the secreted, soluble form of mouse neural agrin (C-Ag4,19; y = 4, Z = 19), using PCR to amplify the 1.5-kb C-terminal half of an alternative isoform of mouse agrin cDNA, which contains 19 amino acids at the z site, and using the first-strand cDNA of the mouse spinal cord (49). The following primers were designed based on the sequence of mouse agrin: 5′-GGGGATCCTGGCCGCTTTGGCCCAACTTGTGCAGATG-3′ and 5′-GCTCTAGAGAGAGTGGGGCAGGGTCTTAGCTC-3′ (50).

The amplified agrin cDNA, with added terminal *Bam*HI and *Xba*I sites, was inserted between the *Bam*HI and *Xba*I sites of the pSecTag vector (Invitrogen Corp.), which contains the ER signal sequence of the mouse Igκ gene (47) and a myc/his-tag sequence at the 3′ site. COS-7 cells were transfected by using Fugene-6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. The secreted recombinant agrin (C-Ag4,19) was concentrated with centrifugal filter devices (Millipore), and the secreted recombinant MuSK-AP and MuSK-Fc proteins were purified by using alkaline phosphatase monoclonal antibodies coupled to agarose (Sigma-Aldrich) and protein A-Sepharose (Amersham Biosciences), respectively, by standard methods (48). Agrin protein was purified from the supernatant of transfected COS-7 cells in a Sephadex G-200 column (Amersham Biosciences). Peak fractions that contained agrin were identified by Western blot analysis with anti-myc antibodies (Invitrogen Corp.), then pooled and concentrated. The purity of the recombinant proteins was determined by SDS-PAGE with silver staining. The concentration of recombinant proteins was determined by using the BCA Protein Assay kit (Pierce Biotechnology) with BSA as the standard. The relative band intensities for MuSK-AP, MuSK-Fc, agrin (z = 19), and known amounts of BSA were compared on Coomassie-stained gels as an independent measure.

Animal experiments and antibodies. The Ehime University Animal Care and Use Committee approved all of the procedures used in the animal experiments. Antiserum against the MuSK ectodomain (the MuSK antibodies) was raised by repeatedly injecting New Zealand White rabbits with 100–400 μg of protein A-Sepharose–purified MuSK-Fc. Three of the 4 injected rabbits manifested fatigable muscle weakness after 3 rounds of immunization. One rabbit developed the symptoms after the fourth round of immunization. Electromyograms were recorded from a paretic rabbit and a normal rabbit. The retroauricular branch of the facial nerve was stimulated at 20 Hz, and records were taken from the retroauricular muscle using Synax 1100 (NEC). Polyclonal antisera against the C terminus of the intracellular domain of the MuSK protein were obtained from rabbits injected with a synthetic peptide (CSIHRILQRMCERAEGTVGV). These rabbits remained healthy even after immunization. The specificity of the antiserum was confirmed by Western blot analysis using a lysate of COS-7 cells that were transfected with an expression construct of receptor-type MuSK cDNA. The IgG fraction of the MuSK antiserum was purified by standard methods using protein A-Sepharose (Amersham Biosciences). The concentration of purified IgG was determined with the BCA Protein Assay Kit (Pierce Biotechnology). Normal and paretic rabbits were anesthetized with sodium pentobarbital (Abbott Laboratories; 60 mg/kg, i.p.) and perfused transcardially with 100 ml of saline, followed by 800 ml of a fixative solution that contained 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the middle portions of the soleus muscles were removed and immersed in the same fixative at 4°C for 24 hours. Cross sections from the soleus muscles of paretic and normal rabbits were stained with H&E for the histological studies and stained with 10 nM rhodamine-conjugated BTX (Invitrogen Corp.) for semi-quantitative measurement of the size and density of AChR clustering at NMJs. AChR clustering was examined at ×400 magnification using a fluorescence microscope (Olympus), and representative images of AChR clusters in random fields were taken with a digital camera (DP70; Olympus). With the unprocessed images, the area and OD of AChR clustering were measured using the Density Slice Command in NIH image analysis software (version 1.62; http://rsb.info.nih.gov/nih-image).

Muscle cell culture. The C2C12 cell line was obtained from the American Type Culture Collection and used for 3–5 passages. Cells were cultured in 6-well plates as myoblasts in DMEM that contained 10% FCS. When the cells reached confluence, myotube formation was induced by replacing the medium with DMEM that contained 2% horse serum. The cells were incubated for 3–4 days, with fresh medium exchange every day, until full differentiation into multinucleated myotubes was observed morphologically.

AChR clustering assay and immunocytochemical staining. AChR clustering was assayed by growing C2C12 myotubes in 6-well plates and then treating them for 16 hours with 1 nM agrin (C-Ag4,19), 40 nM laminin-1 (Kouken), 25 μg/ml VVA-B4 (Sigma-Aldrich), or 0.1 U/ml *Clostridium perfringens* neuraminidase (Sigma-Aldrich) with or without 10 μg/ml purified MuSK antibodies. The optimal dose of each inducer or MuSK antibodies required for maximal AChR clustering or inhibition, respectively, was determined. Inhibition was blocked by absorption of MuSK antibodies with 6.5 nM MuSK-AP before the cells were treated. The optimal dose of MuSK-AP for neutralization of the MuSK antibodies in the inhibition of AChR clustering assay was determined by serially diluting MuSK-AP protein premixed with antibodies at room temperature, followed by treating the C2C12 myotubes with the mixture. AChR clustering was visualized after incubation with 40 nM rhodamine-conjugated BTX (Invitrogen Corp.) in fusion medium for 1 hour at 37°C before fixation. The cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. MuSK on the myotubes was then stained using the MuSK antibodies. The myotubes were incubated with the antibodies and rhodamine-conjugated BTX for 1 hour at 37°C, rinsed, and treated with fluorescein-conjugated goat anti-mouse antibodies (BioSource International) for 1 hour at 37°C. The cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and then mounted using the SlowFade Antifade Kit (Invitrogen Corp.). The myotubes were examined at ×200 magnification using a fluorescence microscope (Olympus), and photographs of representative images of AChR clusters in random fields were taken using PRESTO NEOPAN400 (Fuji Photo Film Co. Ltd.). The numbers of AChR clusters in the photographs were then counted.

Precipitation assay and immunoblot analysis. Tyrosine phosphorylation of MuSK and AChRs induced by agrin or the antibodies was analyzed by treating C2C12 myotubes with either 1 nM agrin or 1 μg MuSK antibodies (purified by protein A-Sepharose) for 30 minutes with or without 3 nM MuSK-AP. As a control, cells were treated for 30 minutes with antibodies against the C terminus of the intracellular domain of MuSK (purified by protein A-Sepharose) at a concentration of 1 μg/ml. Cultured cells were lysed and solubilized in a protease inhibitor cocktail (Complete EDTA-free; Roche Molecular Biochemicals) with 2 mM sodium orthovanadate. The extracts were immunoprecipitated with antibodies to MuSK, and the resulting precipitates were immunoblotted with a mixture of the anti-phosphotyrosine antibodies 4G10 (Upstate USA Inc.) and PY20 (Chemicon International). MuSK was detected by stripping the blots and then reprobing them with the MuSK antibodies. Tyrosine-phosphorylated AChRs were detected by treating the cells with 120 nM biotinylated BTX (Invitrogen Corp.) for 1 hour at 4°C. The cells were then washed with cold phosphate-buffered saline, followed by lysis and precipitation with streptavidin-coupled agarose beads (Sigma-Aldrich). Phosphotyrosine immunoblotting was performed as described above. AChRs were detected by stripping the blots and then reprobing them for the AChR β subunit using a monoclonal antibody (mAb 124; kindly provided by J.M. Lindstrom, University of Pennsylvania, Philadelphia, Pennsylvania, USA) (51).

Statistics. ANOVA was used to analyze the significance of differences between experimental groups. *P* values less than 0.01 were considered statistically significant.

Acknowledgments

We thank 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, and a grant from the Health Science Research Grants for Comprehensive Research on Aging and Health from the Ministry of Health, Labor, and Welfare, Japan. We are also grateful to the staff of the Integrated Center for Science of Ehime University for assistance with the animal care and sequence analysis.

Received for publication March 10, 2004, and accepted in revised form February 7, 2006.

Address correspondence to: Kazuhiro Shigemoto, Department of Preventive Medicine, Ehime University School of Medicine, Sitsukawa, To-on city, Ehime 791-0295, Japan. Phone: 81-89-960-5278; Fax: 81-89-960-5279; E-mail: shigemot@m.ehime-u.ac.jp.

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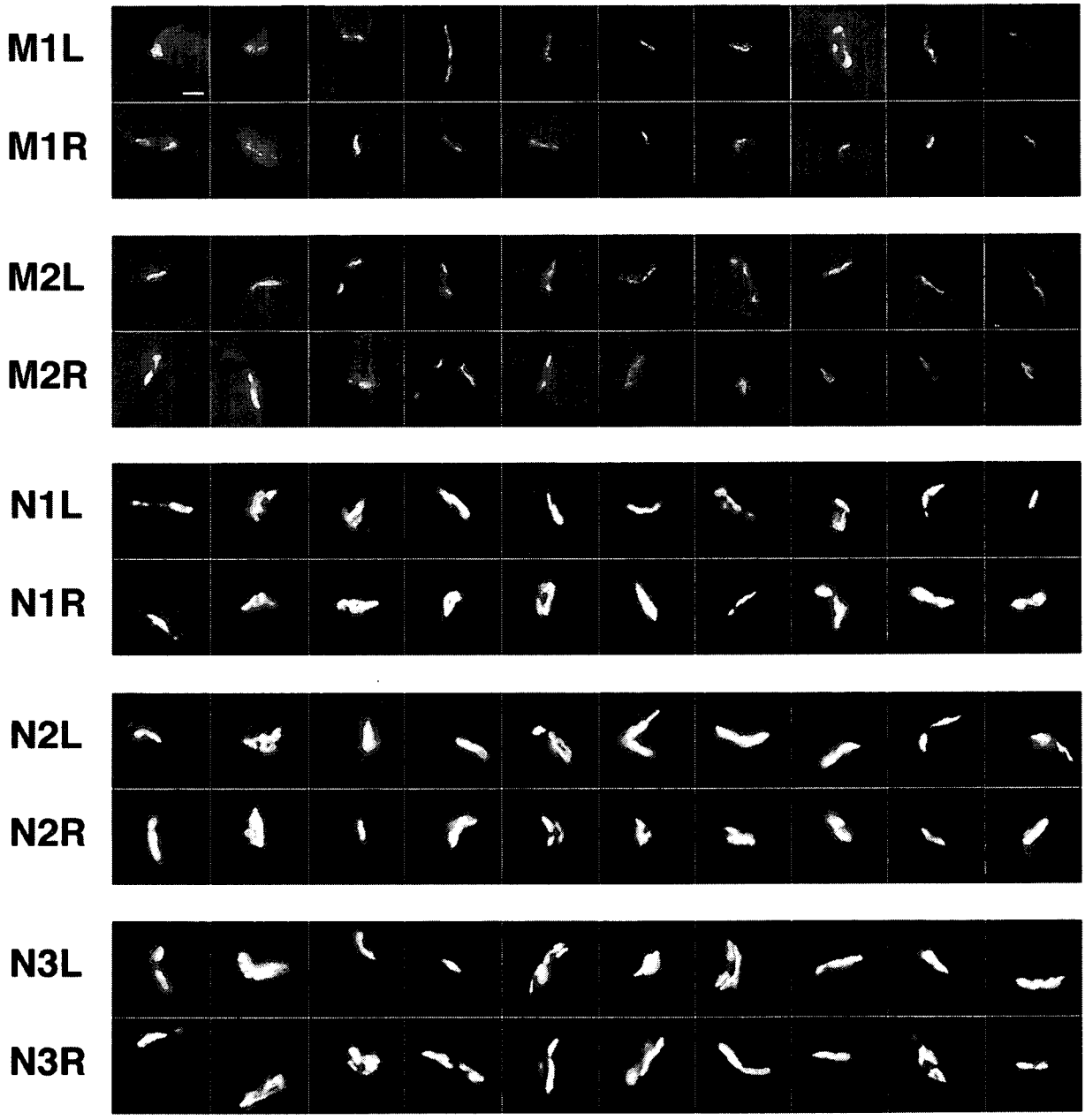
M1L
M1R
M2L
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N1L
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Supplementary Movie 1 and 2

M1 and M2 paretic rabbits manifested myasthenic weakness after immunization with recombinant MuSK protein were recorded in Movie 1 and Movie 2 respectively. M2 paretic rabbit manifested severe exhaustion with muscle weakness.

Supplementary Figure 1

Ten AChR clusters of NMJ randomly stained with 10 nM rhodamine-conjugated BTX chosen from the left and ten from the right soleus muscles in the paretic (M1 and M2) and normal (N1, N2 and N3) rabbits. These unprocessed digitized images were analyzed using NIH image analysis software. Scale bar, 10 μm.

European Journal of Neurology 2007, 14: 1029–1034 doi:10.1111/j.1468-1331.2007.01870.x

Clinical and experimental features of MuSK antibody positive MG in Japan

K. Ohta[a], K. Shigemoto[b], A. Fujinami[c], N. Maruyama[d], T. Konishi[e] and M. Ohta[a,c]

[a]*Clinical Research Center, Utano National Hospital, Kyoto, Japan;* [b]*Department of Preventive Medicine, Faculty of Medicine, Graduate School of Ehime University, Ehime, Japan;* [c]*Department of Medical Biochemistry, Kobe Pharmaceutical University, Kobe, Japan;* [d]*Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan; and* [e]*Department of Neurology, Utano National Hospital, Kyoto, Japan*

Keywords:
clinical features, domain, IgG subclass, muscle specific tyrosine kinase, MuSK antibody, myasthenia gravis, seronegative myasthenia gravis

Received 28 December 2006
Accepted 7 May 2007

We investigated the presence of antibodies (Abs) against muscle-specific tyrosine kinase (MuSK) in Japanese myasthenia gravis (MG) patients. MuSK Abs were found in 23 (27%) of 85 generalized seronegative MG (SNMG) patients but not in any of the ocular MG patients. MuSK Ab-positive patients were characterized as having female dominance (M:F, 5:18), age range at onset 18 to 72 (median 45) years old, and prominent oculobulbar symptoms (100%) with neck (57%) or respiratory (35%) muscle weakness. Limb muscle weakness was comparatively less severe (52%), thymoma absent. Most patients had good responses to simple plasma exchange and steroid therapy. MuSK IgG from all 18 patients was exclusively the IgG 4 subclass and bound mainly with the MuSK Ig 1–2 domain. Serial studies of 12 individuals showed a close correlation between the variation in MuSK Ab titers and MG clinical severity ($P = 0.01$ by Kruskal–Wallis). MuSK Ab titers were sharply decreased in patients who had a good response to early steroid therapy or simple plasma exchange, but there was no change, or a rapid increase on exacerbation after thymectomy. Measurement of MuSK Ab titers aids in the diagnosis of MG and the monitoring of clinical courses after treatment.

Introduction

Muscular weakness in most patients with myasthenia gravis (MG) is caused by an antibody (Ab)-mediated autoimmune response to muscle nicotinic acetylcholine receptors (AChRs), but there is no correlation between the AChR Ab level and degree of muscle weakness. This may be because of AChR Abs heterogeneity and epitope specificity or the presence of Abs against other functionally important muscle antigens. Fifteen percent of patients with generalized MG who have no detectable circulating Abs to AChR are termed seronegative MG (SNMG). Autoantibodies against muscle-specific tyrosine kinase (MuSK) have been identified in that population [1]. The positivity for MuSK Ab in SNMG patients varied from 3.8% to 71% by studies [1–11], which may be due to geographical or ethnic differences. Immunoglobulin allotypes in Caucasian and Chinese MG patients differ from those in Japanese patients [12]. We performed a MuSK Ab survey of a large number of Japanese MG patients and characterized the clinical features of those who were MuSK Ab positive. Furthermore, we investigated the correlation between MuSK Ab titer and disease severity, epitope specificity, and the IgG subclass of MuSK IgG.

Correspondence: Mitsuhiro Ohta, Department of Medical Biochemistry, Kobe Pharmaceutical University, Motoyamakita, Higashinada-ku, Kobe 658–8558, Japan (tel.: +81 78 441 7557; fax: +81 78 441 7559; e-mail: mohta@kobepharma-u.ac.jp).

Patients and methods

Patients

We studied 85 patients (27 men, 58 women, mean age 56 years old, range 18–76 years) who had generalized SNMG and were consistently negative for serum AChR Abs, as well as 272 AChR Ab-positive MG (SPMG) patients (87 men, 185 women, mean age 54 years old; age range 32–74 years); 50 with and 222 without thymoma. The control populations comprised 70 healthy participants (29 men, 41 women; mean age 50 years old, range 27–74 years) and 91 patients (37 men, 54 women; mean age 50 years old, range 32–74 years) with other neurological or immunological diseases (five Lambert-Eaton myasthenic syndrome, six polymyositis, 10 muscular dystrophy, 15 thyroiditis, 10 type 1 diabetes mellitus, five rheumatoid arthritis, 10 multiple sclerosis, five spinal progressive muscular atrophy, five chronic inflammatory demyelinating polyneuropathy, 10 amyotrophic lateral sclerosis, and 10 epilepsy). The study was approved by the ethics committee of Utano National Hospital. All persons gave their informed consent prior to their inclusion in the study.

Preparation of recombinant human MuSK protein

To produce his-tag human MuSK protein, the entire extracellular domain (MuSK 1–4; nucleotides 107–1526,

GenBank/EMBL accession number AF006464) of human MuSK, and MuSK fragments comprised of the first half bearing two Ig-domains (MuSK 1–2; nucleotides 107–700) were linked to the PCR3.1/Myc-His vector (Invitrogen Corporation, Carlsbad, CA, USA) [13]. Membrane-proximal extracellular domains, including Ig-domains 3 and 4 (MuSK 3–4; nucleotides 701–1526), were linked to the pSecTag-His vector (Invitrogen) carrying the ER signal sequence of the mouse Igκ gene. All constructs were transiently transfected to COS7 cells [14]. The recombinant his-tag MuSK secreted was purified in a histidine-affinity column (Clonetech Laboratories, Palo Alto, CA, USA). Recombinant protein purity was determined by SDS-PAGE with silver staining. Recombinant protein concentrations were obtained with a BCA Protein assay kit (Pierce, Biotechnology, Inc., Rockfold, IL, USA) with bovine serum albumin as the standard. The MuSK extracellular domain and MuSK fragments then were labeled with ^{125}I [15].

Detection of MuSK Ab by radioimmunoprecipitation assay

All the sera underwent a radioimmunoassay (RIA) to determine the presence of MuSK Ab. In brief, 5 μl of each sample was incubated overnight at 4°C with 50 μl of ^{125}I-his-tag MuSK (40 000 cpm), after which 50 μl of anti-human IgG was added, and the sample incubated for another 2 h at room temperature. Radioactivity was counted after two washes of the pellets with saline. All positive sera were titrated, and results expressed as nanomoles of ^{125}I-MuSK precipitated per liter of serum.

Epitope mapping

Muscle-specific tyrosine kinase Ab-positive sera were tested by an RIA for the presence of IgG Abs to MuSK 1–2 or MuSK 3–4. In brief, 5 μl of each sample was incubated overnight at 4°C with ^{125}I-his-tag MuSK (40 000 cpm), ^{125}I-his-tag MuSK 1–2 (30 000 cpm), or ^{125}I-his-tag MuSK 3–4 (30 000 cpm), after which 50 μl of anti-human IgG was added. The samples then were incubated for another 2 h at room temperature. Radioactivity was counted after two washes of the pellets with saline.

IgG subclasses of MuSK Ab

Microtiter plates (Breakapart plate, Nunk-Immuno Module, Roskilde, Denmark) were coated with 100 μl of 10 μg/ml of each Ab to IgG subclasses (sheep polyclonal anti-human IgG1, 2, 3 and 4; Binding Site, Birmingham, UK) diluted with 10 mM sodium carbonate-bicarbonate buffer, pH 9.3 and kept for 1 h at room temperature. Nonspecific binding sites were saturated with 200 μl PBS containing 5% skimmed milk and 10% Blockace (Dainippon Seiyaku, Osaka, Japan) for 2 h at room temperature. A serum sample (20 μl), first incubated for 2 h at room temperature with ^{125}I-MuSK (30 000 cpm), was added to a plate, and the whole incubated for 2 h at room temperature. After four washes, ^{125}I was counted in each well.

Statistical analysis

Statistical analysis was performed by regression analysis, Kruskal–Wallis, one-way analysis of variance, and Student *t* test. A *P*-value of <0.05 was considered significant.

Results

MuSK Abs

The cut-off value (0.01 nM) was calculated from the mean + 3SD of the healthy subjects' values obtained by an RIA constructed with ^{125}I-MuSK extracellular domains. MuSK Ab was present in 23 (27%) of the 85 SNMG patients but not in any of the 272 SPMG patients, healthy subjects and patients with other neurological or immunological diseases (Fig. 1). Ab-positive samples were confirmed by serial dilution tests, and titers shown as nanomoles of ^{125}I-MuSK precipitated per liter of serum. MuSK Ab titers ranged from 8.4 to 240 nM (median, 57 nM). All the positive serum samples had extremely high titers on ^{125}I-human MuSK immunoprecipitation.

Clinical features of patients with MuSK Abs

Table 1 shows the clinical features of 23 MuSK Ab-positive patients. MuSK Ab in generalized SNMG showed female predominance (five men, 18 women) but not in ocular MG. Age at onset ranged from 18 to 72 years old (median 45 years). Clinical features of MuSK Ab-positive patients were confined to ocular [ptosis, 13/23 (57%) and double vision, 18/23 (78%)]; bulbar [dysphagia: 23/23 (100%), dysarthria: 19/23 (83%)]; neck extensor, 13/23 (57%); respiratory 8/23 (35%) muscle weaknesses. Prevailing weaknesses affected the oculobulbar and respiratory muscles of MuSK Ab-positive patients. About 48% (11/23) had no limb weakness. No thymomas were detected by CT. Six (26%) of the 23 MuSK Ab-positive patients who were thymectomized, had histological abnormalities including small hyperplastic features.

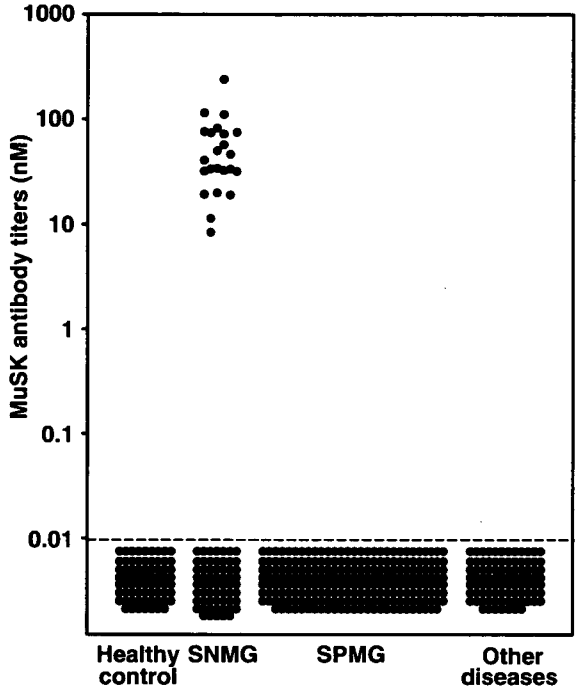

Figure 1 RIA-detected MuSK Ab titers of 85 patients with SNMG, 272 patients with SPMG, 91 patients with other neurological or immunological diseases, and 70 healthy participants. Broken line, the cutoff (0.01 nM) for MuSK Abs.

Table 1 Clinical features of MuSK Ab-positive patients

MuSK Ab positivity in SNMG	23/85 (27%)
MuSK Ab titers	8.4–239 (median 57 nM)
F:M	18:5
Age at onset	18–72 years (median 45 years)
Distribution of weakness	
Ptosis	13/23 (57%)
Ocular motor dysfunction	18/23 (78%)
Bulbar	23/23 (100%)
Neck	13/23 (57%)
Respiratory (crises)	8/23 (35%)
Limb	12/23 (52%)
Thymus	
Thymoma	0/23 (0%)
Hyperplasia	6/23 (26%)

Serial studies of clinical status and MuSK Abs

We measured MuSK Ab titer serially during the disease's course. Table 2 shows anti-MuSK Ab titers in relation to disease severity and duration, and immunosuppressive treatment (A), plasma exchange (B), or thymectomy (C). Disease severity was graded according to the Myasthenia Gravis Foundation of America (MGFA) classification [16] at the onset of myasthenic symptoms, in the maximally deteriorated state, and at

Table 2 Changes in MuSK Ab titers and in clinical status in MuSK Ab-positive patients

Case	Gender	Age at onset (years)	Duration (days)	MuSK Ab (nM)	MGFA classification	treatment
(A) Early steroid therapy						
P-1	F	18	0	39.3	IIIb	
			56	39.0		Pred
			82	40.2		Pred
			138	38.0		Pred
			175	35.0	IIb	Pred
			313	33.0	PR	Pred
			577	21.0		Pred
P-2	F	32	0	113.0	IVb	Pred
			141	17.0		Pred
			261	15.0	IIb	Pred
			409	16.0		Pred
P-3	F	48	0	80.0	IVb	Pred
			46	28.0		Pred
			101	5.0		Pred
			1,641	4.2	PR	Pred
P-4	F	53	0	36.8	IIIb	
			41	31.0		Pred
			97	15.2		Pred
			111	10.0	IIb	Pred
P-5	F	52	0	240.0	V	Pred
			49	57.0		Pred
			77	22.9		Pred
			101	8.4		Pred
			129	3.0	IIb	Pred
P-6	F	76	0	33.0	IIb	Pred
			83	0.5		Pred
			118	0.2	PR	Pred
(B) Simple plasma exchange (PE)						
P-7	M	53	0	74.4	V	
			42	59.0		
			52	47.0		
			PE ⟶			
			62	28.5	IIb	
			PE ⟶			
			67	17.2		
			125	16.0		Pred
			132	11.5		Pred
			138	9.0	IIb	Pred
P-8	M	71	0	113.9	IVb	
			PE ⟶			
			11	32.1		
			45	32.0		Pred
			219	31.0		Pred
			616	28.0	IIb	Pred
P-9	F	66	0	30.0	IIIb	
			45	40.5		
			361	32.0		
			PE ⟶			
			374	14.0	IIb	
			379	21.2		
			389	29.9		
			403	35.5	IIIb	Pred, Cyclo
			441	25.0		Pred, Cyclo
			476	20.5	IIb	Pred, Cyclo

Table 2 (Continued)

(C) Thymectomy (Tx)						
P-10	F	47	0	20.0		
			47	26.0	IIb	
			Tx ——→			
			95	47.2	IIIb	Pred
			270	19.8	IIb	Pred
P-11	F	52	0	22.6	IIb	
			58	23.2		
			Tx ——→			
			170	21.1		Pred
			255	25.0	IIb	
P-12	F	48	0	16.5	IIb	
			95	17.6		
			Tx ——→			
			210	15.7		Pred
			274	17.0	IIb	

PR, Pharmacological Remission; Pred, Predonisolone; Cyclo, Cyclosporine.

the last clinic visit after or during treatment. As shown in Table 2a, six patients (P1–P6) who underwent early steroid therapy showed impressive clinical improvement associated with a sharp decrease in anti-MuSK Ab titer; from 39.3 to 21.0 nM (P-1), 113.0 to 16.0 nM (P-2), 80.0 to 4.2 nM (P-3), 36.8 to 10.0 nM (P-4), 240.0 to 3.0 nM (P-5), and 33.0 to 0.2 nM (P-6). MG severities showed clinical improvement from class IIIb to pharmacological remission (PR) (P-1), class IVb to IIb (P-2), class IVb to PR (P-3), class IIIb to IIb (P-4), class V to IIb (P-5), and class IIb to PR (P-6).

Muscle-specific tyrosine kinase Ab titers of three patients were measured in serial samples taken before and after simple plasma exchange (Table 2b). The patients responded dramatically to that therapy, Ab titers decreasing from 74.4 to 9.0 nM (P-7), 113.9 to 28.0 nM (P-8), and from 30.0 to 20.5 nM (P-9), indicative of clinical improvement from class V to IIb (P-7), class IVb to IIb (P-8), and class IIIb to IIb (P-9). Moreover, conventional immunosuppression maintained the clinical improvement initially achieved by plasma exchange. In one patient (P-9), the effect had tapered off 45 days after plasma exchange, and Ab titer and disease severity returned to the level before treatment. Prednisolone and cyclosporin administered after MG relapse resulted in slower improvement.

Three patients who had histological abnormalities, including a small hyperplastic thymus, underwent thymectomies (Table 2c). After surgery one patient (P-10) immediately had worsening of dysphagia from class IIb to IIIb associated with a rapid increase in MuSK Ab titer from 26.0 to 47.2 nM. Thymectomy was not effective for the other two patients (P-11, P-12) who showed no change in disease severity and MuSK Ab titer.

We analyzed MuSK Ab titers in relation to quantitative clinical scores on the MGFA scale in serial studies of 12 individuals. MuSK Ab titers and disease severity were correlated ($P = 0.01$ by Kruskal–Wallis).

Epitopes in the extracellular domains of human MuSK

Eighteen sera with MuSK Abs were examined for ^{125}I-MuSK 1–2 and ^{125}I-MuSK 3–4 binding. All predominantly bound to ^{125}I-MuSK 1–2, range 68–97%. Only five of the 18 sera also showed slight binding (20–30%) to ^{125}I-MuSK 3–4 (Table 3).

IgG subclasses of MuSK Abs

In a solid phase RIA with sheep polyclonal antibodies to human IgG subclasses, in all the 18 sera tested MuSK Abs were exclusively IgG4 (Table 4).

Discussion

The MuSK Ab-positive rate found for generalized SNMG patients in Japan was 27% with female predominance (M:F = 5:18). This rate is lower than the 70% positivity originally reported [1] and the 40–50% recently reported [2–7]. It is consistent with the 27–33% reported for Japanese and Korean population [8–10] but significantly higher than the 3.8% Chinese positivity rate [11]. Age at onset ranged from 18 to 72 years old (median, 45 years); 61% of the patients presenting at >40 years of age, later than for Caucasians, and 57–71% of patients presenting at <40 years of age, but the differences was not significant [3,7,17,18].

Table 3 Ratio of MuSK Ig 1–2 and 3–4 Ab in MuSK 1–4 Ab titers

Case	MuSK Ab (nM)	Ig 1–2 domain (%)	Ig 3–4 domain(%)
1	8.4	97.1	2.9
2	19.3	68.1	31.9
3	32.5	81.2	18.8
4	50.0	82.7	17.3
5	33.4	97.3	2.7
6	31.8	95.7	4.3
7	19.9	91.0	9.0
8	11.4	95.2	4.8
9	40.7	87.9	12.1
10	32.0	91.0	9.0
11	33.6	95.0	5.0
12	74.7	95.4	4.6
13	72.0	76.2	23.8
14	46.2	80.8	19.2
15	74.7	96.9	3.1
16	82.2	74.2	25.8
17	110.7	89.5	10.5
18	114.6	71.2	28.8

Table 4 Ratio of IgG subclasses of MuSK Abs

Case	MuSK Ab (nM)	IgG 1 (%)	IgG 2 (%)	IgG 3 (%)	IgG 4 (%)
1	114.6	0.0	0.0	0.0	100.0
2	110.7	0.0	0.0	4.9	95.1
3	82.2	0.0	0.0	0.0	100.0
4	74.7	11.0	21.0	19.0	49.0
5	74.0	4.1	5.0	5.6	85.3
6	72.0	0.0	1.0	0.0	99.0
7	46.2	0.0	0.0	0.0	100.0
8	40.7	5.3	0.0	7.1	87.6
9	33.6	15.1	15.7	0.0	69.2
10	33.4	0.0	0.0	0.0	100.0
11	32.5	5.3	0.0	0.0	94.7
12	32.0	4.2	2.6	1.2	92.0
13	31.8	6.7	6.7	8.3	78.3
14	19.9	0.0	0.0	1.7	98.3
15	19.5	0.0	0.0	0.0	100.0
16	19.3	0.0	0.0	0.0	100.0
17	11.4	0.0	28.9	26.1	45.0
18	8.4	0.0	0.0	0.0	100.0

All the Ab-positive patients had similar patterns of muscle weakness, with prevalent involvement of the bulbar muscles in 100%, ocular symptoms (blepharoptosis and/or double vision) in 80%, and of the respiratory muscles in 35% with frequent myasthenic crises. Limb muscle involvement was comparatively less severe and inconsistent. Japanese MuSK Ab-positive patients therefore have clinical features similar in terms of the predominance of bulbar involvement to those reported for Caucasians.

We evaluated the correlation between MuSK Ab titers and disease severity. Table 2 shows patients who had a good response to early immunosuppressive therapy or simple plasma exchange. Their MuSK Ab titers sharply decreased in parallel with clinical improvement, whereas their Ab titers remained positive. We evaluated the effect of thymectomy in three individuals by measuring MuSK Ab titers in serum samples taken pre- and post-thymectomy. One patient's condition deteriorated after thymectomy and her Ab titer greatly increased. The two others showed neither progression nor Ab titer change during the observation period. Thymectomy therefore did not produce good results. Histological changes in the thymus of MuSK Ab-positive subjects are reported to be minimal and to include rare small germinal centers [19,20] in contrast to SPMG patients who had lymph node-type infiltrates. These findings, together with the lack of benefit of thymectomy, are evidence against a role for the thymus in antigen presentation and antibody production.

Serial studies showed a statistically close correlation between MuSK Ab titers and disease severity. MuSK Ab titers also recently were found to correlate with MG severity [21]. MuSK Ab titers were extremely high in all the positive cases (Fig. 1). The close relationship between clinical status and MuSK Ab, found by monitoring Ab titers, suggests that MuSK Ab has a significant pathogenic role in MG patients. Circulating MuSK Abs, however, are reported not to cause a MuSK or AChR deficiency at the endplates [22]. Recent experimental models (rabbits [13] and mice [23]), developed by immunization with recombinant MuSK ectodomain protein, produced MG-like muscle weakness with reduced AChR clustering at neuromuscular junctions. These findings clarified the pathogenic MG mechanisms produced by MuSK Ab.

The paramount MuSK Ab IgG subclass in our eighteen patients was IgG4. Limb and intercostal muscle biopsies found neither reduction in AChR numbers nor complement deposition [9,24]. The absence of complement deposits at a patient's end plates is explained by the fact that MuSK Ab is mainly IgG class 4 which does not fix complement [5,25]. The MuSK extracellular domain consists of four MuSK immunoglobulin-like (Ig) domains. Binding analysis of MuSK Abs to ^{125}I-MuSK Ig 1–2 or ^{125}I-MuSK Ig 3–4 showed that the eighteen sera tested predominantly bound to the ^{125}I-MuSK Ig 1–2 domain. The epitope was the N-terminal of the extracellular domain of human MuSK as described previously [5]. Furthermore, MuSK Abs have been shown to inhibit agrin-induced clustering of AChRs [26]. In fact, MuSK Ig 1–2 domains are more responsible for agrin responsiveness of MuSK, in contrast to Ig 3-4 domains which are more responsible for rapsyn association. We postulate that this is relevant to our findings of predominant binding analysis to MuSK Ig 1–2. The characteristics of the MuSK IgG subclass and Ab binding epitope in Japanese patients therefore are similar to those of Caucasians.

Muscle-specific tyrosine kinase Ab-positive patients often suffer facial and tongue muscle atrophy [3,27]. Benveniste *et al.*[28] reported that MuSK Ab plasma may affect the expression of atrophy-related protein and that a facial muscle, the masseter, is the most susceptible. Amongst our MuSK Ab-positive patients, four patients had detectable tongue atrophy from a relatively early phase of illness; weakness was moderate in 2 patients and mild in two patients. More *in vitro* and *in vivo* studies are needed to clarify the pathologic mechanisms that cause the muscle weakness produced by MuSK Ab. MuSK Ab detection provides a valuable biological means of support for the clinical diagnosis of MG and a way to monitor its clinical course.

Acknowledgements

This study was supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and

Technology, Japan, and grants from the High-Tech Research Center, the Kobe Pharmaceutical University Collaboration Fund, and the Science Research Promotion Fund of the Japan Private School Promotion Foundation.

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Anti-alkaline phosphatase antibody positive myasthenia gravis

Tetsuro Konishi [a,*], Kiyoe Ohta [b], Kazuhiro Shigemoto [c], Mitsuhiro Ohta [d]

[a] *Department of Neurology, National Hospital Organization Utano National Hospital, 8, Ondoyama-cho, Narutaki, Ukyo-ku, Kyoto 616-8255, Japan*
[b] *Clinical Research Center, National Hospital Organization Utano National Hospital, Kyoto, Japan*
[c] *Department of Preventive Medicine, Ehime University School of Medicine, Ehime, Japan*
[d] *Department of Medical Biochemistry, Kobe Pharmaceutical University, Kobe, Japan*

Received 17 January 2007; received in revised form 19 April 2007; accepted 8 June 2007
Available online 12 July 2007

Abstract

Anti-alkaline phosphatase antibody (AP Ab) was specific in 9% of 249 anti-acetylcholine receptor (AChR) Ab-positive myasthenia gravis (MG) (SPMG) patients but not in patients with AChR Ab-negative MG (SNMG), other neurological and immunological diseases, or healthy volunteers. No cross-reactivity and no significant titer correlation were found between AP Ab and AChR Ab. We confirmed immunologically by radioimmunoassay and western blot analysis the presence of antibodies directed against AP. AP Ab-positive SPMG patients were characterized clinically as having female predominance and a more severe form of generalized MG than AP Ab-negative SPMG patients, and about half required artificial ventilation at maximum severity. AP Ab's pathogenic role in MG is yet unclarified, but our findings show AP to be a novel antigen among the various autoantigens present in MG patients and in whom AP Ab may modify clinical symptoms.

Keywords: Alkaline phosphatase antibody; AChR antibody; Myasthenia gravis

1. Introduction

Myasthenia gravis (MG), a T-cell-dependent chronic autoimmune disorder, is induced by sustained production of an antibody (Ab) to nicotinic acetylcholine receptor (AChR) at neuromuscular junctions [1]. Unexpectedly, we recently found alkaline phosphatase (AP) Ab in AChR Ab-positive MG (seropositive MG: SPMG) sera in a radioimmunoassay (RIA) for the muscle-specific receptor tyrosine kinase (MuSK) Ab, in which a recombinant MuSK protein was fused with placental AP. AP Ab was detected in 9% (20/229) of the SPMG patients, but was absent in the AChR Ab-negative MG (seronegative MG:SNMG) patients studied [5].

Here, we show immunologically the presence of AP Ab in SPMG and investigate the disease specificity and clinical features of AP Ab-positive patients.

2. Materials and methods

2.1. Subjects

Serum samples were obtained from 319 MG (249 SPMG, 70 SNMG) patients and from 136 patients with other neurological or immunological diseases (10 Lambert–Eaton myasthenic syndrome, 10 polymyositis, 15 multiple sclerosis, 5 spinal progressive muscular atrophy, 5 chronic inflammatory demyelinating polyneuropathy, 28 ALS, 10 epilepsy, 15 progressive muscular dystrophies, 15 thyroiditis, 10 type 1 diabetes mellitus, and 13 rheumatoid arthritis). Positive diagnostic features of MG was confirmed by electromyography (>10% decrement on repetitive nerve stimulation at 3 Hz in a hand or shoulder muscle), and an unequivocally positive response to edrophonium or other anticholinesterase agents.

Abbreviations: AChR, acetylcholine receptor; AP, alkaline phosphatase; MuSK, muscle-specific receptor tyrosine kinase; MG, myasthenia gravis.

* Corresponding author. Tel.: +81 75 461 5121; fax: +81 75 464 0027.
E-mail address: konishi@unh.hosp.go.jp (T. Konishi).

0022-510X/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jns.2007.06.006

MG patients were classified according to the MG Foundation of America (MGFA) [3]. The control population comprised 70 healthy volunteers.

Ninety-nine SPMG patients, whose medical records were detailed, were divided into two groups for comparison; ten with AP Ab-positive MG and eighty-nine AP Ab-negative MG. Clinical features investigated were gender, age at disease onset, disease duration, MGFA clinical classification at maximum severity, thymic pathology, and the use of mechanical ventilation, steroids or other immunosuppressants. The study was approved by the ethics committee of Utano National Hospital. All persons gave their informed consent prior to their inclusion in the study.

2.2. AChR Ab assay

AChR Abs were measured with a standard AChR Ab kit (RSR Limited, Cardiff, UK).

2.3. Human MuSK and AP Ab assay

Recombinant MuSK proteins were constructed from the extracellular domains of human MuSK. AP-tag-MuSK was the fusion protein of MuSK and human placental alkaline phosphatase (AP). AP-MuSK and His-MuSK were secreted from COS 7 cells. These recombinant MuSK (rMuSK) proteins were purified in a nickel affinity column then labeled with ^{125}I. Anti-MuSK Ab were measured by the previously described radioimmunoprecipitation method [5]. AP Ab was detected by an RIA. In brief, 5 μL of each serum sample was incubated with ^{125}I-AP-MuSK (40,000 cpm) overnight at 4 °C, after which 50 μL of anti-human IgG was added, and the sample were incubated for 2 h at room temperature. After two washes of the pellets with saline, radioactivity was counted. AP antibodies were also detected by an RIA using ^{125}I-AP. Human placental AP was purchased (Calzyme Laboratories, CA) and further purified (95% purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) by affinity chromatography on anti-AP monoclonal antibody coupled to agarose (Sigma, St. Louis, MO). AP was assayed in 5 μL of serum samples with ^{125}I-AP (40,000 cpm). Results expressed as nanomoles of ^{125}I-AP-MuSK precipitated per liter of serum. Specific radioactivity of ^{125}I-AP-MuSK was 3100 cpm per pM. Furthermore, the assay was modified to demonstrate inhibition of AP specific counts employing unlabeled AP.

2.4. Western blot analysis

Alkaline phosphatase (AP) (Calzyme Laboratories Inc. California) was separated in 4–20% sodium dodecyl sulfate polyacrylamide gels at a constant current of 40 mA then transferred to a nitrocellulose membrane at 80 V for 1.5 h. After non-occupied protein binding sites had been blocked by 5% skim milk, the membrane was cut into small strips (3 × 70 mm), which were incubated at room temperature for 2 h with the test serum diluted 1:500. The strips were washed four times and again incubated at room temperature for 2 h with horseradish peroxidase-conjugated anti-human IgG (Amersham Biosciences, Tokyo, Japan) diluted 1:5000. After a final wash, the membrane was soaked in chemiluminescence solution containing ECL Western blotting detection reagents (Amersham Biosciences, Tokyo, Japan).

2.5. Statistical analysis

All p-values given were 2-tailed by Fisher's exact test. The frequencies of the MGFA clinical classifications at maximum severity were compared for AP status for trend by the Mann–Whitney U test. $p<0.05$ was considered statistically significant.

3. Results

The AP Ab titer cut-off value (0.01 nM) was calculated from the mean+3SD (0.0025+0.0075) of the values of the

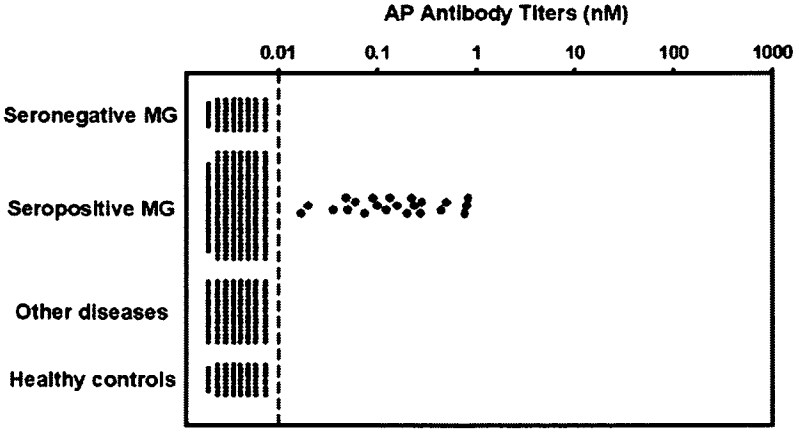

Fig. 1. Alkaline phosphatase antibody titers detected by ^{125}I-human placental AP assays. Titers of 70 patients with seronegative MG, 249 patients with seropositive MG, 136 patients with other neurological and immunological diseases, and 70 healthy volunteers. The dashed line denotes the cut off value (0.01 nM) calculated from the mean+3SD of values for the 70 healthy control participants.