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Passive transfer models of myasthenia gravis with muscle-specific kinase antibodies

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Abstract

Myasthenia gravis (MG) with antibodies to muscle-specific kinase (MuSK) is characterized by fluctuating fatigable weakness. In MuSK MG involvement of bulbar muscles, neck, shoulder and respiratory weakness is more prominent than in acetylcholine receptor (AChR) MG.

MuSK autoantibodies are mainly of the IgG4 subclass, which is unable to activate complement, has low affinity for Fc-receptors, and is functionally monovalent. Therefore the pathogenicity of IgG4 MuSK autoantibodies was initially questioned.

A broad collection of *in vitro*, active immunization and passive transfer models has been developed that have shed light on the pathogenicity of MuSK autoantibodies. Passive transfer studies with purified IgG4 from MuSK MG patients confirmed that IgG4 is sufficient to reproduce clear clinical, electrophysiological as well as histological signs of myasthenia. *In vitro* experiments revealed that MuSK IgG4 autoantibodies preferably bind the first Ig-like domain of MuSK, correlate with disease severity and interfere with the association between MuSK and low density lipoprotein receptor-related protein 4 (Lrp4) and Collagen Q. Some patients have additional IgG1 MuSK autoantibodies, but their role in the disease is unclear. All together this provides a rationale for epitope-specific or IgG4-specific treatment strategies for MuSK MG, and emphasizes the importance of the development of different experimental models.

Introduction

Autoimmune myasthenia gravis (MG) with antibodies to muscle-specific kinase (MuSK) is a neuromuscular disorder, which is clinically and immunologically different from the more prevalent MG with acetylcholine receptor (AChR) antibodies. Key clinical features are muscle weakness with prevalent involvement of cranial and bulbar muscles and more frequent respiratory crises than in AChR MG¹. Compared to generalized AChR MG, a higher proportion of MuSK MG patients is reported to have a refractory course of the disease. More than 95% of the patients have ocular manifestations at some time during their disease, but bulbar muscles remain the most severely affected throughout the course of the disease. In contrast to the bulbar weakness, the ocular weakness in most cases quickly resolves with immunosuppressive treatment, and often do not reappear during MG relapses when the patients do suffer from severe bulbar weakness². Interestingly, the observed pronounced sensitivity of bulbar muscles might be related to low endogenous levels of MuSK as compared to limb muscles such as the soleus muscle³.

The diagnosis of MuSK MG is based on the combination of distribution of clinical weakness, results of electromyographical tests, and the presence of serum MuSK antibodies. The effect of acetylcholinesterase inhibitors, edrophonium or neostigmine, for diagnostic purposes is disappointing in one third of the patients. Also during treatment patients tend to have a poor response to oral acetylcholinesterase inhibitors, ranging from mild benefit to overt intolerance¹. Most patients can be adequately managed with immunomodulatory therapies, using corticosteroids, and other immunosuppressive agents. Plasmapheresis or rituximab therapy have been shown to be very successful in the treatment of MuSK MG patients⁴⁻⁶.

MuSK is a transmembrane tyrosine kinase that is expressed predominantly in skeletal muscle and concentrated at the postsynaptic membrane of the neuromuscular junction (NMJ). For a comprehensive review on the role of MuSK in NMJ development and maintenance see Burden *et al.*⁷. MuSK is concentrated at the central part of the muscle fiber and together with low density lipoprotein receptor-related protein 4 (Lrp4), docking protein 7 (or downstream of tyrosine kinase 7, Dok-7) and pre-synaptically released agrin stimulates AChR clustering and establishes a single NMJ at each muscle fiber. When MuSK is absent, synapses fail to form and mice die perinatally⁸. MuSK remains important in postnatal life for the maturation and maintenance of the adult neuromuscular synapse. Postnatal inactivation of *MuSK* causes loss of AChRs, disassembly of the postsynaptic organization and loss of innervation⁹. Moreover, mutations in *MuSK* cause congenital MG in humans^{10;11}.

The first clinical observations for a role of MuSK in myasthenia gravis

The discovery of MuSK in 1993 and growing knowledge in the following years on its importance for NMJ formation and maintenance lead to testing MuSK as a candidate antigen in seronegative MG^{8;12;13}. Indeed in 2001, *in vitro* experiments confirmed binding of serum antibodies to extracellular MuSK epitopes¹⁴. These early studies furthermore showed that these AChR antibody negative, MuSK antibody positive sera inhibited agrin-dependent AChR clustering in C2C12 myotube cultures suggesting their involvement in the pathogenesis of MG. Moreover, active immunization with the extracellular part of the human MuSK protein induced muscle weakness and associated changes in the neuromuscular junction in mice or rabbits^{15;16}. In contrast, the first studies using biopsies of MuSK MG patients surprisingly failed to demonstrate clear evidence of synaptic impairment or loss of AChR^{17;18}. The study of Selcen *et al.* described one male patient who had typical facial and bulbar weakness and was positive for MuSK antibodies, but his endplates in the intercostal muscle biopsy, taken 13 years after disease onset, showed no AChR or MuSK loss at the NMJs. Another case report did find abnormalities in an intercostal muscle biopsy of a 29-year old female MuSK MG patient. In this case the biopsy was taken early in the disease at only nine months after the start of the disease¹⁹. *In vitro* electrophysiological and histological studies showed pre- and postsynaptic abnormalities. Histology revealed partially denervated postsynaptic areas and some degeneration of

postsynaptic folds. However, only a qualitative analysis was possible due to the low number of synapses that were available.

In a study using muscle biopsies of 10 Japanese MuSK MG patients the endplate AChR density was not significantly decreased, and complement C3 was detected at the endplate in only two of eight cases¹⁸. The authors concluded that MuSK antibodies did not appear to cause substantial AChR loss, complement deposition, or morphological damage. A possible drawback of this study was that it used motor point biopsies from the biceps brachii, a muscle that usually is less severely affected in MuSK MG.

Importantly, serum IgG MuSK autoantibodies correlate with disease severity²⁰⁻²² and placental transfer of antibodies from mother to her child induces transient MG in the neonate^{23;24}.

In conclusion, clinical correlations, experimental data on the function of the MuSK protein in animals and *in vitro* preparations, as well as the results of active immunization experiments form clear evidence for pathogenicity of MuSK antibodies in autoimmune MG. In contrast, the sparsely available histological studies of human endplates failed to convincingly show a decrease in MuSK or AChR to an extent that it could explain the electrophysiological dysfunction found in the same neuromuscular synapses. Passive transfer studies, using human MuSK serum antibodies, have proven essential in obtaining more detailed understanding of the pathophysiology of MuSK MG and will be discussed below (Table).

Passive transfer of IgG from “seronegative” patients

Before the discovery of MuSK antibodies in 2001¹⁴, there were already indications that some MG patients might have other serum antibodies causing similar patterns of muscle weakness. In 10-15% of the MG patients no AChR antibodies were found, while these patients had characteristic features of MG on electromyographical examination or showed clinical improvement after plasmapheresis or immunosuppressive treatment. One of the earliest studies used plasma obtained by plasmapheresis from 8 patients with signs and symptoms of MG, but in whom serum AChR antibodies had been persistently undetectable by radioimmunoprecipitation assay or by testing inhibition of α -bungarotoxin binding²⁵. Remarkably, six of these patients had bulbar weakness, of whom four with severe bulbar weakness and four patients required assisted ventilation at some time point during their disease. This pattern fits well with the now well-known clinical features of MuSK MG^{1;5;26}. Indeed, a later study from the same group confirmed that most sera were subsequently tested positive for MuSK antibodies²⁷.

Crude immunoglobulin fractions prepared by precipitation with 45% ammonium sulphate followed by extensive dialysis were used for passive transfer studies to BKTO mice, an outbred strain²⁵. Pooled IgG from healthy individuals was used as a negative control, and IgG from AChR MG patients as positive control. Mice were injected intraperitoneally with 60 mg IgG per day for 3 days. Eighteen hours after the last injection the diaphragms were studied. The diaphragms of the mice were studied using *in vitro* contraction tests, in which supramaximal stimuli at 3 Hz were given through the nerve ending attached to the muscle. Using a normal perfusion solution no decrement of the twitch contraction force was found comparing the amplitude of the fifth response with that of the first in that train of stimuli. However, using a low dose of tubocurarine of 0.6 μ g/ml a clear contraction force decrement was seen in the diaphragms of the mice treated with IgG from the “seronegative” patients. The number of AChRs was lower than in the control mice, but the decrease was much less than that of the AChR MG IgG treated mice, suggesting it might be a secondary effect due to antibodies interfering with other molecules than the AChR itself, e.g. MuSK or Lrp4. These early experiments suggested that MG patients without AChR serum antibodies do have antibodies binding to determinants at the neuromuscular junction other than the AChR. In these experiments, mice were treated for only three days, and in retrospect that is probably too short to see a profound effect. None of the mice showed clinical signs of disease. Also in a second set of experiments, in which mice were treated up to 15 days with either purified IgG or whole plasma, no clinical weakness was observed²⁸. In these latter experiments again plasma or IgG fractions were used for

passive transfer studies to BKTO mice. With IgG from 5 of 6 patients considerably reduced miniature endplate potential (MEPP) amplitudes were found in the mice after 15 days of injection. After 3 days of treatment, less or no MEPP amplitude reduction was found, indicating that for most IgG preparations or plasmas at least 7 to 15 days were necessary before an effect on the NMJs could be detected by *ex vivo* electrophysiology assays.

Passive transfer of IgG from MuSK MG patients

Several years after the discovery of MuSK auto-antibodies passive transfer studies from well-documented MuSK MG patients were described²⁹. Total IgG purified from plasmapheresis material from three patients was used to perform passive transfer studies in two strains of mice. Mice were treated with 45 mg/day of IgG during up to 14 days. Passive transfer of IgG to FVB/NJ mice was not able to induce clinical muscle weakness nor weight loss, while C57BL/6J mice showed clear weakness, including chin down, flaccid tail and limb weakness. Initially, this appeared only after exercise, but later on also at rest. Weight loss appeared typically after 7 days of daily IgG injections. In active immunization models some strains of mice were reported to be more susceptible to MG than others¹⁵. Interestingly, mice injected for 14 days with MuSK MG patient IgG displayed reduced levels of post-synaptic AChR and a reduced apposition of presynaptic and postsynaptic neuromuscular structures. These findings were more pronounced in mice that showed more muscle weakness²⁹.

A passive transfer study with intramuscular injections found that MuSK MG plasma decreased the size of new endplates formed upon muscle regeneration after pretreatment with notexin, a myotoxic snake toxin. Furthermore, MuSK plasma injections caused impairment of muscle contraction *in vitro* in the presence of reduced calcium in the medium³⁰.

These findings together with the results of the active immunization studies, correlation with disease severity and therapeutic effect of plasmapheresis and immunosuppressive treatment in MuSK MG patients, contribute to the evidence on MuSK autoantibody pathogenicity and identify them as a helpful diagnostic tool.

The exact target and pathophysiological mechanisms of MuSK MG remained elusive, until in 2011 and 2012 several papers provided new information. Using *in vitro* studies, Kawakami *et al.* found that MuSK IgG blocks the binding of Collagen Q (ColQ) to MuSK³¹. In their passive transfer model they injected purified total IgG from a MuSK MG patient to C57BL/6J mice during 14 days. No weakness or weight loss was described, but they found that anchoring of ColQ was compromised, while they observed a less prominent effect on the expression of MuSK and AChR at the endplates. These results would predict endplate acetylcholinesterase (AChE) deficiency in MuSK MG patients, and might explain the increased sensitivity of MuSK MG patients for acetylcholinesterase inhibitors¹. However, a previous biopsy study on intercostal muscles did not report an AChE deficiency¹⁷. To explain the various results of the experimental studies *in vitro*, in mice and the human biopsies, it was suggested that MuSK IgG behaves differently in humans and does not block binding of ColQ to the NMJ to such an extent that it induces clinical symptoms.

Around the same time we showed that purified IgG4 from MuSK MG patients binds to mouse NMJs and can induce severe paralysis³². MuSK antibodies are predominantly of the IgG4 isotype, and since IgG4 (MuSK) antibodies are functionally monovalent and do not activate complement, this pathogenic effect was surprising³³⁻³⁵. Purified MuSK MG patient IgG4 was passively transferred to immunodeficient NOD/SCID mice for up to 14 consecutive days. MG symptoms developed usually after 7-9 days depending on the patient and the dose. This model has several advantages compared to the abovementioned animal models. Due to their immunodeficiency these mice do not need any additional immunosuppressive drug treatment with potential toxic side effects to prevent the formation of neutralizing antibodies. The use of purified IgG4 allows more variation in dosing of the antibodies. Furthermore, the non-IgG4 fraction of the same patients can be tested in parallel. Passive transfer models using purified total IgG are limited by the maximum volume of 1 ml that is allowed for intraperitoneal injection in mice. This again limits the amount of IgG to typically 45-60

mg that can be injected at one time point, as higher IgG concentrations are generally not soluble. IgG4 constitutes about 5-14% of the total IgG, from which one can calculate that the preparations of total MuSK IgG contain about 2 to 8 mg of MuSK IgG4. Indeed, 4 mg of purified MuSK IgG4 induced overt and consistent clinical weakness in mice³². All together these studies identify mouse strain, dosing and the duration of treatment, as important reasons why earlier experiments using total purified IgG were not always successful in inducing clinical disease in mice.

In line, Viegas *et al.* observed no weight loss or overt weakness in mice that received 5 days of 50 mg of total IgG from MuSK MG patients³⁶. Interestingly, they described that MuSK antibody levels in these mice were very similar to patient plasma levels, although five to 10-fold lower than that of actively MuSK immunized mice. It has not been studied in detail if treatment for 3 to 5 days with a high dose MuSK autoantibodies is sufficient to induce disease. Because the indirect effect of MuSK on AChR function, a period of at least one week could be the minimum time needed for MuSK antibodies to have a clinical overt effect on the muscles in mice after passive transfer. Pulse-labelling of NMJs with α -bungarotoxin of mice treated with MuSK MG patient IgG showed a reduction of 6.6% of AChR per day amounting to 40% after 6 days³⁷. The decline of AChR density corresponded with the progressive decline of electromyographic read outs that represent MG³⁸. All together, these experiments suggest that a dose of at least 45 mg/day per mouse of total IgG for a duration of at least 7 days is necessary to induce weakness and weight loss in the mice (Table).

The passive transfer studies in NOD/SCID mice showed that the purified IgG4 fraction from MuSK MG patients induced clinical disease, reduced the density and increased fragmentation of the AChRs (figure 1), and caused severe reduction of postsynaptic acetylcholine sensitivity³². Interestingly, the normal presynaptic homeostatic response, consisting of a compensatory transmitter release upregulation, as normally seen in AChR MG³⁹, was absent. Another interesting finding was that the IgG1-3 fraction of the same MuSK MG patient did not induce any signs of *in vivo* disease and functional or histological NMJ aberrations in dissected muscles. A possible explanation for the lack of effect from IgG1-3 IgG from MuSK MG patients could be the absence of complement. Therefore healthy human serum was added as a source of complement into NOD/SCID mice that were treated for 10-18 days. This however also did not result in any signs of weakness or *in vitro* electrophysiological abnormalities. The fact that the IgG1-3 fraction contains lower MuSK antibody titres forms an additional explanation.

Thus, IgG4 plays a major role in the pathogenesis of MuSK MG. Still, some MuSK binding capacity is present in the IgG1-3 serum fraction of some MuSK MG patients, and these antibodies are also capable of inhibiting agrin-induced AChR clustering⁴⁰. Therefore complement activation and a role for IgG1-3 MuSK autoantibodies in the disease cannot be excluded^{40;41}. However, in general the concentration of MuSK binding IgG1-3 autoantibodies appears to be too low to have a clinically relevant impact.

Treatment of MuSK MG in passive transfer models

Pyridostigmine and 3,4-diaminopyridine (3,4-DAP) were tested in a passive transfer model of MuSK MG⁴². Pyridostigmine is used as a first line symptomatic treatment for patients with MG⁴³. It inhibits the action of acetylcholinesterase, thereby prolonging the action of acetylcholine on postsynaptic AChRs. 3,4-DAP enhances acetylcholine release from the presynaptic nerve terminal, and is used in Lambert-Eaton myasthenic syndrome or in patients with congenital MG⁴⁴. Pyridostigmine prolongs the activity of acetylcholine in the synaptic cleft, while 3,4-DAP increases the number of quanta that are released presynaptically. This results in different and to some extent opposing effects on the NMJ.

The activation of MuSK by neural agrin and Lrp4, and that of the AChR by acetylcholine, deliver competing signals to the NMJ that regulates assembly or disassembly of AChR clusters. MuSK contributes to the assembly and activation of AChR clusters, while activation of the AChRs drives intracellular pathways that promote dismantling of AChR clusters. Morsch *et al.* showed that systemic delivery of pyridostigmine to mice receiving daily injections of total IgG from MuSK MG

patients exacerbated the disease, and induced structural alterations and functional impairment at diaphragm NMJs⁴². Mice receiving lower doses of MuSK auto antibodies (25 mg per day), that usually does not lead to clinically overt weakness, showed generalized weakness after a 9 day treatment with pyridostigmine. The same dose of pyridostigmine did not harm control mice receiving normal human IgG. Interestingly, MuSK autoantibodies have been demonstrated to disrupt the interaction between MuSK and ColQ³¹. Loss of endplate acetylcholinesterase could potentiate any harmful prolonged activation of endplate AChRs by pyridostigmine mediated increase in available acetylcholine in MuSK MG. In contrast, 3,4-DAP improved neuromuscular transmission in the diaphragm after one week of treatment⁴². A subcutaneous minipump delivered an effective dose of about 16 mg/kg/day pyridostigmine or 3,4-DAP at 8 mg/kg/day. A MG patient using 6 x 60 mg of pyridostigmine per day, uses typically about 5 mg/kg per day, while 3,4-DAP at a daily dose of 70 mg would result in 1 mg/kg/day. It is difficult to directly compare mice and men, but the dose of pyridostigmine or 3,4-DAP in mice was likely at a therapeutically relevant level.

Drugs that activate beta-adrenergic receptors, among which ephedrine and albuterol, offer a therapeutic benefit in certain forms of congenital MG^{45;46}. Ephedrine has also been used in autoimmune MG⁴⁷ and recently it was successful in a small n-of-1 trial⁴⁸. Ghazanfari *et al.* also used the passive transfer MuSK MG mouse model to test the effect of a beta2-adrenoceptor agonist albuterol⁴⁹. Mice were treated with 25 mg total IgG from a MuSK MG patient during 15 days. In this model weight loss and weakness typically developed between 12 and 15 days. Treatment with albuterol (8 mg/kg/day) during two weeks reduced the clinical signs of disease. Surprisingly, endplate potential amplitudes did not increase in the treated group and loss of AChRs was not prevented. The possibility was considered that albuterol reduced the clinical defects through a direct effect on the contractility of the muscle fibers with still intact neuromuscular synaptic transmission. On the other hand, albuterol significantly reduced the degree of fragmentation of AChR clusters and increased the extent to which the remaining receptor clusters were covered by presynaptic nerve terminals.

Further experiments suggested that forced expression of MuSK could help to slow down the loss of postsynaptic AChRs in MuSK MG⁵⁰. This was tested by injecting an adeno-associated viral vector encoding MuSK in the tibialis anterior muscle of mice. Next, the mice were challenged with 35 mg of total IgG from MuSK MG patients during 11 days. In the treated mice more MuSK and AChR were retained at the endplates. Electromyographical recordings revealed less impairment in the treated muscle compared to the contralateral leg.

In conclusion, the passive transfer model of MuSK MG has proven very useful to gain more insight in the pathogenesis of the disease, and in testing the potential therapeutic benefit of several readily available drugs known for their beneficial effect in other forms of MG.

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Table. Summary of published passive transfer models for MuSK MG

Study	Material	Dose	Duration of treatment	Clinical signs of MG in mice	Laboratory signs of MG in mice	MG variant in donor patients
Mossman, 1986	IgG	60 mg/day	3 days	None	Reduced safety factor	SNMG
Burges, 1994	IgG Plasma	60 mg/day 22 mg/day	3 to 7 days 7 to 15 days	None	Reduced MEPP amplitude	SNMG
Cole, 2008	IgG	45 mg/day	14 days	Weakness and weight loss in C57BL/J6 mice None in FVB/NL mice	Reduced and fragmented endplate AChR staining Reduced synaptic alignment Denervation	MuSK MG
ter Beek, 2009	plasma	40 microliter	21 days, twice daily, on weekdays	None	Reduced safety factor Reduced endplate size	MuSK MG
Kawakami, 2011	IgG	40 mg/day	15 days	None described	Reduced endplate AChR area Reduced AChE and ColQ staining	MuSK MG
Morsch, 2012	IgG	45 mg/day	14 days	Weakness Weight loss	Reduced MEPP and EPP amplitudes Decrement of CMAP at 3 Hz	MuSK MG
Viegas, 2012	IgG	50 mg/day	5 days	None	Reduced MEPP and EPP amplitudes (in weak mice)	MuSK MG
Klooster, 2012	IgG4, IgG1-3	0.5-6 mg/day	14 days	Weakness Weight loss	Decrement of CMAP at 10 Hz Reduced MEPP and EPP amplitudes Reduced safety factor Reduced and fragmented AChR staining	MuSK MG
Ghazanfari, 2014	IgG	45 mg/day	14 days	Weakness	Reduced phosphorylation Loss of AChR from endplate	MuSK MG