Experimental Neurology 270 (2015) 29–40

Contents lists available at ScienceDirect

Experimental Neurology

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

# Guidelines for pre-clinical animal and cellular models of MuSK-myasthenia gravis

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ARTICLE INFO

*Article history:*
Received 29 October 2014
Revised 12 December 2014
Accepted 17 December 2014
Available online 24 December 2014

*Keywords:*
Preclinical
Neuromuscular junction
Muscle specific kinase
Autoantibodies
Rodent models
Consensus protocol
Experimental design
Antibody-mediated
Myasthenia gravis

ABSTRACT

Muscle-specific tyrosine kinase (MuSK) autoantibodies are the hallmark of a form of myasthenia gravis (MG) that can challenge the neurologist and the experimentalist. The clinical disease can be difficult to treat effectively. MuSK autoantibodies affect the neuromuscular junction in several ways. When added to muscle cells in culture, MuSK antibodies disperse acetylcholine receptor clusters. Experimental animals actively immunized with MuSK develop MuSK autoantibodies and muscle weakness. Weakness is associated with reduced postsynaptic acetylcholine receptor numbers, reduced amplitudes of miniature endplate potentials and endplate potentials, and failure of neuromuscular transmission. Similar impairments have been found in mice injected with IgG from MG patients positive for MuSK autoantibody (MuSK-MG). The active and passive models have begun to reveal the mechanisms by which MuSK antibodies disrupt synaptic function at the neuromuscular junction, and should be valuable in developing therapies for MuSK-MG. However, translation into new and improved treatments for patients requires procedures that are not too cumbersome but suitable for examining different aspects of MuSK function and the effects of potential therapies. Study design, conduct and analysis should be carefully considered and transparently reported. Here we review what has been learnt from animal and culture models of MuSK-MG, and offer guidelines for experimental design and conduct of studies, including sample size determination, randomization, outcome parameters and precautions for objective data analysis. These principles may also be relevant to the increasing number of other antibody-mediated diseases that are now recognized.



## Introduction

### *Pathophysiology in MuSK-MG patients*

MuSK-MG patients are most reliably diagnosed by detection of the MuSK autoantibody, which is predominantly, but not exclusively, of the IgG4 type (McConville et al., 2004; Ohta et al., 2007). A key clinical feature is bulbar and facial muscle weakness. Slurred and fatiguing speech may be combined with atrophy of tongue and facial muscles. A decrement in the amplitude of the compound muscle action potential (CMAP) during repetitive stimulation of the nerve is the most common test for impaired neuromuscular transmission. However such a decrement is seen in only approximately 60% of clinically affected proximal muscles in MuSK-MG (Guptill et al., 2011). Single fiber electromyography (SFEMG) provides a more reliable test for detecting impaired neuromuscular transmission. It can detect trial-to-trial variations in the timing between action potentials in two or more fibers belonging to the same motor unit. Such 'jitter' reflects impaired transmission at one or more of the neuromuscular junctions (NMJs) from which the action potentials originate. Studies of MuSK-MG patients using SFEMG show that facial and neck muscles are more consistently affected by impaired neuromuscular transmission, compared to more distal muscles (Farrugia et al., 2006; Guptill et al., 2011).

There have been few biopsy studies of MuSK-MG cases (Niks et al., 2010; Selcen et al., 2004). Shiraishi and colleagues studied motor point biopsies from the biceps brachii of 8 MuSK-MG patients

*Abbreviations:* AChR, acetylcholine receptor; CMAP, compound muscle action potential; DOK-7, downstream of tyrosine kinase 7; EPP, endplate potential; i.p., intraperitoneal; LRP4, low-density lipoprotein receptor-related protein 4; mEPP, spontaneous miniature endplate potential; MuSK, muscle specific (tyrosine) kinase; MG, myasthenia gravis; NMJ, neuromuscular junction; SFEMG, single fiber electromyography; s.c., subcutaneous.

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http://dx.doi.org/10.1016/j.expneurol.2014.12.013
0014-4886/

(Shiraishi et al., 2005). Immunoperoxidase-stained sections in only 2/8 biopsies showed reduced staining intensity for acetylcholine receptors (AChR) at their endplates, when compared to non-myasthenic controls. This differed markedly from results in animal models (described below) where reductions in endplate AChR staining intensities or AChR numbers, were consistently associated with muscle weakness. This anomaly might reflect the relative lack of involvement of the biceps muscle in MuSK-MG patients. Given what we have since learnt from animal models, further biopsy studies should employ quantification of AChR by α-bungarotoxin binding (radioactive), and confocal microscopy (using fluorescently-tagged α-bungarotoxin) to test for reductions in the AChR-rich area of the endplate and for nerve terminal misalignment. If possible, samples from the motor point of the most severely affected bulbar or neck muscles should be examined.

Two studies compared myopathic changes in muscle biopsies from MuSK-MG patients and patients with AChR antibodies (AChR-MG) (Martignago et al., 2009; Rostedt Punga et al., 2006) and reported mitochondrial abnormalities in MuSK-MG. The myopathic pattern on muscle biopsies and EMG changes were typical of disturbed neuromuscular transmission rather than a primary muscle disorder (Rostedt Punga et al., 2006). Further work is needed to determine whether the observed myopathy and mitochondrial abnormalities were directly caused by MuSK autoantibodies.

### *Function of MuSK at the neuromuscular junction*

MuSK is a postsynaptic tyrosine kinase essential for the development of the NMJ. Neural agrin, low-density lipoprotein receptor-related protein 4 (LRP4), MuSK, downstream of tyrosine kinase 7 (DOK-7) and rapsyn are all proteins in a signaling pathway that stabilizes postsynaptic clusters of AChRs and the overlying presynaptic nerve terminals (reviewed by Ghazanfari et al., 2011). Targeted inactivation of the genes encoding agrin, MuSK, DOK-7 or rapsyn in each case prevented the formation of AChR clusters in embryonic mouse muscles (DeChiara et al., 1996; Gautam et al., 1995, 1996; Okada et al., 2006). Within the muscles of these embryos motor axons failed to stop and differentiate at the normal endplate zones, suggesting an additional role for MuSK in directing the location and stability of the NMJ.

Neural agrin, which is secreted by the motor nerve terminal, binds to LRP4 on the muscle cell surface, thereby activating MuSK. Cell culture studies show that LRP4 and MuSK form a tetrameric complex that can be activated when neural agrin binds to LRP4 (Kim et al., 2008; Zhang et al., 2008). Agrin-induced activation of this pathway involves autophosphorylation of several tyrosine residues on MuSK (Till et al., 2002). The active MuSK dimer is then stabilized by the phosphotyrosine-binding adaptor protein, DOK-7 (Bergamin et al., 2010; Inoue et al., 2009). In turn, MuSK activates non-receptor tyrosine kinases including Abl and Src that phosphorylate postsynaptic target proteins, one of which is the AChR itself (Finn et al., 2003; Mittaud et al., 2004). Phosphorylation of the AChR β-subunit (Y390) recruits rapsyn, which cross-links AChRs and helps to stabilize AChR clusters (Borges et al., 2008). Loss of MuSK tyrosine kinase signaling inhibited NMJ formation while expression of MuSK in parts of the embryonic muscle that do not normally express MuSK led to formation of ectopic AChR clusters, some of which became innervated by motor axons (Kim and Burden, 2008).

There is reason to think that this pro-synaptic MuSK signaling pathway remains important for maintaining the postnatal NMJ. Conditional knockdown of MuSK expression starting from 10 days postnatal caused a progressive loss of endplate AChR density. Nerve terminals became partially or totally displaced from the endplate, and the mice died by one month postnatal (Hesser et al., 2006; Kong et al., 2004). Conditional knock-down of agrin expression in a subset of motor neurons in adult mice led to the loss of AChRs, rapsyn and dystroglycan from the endplate, together with a variety of structural abnormalities at a subset of the affected nerve terminals (Samuel et al., 2012). Finally, congenital myasthenic syndrome (CMS) can be caused by mutations in the genes encoding rapsyn, DOK-7, MuSK or agrin: rapsyn and DOK-7 being most common. The patients are clinically variable in presentation, but weakness is usually persistent throughout life, suggesting that the MuSK pathway is important for maintaining the postnatal human NMJ (Beeson et al., 2006; Ohno et al., 2002). These observations support the hypothesis that disruption of the physiological signaling role of MuSK by autoantibodies is sufficient to cause structural NMJ abnormalities without involvement of other immunologic effector mechanisms.

### *Treatment of MuSK-MG*

Many immunosuppressive drugs that are successfully used in AChR-MG, such as corticosteroids, azathioprine and rituximab, are also effective in MuSK-MG (for a review see Reddel et al., 2014). An interesting and unexplained observation is that MuSK-MG patients are often refractory or hypersensitive to acetylcholinesterase inhibitor drugs that are routinely used in AChR-MG (Evoli et al., 2003; Guptill et al., 2011; McConville et al., 2004; Pasnoor et al., 2010). This disparate effect of cholinesterase inhibitors is also observed in different forms of CMS: acetylcholinesterase inhibitors are effective against CMS caused by rapsyn or AChR deficiency, while they are ineffective or even counterproductive in CMS with AChE defects or slow-channel syndromes (Engel, 2007) and DOK-7 mutations (Müller et al., 2007; Palace et al., 2007). In one patient with CMS caused by MuSK mutations, pyridostigmine was ineffective but 3,4-diaminopyridine had a beneficial effect (Chevessier et al., 2004). Recent findings in a mouse model of MuSK-MG showed that pyridostigmine exacerbated endplate AChR loss, while 3,4-diaminopyridine enhanced neuromuscular transmission (Mori et al., 2012a; Morsch et al., 2013). Interestingly albuterol, which has been successful in treating DOK-7 CMS patients, also ameliorated weakness in mice caused by MuSK antibodies, though the mechanism was unclear (Ghazanfari et al., 2014b). This example underscores the relevance of testing novel drugs in animal and culture models of MuSK-MG.

## Cell culture models of MuSK-MG

### *Contribution of cell culture models to pre-clinical assessment*

Postmitotic, multinucleate myotubes are most often used for studying the effects of MuSK antibodies in culture (Table 1). Cultured myotubes express on their surface the key components of the MuSK signaling pathway: LRP4, MuSK and AChR. Several groups have shown that, when added to cultured myotubes, plasma or IgG from MuSK-MG patients can inhibit the formation of AChR clusters and/or cause disassembly of pre-existing AChR clusters (Cole et al., 2010; Farrugia et al., 2007; Hoch et al., 2001; Huijbers et al., 2013; Koneczny et al., 2013). Cell culture assays using multiwell trays lend themselves to the testing of plasma/serum samples from large numbers of MuSK-MG patients. Moreover, only a small amount of plasma from each patient is required for such assays, given the small culture volume ($\leq$1 ml per well). Cell culture assays therefore facilitate quantitative comparison of the effects of the autoantibodies from multiple MuSK-MG patients in a well-established model of AChR clustering.

Cell culture models have suggested several alternative pathogenic mechanisms in MuSK-MG. Early studies with mononucleate myoblasts reported that MuSK-MG patient serum inhibited cell proliferation, or caused changes in gene expression that are normally associated with denervation and muscle atrophy (Benveniste et al., 2005; Boneva et al., 2006). The significance of these myopathic-like effects remains to be further investigated. More recent studies have examined the impact of anti-MuSK IgG upon MuSK signaling. Mori et al. used C2C12 myotubes to show that divalent, murine and rabbit anti-MuSK IgG caused activation (phosphorylation) of MuSK kinase. Monovalent Fab fragments had no such activating effect. They suggested that anti-MuSK IgG cross-links MuSK monomers to form activated dimers. However, both the divalent and monovalent forms of anti-MuSK IgG