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Animal models of antimuscle specific kinase myasthenia

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Abstract

Antimuscle specific kinase (anti-MuSK) myasthenia (AMM) differs from antiacetylcholine receptor myasthenia gravis in exhibiting more focal muscle involvement (neck, shoulder, facial, and bulbar muscles) with wasting of the involved, primarily axial, muscles. AMM is not associated with thymic hyperplasia and responds poorly to anticholinesterase treatment. Animal models of AMM have been induced in rabbits, mice, and rats by immunization with purified xenogeneic MuSK ectodomain, and by passive transfer of large quantities of purified serum IgG from AMM patients into mice. The models have confirmed the pathogenic role of the MuSK antibodies in AMM and have demonstrated the involvement of both the presynaptic and postsynaptic components of the neuromuscular junction. The observations in this human disease and its animal models demonstrate the role of MuSK not only in the formation of this synapse but also in its maintenance.

Keywords

animal models; autoimmune; muscle-specific kinase; muscle wasting; MuSK; myasthenia; neuromuscular junction; synapse

Antimuscle specific kinase myasthenia

Ninety percent of patients with generalized myasthenia gravis (MG) have measurable acetylcholine receptor (AChR) antibodies (Abs) circulating in blood. The remainder, referred to as having seronegative MG, have clinical characteristics that are similar, but not necessarily identical, to those with AChR Abs, that is, seropositive MG.¹ Vincent *et al.* and others have identified circulating Abs to the extra cellular domain of antimuscle specific kinase (anti-MuSK) in approximately 40% of seronegative patients.^{2–7} This MuSK Ab-positive subgroup of seronegative patients, which we have referred to as anti-MuSK myasthenia (AMM), tends to differ from seropositive MG in demonstrating more focal muscle involvement (neck, shoulder, facial, and bulbar muscles), but with considerable

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Conflicts of interest

The authors declare no conflicts of interest.

variability from patient to patient.^{2,3,6-9} Unlike seropositive MG, many patients have wasting of these muscles.^{7,9-17} The data available, including those from electrophysiologic studies of the involved muscles and a small number of histologic studies of (less involved) extremity muscles, have failed to show evidence of denervation, but rather have suggested a myopathic process, perhaps involving mitochondrial function.^{8,11,15,18-23} Other differences from MG include poor response to treatment with cholinesterase inhibitors, intravenous Ig, or thymectomy.^{3,6,24} Also, only a rare AMM patient has been found to have thymic lymphoid hyperplasia (commonly found in MG).²⁵⁻²⁷ Histologic studies of the neuromuscular junction (NMJ) in AMM carried out to date,²⁸⁻³⁰ have observed³¹ mild simplification of the EP pretzel architecture. Microelectrode analysis of two of these specimens revealed a mild decrease in miniature endplate potential (MEPP) amplitudes^{29,30} and quantal content.³⁰

Few studies^{18,32-35} have addressed the pathogenesis of AMM. While it is a very reasonable hypothesis that MuSK Abs represent the etiologic factor in AMM, until recently, the importance or precise role of these Abs has been poorly understood. In fact, there have been a number of publications questioning their role in AMM—but rather suggesting that they may occur secondary to some other process or even that they are an epiphenomenon.^{29,36} However, recent experimental data have supported the above hypothesis by observing weakness and NMJ changes in animals actively³⁷⁻⁴⁴ or passively immunized with MuSK.^{33,34,42,45} A number of investigators have found both MuSK-activating (agonist) and MuSK-blocking (antagonist) effects of polyclonal serum MuSK Ab in muscle cell cultures, assaying both phosphorylation of various components of the downstream MuSK phosphorylation pathway (see later) and AChR clustering.^{4,33,40,41,46,47} In addition, polyclonal human AMM serum added to muscle cultures appears to induce endocytic internalization of MuSK leading to partial reduction in membrane MuSK.³³

Role of MuSK in the NMJ

MuSK is a 100 kD transmembrane receptor tyrosine kinase. It is a single-pass integral membrane protein with an N-terminal extracellular domain followed by a short transmembrane domain and then a C-terminal cytoplasmic domain.⁴⁸⁻⁵⁰ The extracellular domain, which appears to be required for interaction with agrin and low density lipoprotein receptor-related protein 4 (lrp4; see later), comprises three immunoglobulin (Ig)-like domains⁵¹⁻⁵⁴ and a cysteine-rich (frizzled-like) C6 box region.^{48,49,53,55} The cytoplasmic domain contains the kinase activity and signaling components of the molecule that lead to the development of the postsynaptic apparatus.⁵⁶⁻⁵⁸ Studies employing both rat and human MuSK have determined that it is only the extracellular domain of the molecule that is the target of the AMM Abs.^{4,5} We have recently identified a splicing variant of MuSK, MuSK 60, containing an additional 20-residue domain located between Ig-2 and Ig-3 expressed primarily in adult muscle, which appears from this study to be an important antigen in AMM.^{59,60}

MuSK plays a major role in the development of the NMJ. The synapse begins to form when the axon growth cone of a developing motor neuron encounters a developing myotube and begins to secrete the glycoprotein agrin.^{31,61-63} The secreted agrin induces dense clustering of the AChRs in the postsynaptic endplate membrane as well as the elaboration of this structure, including its pretzel-like topographic profile and the marked folding and specialization of that membrane at the ultrastructural level. Both the initial spontaneous AChR clustering⁶⁴ and the agrin-induced more extensive clustering require the presence of both MuSK and a second postsynaptic protein, lrp4.^{51,54,65,66} However, the processes involved in the further maturation of the NMJ and, in particular, the mechanisms involved in

the maintenance of the mature NMJ, and the role of MuSK in these mechanisms, have been much less well delineated.^{67–72}

NMJ histologic and electrophysiologic abnormalities in AMM

Histologic studies of the NMJ in AMM carried out to date, have observed mild simplification of the EP pretzel architecture. Microelectrode analysis of two of these specimens revealed a mild decrease in MEPP amplitudes and, in one, reduced quantal content.^{28–30} In contrast, an examination of a patient with very severe acute disease, revealed simplified endplate membranes and poor registration between the nerve terminal and the abnormal endplate.³⁰

Muscle wasting in AMM

Not only does the distribution of muscles involved in AMM differ from that of seropositive MG, but frequently those muscles are wasted. The mechanism of the development of the prominent axial muscle wasting in these patients also remains unclear. The data available, including those from electrophysiologic studies of the involved muscles and a small number of histologic studies of (less involved) extremity muscles, have failed to show evidence of denervation, but rather have suggested a myopathic process, perhaps involving mitochondrial function.^{8,11,15,18–23} Such myopathic changes support a role for MuSK in mediating trophic effects on muscle, perhaps through complex two-way communication between nerve and muscle at the NMJ. On the other hand, focal denervation with accompanying reinnervation, as suggested by the morphologic studies described the rat model of AMM (see later), might also lead to muscle wasting.

Rabbit and mouse models of AMM

In rabbits and mice repeatedly immunized with MuSK over extended periods of time, weakness has been observed^{37–43} along with mild electrophysiologic evidence of disordered NMJ transmission (Table 1). NZW strain rabbits immunized with 100–400 µg, repeated times three, of ectodomain of mouse MuSK exhibited severe weakness.⁴¹ Three immunizations of various mouse strains with the ectodomain of rat MuSK (minus the third Ig-like domain) produced mild to severe weakness and decremental responses, most severe in A/J and B6 strain mice but not in BALB/c mice. In additional mouse studies using either the same immunogen, or intact rat ectodomain, in C57BL/6 strain mice, 3–4 repeated immunizations resulted in mild disease.^{39,42,43} In contrast, A/WySnJ strain mice, which are deficient in both complement C5 and BAFF receptor, injected twice with 20 µg of rat ectodomain developed severe weakness.³⁹

In addition, in immunosuppressed mice passively injected with xenogeneic (human) AMM IgG repeatedly over 14 days (total of 0.68 g), mild–moderate weakness occurred in conjunction with reduced AChR staining and reduced registration between nerve terminals and EPs.³⁴ In a similar study using a total of 250 µg of AMM IgG, weakness was not observed but microelectrode analysis revealed reduction in both MEPP and endplate potential amplitudes.⁴²

EAMM: AMM model in Lewis rats

The strategy for the development of this model was to use a xenogeneic immunogen, mouse MuSK, that differed by a minor degree from the target of the autoimmune attack, native Lewis rat MuSK. In the course of purifying mouse MuSK for this purpose, we identified in adult mouse muscle a pair of new splice variants of this protein.^{59,60} In fact, nearly half of the MuSK cDNAs cloned from adult mouse muscle encoded one of the variant isoforms,

which we designated MuSK 60. We have found that MuSK 60 is expressed in progressively greater amounts during muscle differentiation, such that it represents on the order of 50% of the expressed MuSK in adult muscle.^{59,60} Moreover, *in vitro* studies with this isoform demonstrated that it has a reduced ability to induce AChR clustering in response to the addition of agrin. Taken together, the latter two observations suggest that this “adult” form of MuSK has a distinct function from that of the other isoforms, which are found primarily in developing muscle.

Immunization with the MuSK 60 isoform produced a very severe acute model of AMM resulting from a single injection in Lewis rats.⁴⁴ Unlike the disease induced in mice and rabbits, this severe form of experimental AMM (EAMM) reproduces all the major characteristics of the human disease: fatigable weakness, disordered neuromuscular transmission, and wasting of axial musculature. A single injection of 100 µg of the purified extracellular domain of the MuSK 60 isoform induces very high anti-MuSK Ab titers ($>1:10^6$) and severe weakness that is lethal by Day 27 after immunization. Immunization with lower doses of this antigen produces a more chronic disease with lower anti-MuSK titers. Analysis of NMJ morphology in these animals suggests that Ab attack on MuSK affects both postsynaptic and presynaptic components of this synapse. The animals exhibit marked weight loss and axial muscle wasting not observed in other models of AMM or in EAMG. The ability of these animals to eat, drink, and chew is not observably abnormal until the last two days of life. As the disease progresses, the axial weakness/wasting leads to a striking kyphotic posture, eventually in association with the inability of the forelimbs to lift the chest from the floor of the cage (Fig. 1). It is of note that similar posture and gait abnormalities have been observed in adult mice in which MuSK expression was turned off using Cre recombinase-mediated MuSK gene deletion. The reproduction of all the characteristics of AMM in the current form of EAMM strongly supports the hypothesis that the autoimmune response to MuSK in AMM is pathogenically important in this disease, rather than representing an epiphenomenon. Moreover, these observations highlight the potential usefulness of this model for studying the pathogenesis and future treatments of AMM. Moreover, the correlation between anti-N-MuSK 60 Ab titer and disease severity also supports the hypothesis that EAMM and AMM are the result of the action of the MuSK Abs.

The form of EAMM induced in this study is more severe than the forms induced in mice and rabbits by active immunization with rat or human MuSK and mouse MuSK, respectively. In those studies, relatively mild disease, as determined clinically and histologically, only occurred after three injections of antigen with adjuvant over an extended period, eight weeks. It is unclear why the present disease is so much more severe than that induced in those systems. One possibility is that the differences relate to species sensitivity to the anti-MuSK attack. For example, Lewis rats develop more severe EAMG, the animal model of seropositive MG induced by immunization with AChR, than do various mouse strains,⁷³ an observation previously ascribed to differences in the level of the safety factor of neuromuscular transmission, known to be quite high in mice.⁷⁴ A second possibility is that the Lewis rats are more prone to autoimmune disease induced by immunization with xenogeneic antigens. Such differences have been demonstrated, for EAMG, among various inbred mice strains, but in none of those strains is the EAMG as severe as that in Lewis rats.^{73,75} This possibility seems less likely to explain the differences in severity between the NZW strain rabbit and Lewis rat forms of EAMM because both strains develop equally severe EAMG when immunized with AChR.⁷⁶ A third possibility relates to the differences in the antigens used to induce the disease and, hence, the epitope targets of the disease-inducing Abs. The MuSK 60 isoform, used as immunogen in the current study, appears to be an adult form of the protein,^{59,60} which may be an important target of the auto-Abs in EAMM and AMM. For the rabbit and mouse forms of EAMM, the immunogens have been

either the fetal isoform or another splicing variant of the protein that is missing not only the 20 amino acid extra domain of MuSK 60 but also the entire third Ig domain.³⁷ The severe disease observed here in adult animals resulting from Abs to an adult form of MuSK, supports the possibility of a role for MuSK, perhaps through its MuSK 60 isoform, in the maintenance and functioning of the mature NMJ.³³

Animals immunized with a lower dose of N-MuSK 60 experience a less severe form of EAMM along with lower serum titers of MuSK Abs, suggesting that, in EAMM, Ab titer correlates with disease severity.⁴⁴ However, the animals receiving lower doses of N-MuSK 60 demonstrate higher degrees of diminished neuromuscular transmission, albeit at later times following the immunization. These observations suggest that the weakness, weight loss, and muscle wasting associated with rapidly fatal disease may not correlate with neuromuscular transmission *per se*. If this is the case, then it is possible that interference with other physiologic activities at the NMJ is playing a role in AMM.

The histologic changes observed here support the hypothesis that factors other than reduced neuromuscular transmission play a role and suggest possible mechanism by which this may occur. In particular, the animals receiving 100 µg of MuSK exhibit varying degrees of disruption of the normal NMJ architecture. The architecture of some endplates is altered at the ultrastructural level by hypersegmentation consisting of multiple small axon terminals with marked simplification of postsynaptic membranes. The nerve terminals at some NMJs are more severely affected, with complete or partial loss of these structures. In addition, some axons have an abnormal globular appearance and others exhibit local extension beyond the NMJ, or even frank terminal axon sprouting. In other NMJs, there is misalignment between the presynaptic and postsynaptic portions of these synapses. The latter findings, some of which were also observed in the study of passive transfer of human AMM serum into mice³⁴ and in one of the three studies of AMM,³⁰ suggest that there is abnormal “signaling” between nerve terminal and muscle endplate in both directions, independent of neuromuscular transmission, resulting in failure of maintenance of the mature synapse in these animals.

In addition, cholinesterase staining of teased muscle bundles from EAMM animals reveals abnormal dispersion of paraformaldehyde-resistant cholinesterase activity throughout the length of the muscle fibers. The observed segmented and dispersed cholinesterase-stained patches, away from the compact synaptic region (motor point),⁴⁴ is reminiscent of newly formed synapses, raising the possibility that the Ab attack leads first to frank denervation at some NMJs with subsequent and, possibly, ongoing attempts at reinnervation. The observation, also noted quantitatively in passively induced EAMM in mice,³³ that some NMJs are intact, while neighboring junctions are mildly disrupted and yet others are markedly abnormal supports that possible scenario. It is of special note that severe damage to the endplate membrane, as is seen in acute forms of EAMG and MG, produces essentially no nerve terminal abnormalities and no denervation/reinnervation. Hence, the Ab attack on MuSK appears to have wider ranging effects on the NMJ, as described here and in passively induced EAMM,³³ than is seen with attack on the more abundant endplate AChRs.

The mechanism of the development of the prominent axial muscle wasting in these animals, not seen in EAMG and MG, also remains unclear. Both histologic and electrophysiologic studies in human AMM suggest that the muscle wasting is not the result of denervation but rather is the consequence of a primary myopathy.^{10,11,15,18,20,30,32} Such myopathic changes support a role for MuSK in mediating trophic effects on muscle, perhaps through complex two-way communication between nerve and muscle at the NMJ. On the other hand, focal denervation with accompanying reinnervation, as suggested by the morphologic studies described above, might also lead to muscle wasting.

The observations in this model of AMM provide substantial support for the view that the Abs to MuSK play a major role in the pathogenesis of AMM. However, the molecular mechanisms by which the Abs induce the morphologic and physiologic changes are poorly understood. Data from EAMM passively induced in mice by repeated injections of very large quantities of serum IgG from AMM patients have demonstrated reduced quantities of MuSK (via internalization) and dispersion of AChR clusters in the endplate membrane.³³ On the other hand, the data obtained to date (including our own)^{34,41,46} have failed to identify in EAMM a role for the major mechanism important in MG and EAMG, a widespread attack on the NMJ involving complement mediated mechanisms.

Finally, the observations presented here demonstrate that an immune attack on MuSK can result in weakness, muscle wasting and severe disruption of the architecture of both the postsynaptic and presynaptic portions of the mature NMJ, thereby supporting the hypothesis that, in addition to its role in the developing NMJ, this protein plays a significant role in the maintenance and function of the mature structure.

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Figure 1.

Clinical findings in EAMM. (A) Rat immunized with 100 µg of N-MuSK 60 (right), Day 25, with significant weight loss, flank and neck muscle wasting, extremity weakness, kyphotic posture, and ruffled, ungroomed fur. Adjuvant control (left) is normal. (B) Lateral view of N-MuSK 60-immunized rat from panel A (with permission from Archives of Neurology).

Table 1

Animal models of AMM

Immunization Type	Species	Strain	Mutation	Immunogen/number of immunizations/serum IgG transferred	Clinical severity	Muscle wasting	References
Active	Rabbit	NZW	No	Mouse ectodomain/3-4	Severe	No	41
	Mouse	A/J	No	Rat ectodomain Δ Ig3/2-3	Mild-moderate	No	37
		BALB/c	No	Rat ectodomain Δ Ig3/3	Mild-moderate	No	
	Mouse	C57BL/6	No	Rat ectodomain/3	Mild	Yes	43
	Mouse	A/WySnJ	C5 and BAFF-R deficient	Rat ectodomain/2	Moderate	No	39
	Mouse	C57BL/6	No	Human ectodomain/3	Mild (variable)	No	42
	Rat	Lewis	No	Mouse MuSK-60 ectodomain/1	Severe	Yes	72
Passive	Mouse/human	C57BL/6	No	500-600 mg	Mild	No	34
	Mouse/human	C57BL/6	No	250 mg	None	No	42

Ig = immunoglobulin-like domain; Δ Ig3 = minus the Ig3 domain.