Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies

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Myasthenia gravis (MG) is an antibody-mediated autoimmune disease of the neuromuscular junction. In approximately 80% of patients, auto-antibodies to the muscle nicotinic acetylcholine receptor (AChR) are present[1]. These antibodies cause loss of AChR numbers and function, and lead to failure of neuromuscular transmission with muscle weakness[2]. The pathogenic mechanisms acting in the 20% of patients with generalized MG who are seronegative for AChR-antibodies (AChR-Ab)[3] have not been elucidated, but there is evidence that they also have an antibody-mediated disorder[4,5], with the antibodies directed towards another, previously unidentified muscle-surface–membrane target[6–8]. Here we show that 70% of AChR-Ab–seronegative MG patients, but not AChR-Ab–seropositive MG patients, have serum auto-antibodies against the muscle-specific receptor tyrosine kinase, MuSK. MuSK mediates the agrin-induced clustering of AChRs during synapse formation, and is also expressed at the mature neuromuscular junction[9–12]. The MuSK antibodies were specific for the extracellular domains of MuSK expressed in transfected COS7 cells and strongly inhibited MuSK function in cultured myotubes. Our results indicate the involvement of MuSK antibodies in the pathogenesis of AChR-Ab–seronegative MG, thus defining two immunologically distinct forms of the disease. Measurement of MuSK antibodies will substantially aid diagnosis and clinical management.

We first tested coded plasmas from AChR-Ab–seronegative MG patients and healthy individuals using COS7 cells transfected with rat MuSK constructs (Fig. 1*a*). IgG from all five AChR-Ab–seronegative MG plasmas (Fig. 1*b*, AChR-Ab–neg/MuSK), but not from the three healthy control plasmas, bound to MuSK aggregates on the cell surface at dilutions of up to 1:1000. The pattern of immunoreactivity was indistinguishable from that observed with polyclonal antibodies raised against recombinant MuSK in rabbits[13]. Each of the AChR-Ab–seronegative MG plasmas recognized the extracellular domains of MuSK, as we observed no immunoreactivity with COS7 cells expressing the transmembrane and cytoplasmic domains only (Fig. 1*b*, AChR-Ab–neg/MuSKΔIg1-4).

Immunoprecipitation experiments confirmed that the IgG antibodies in AChR-Ab–seronegative MG plasmas recognized the native MuSK protein. As a source of MuSK, we used detergent extracts from either MuSK-expressing COS7 cells or mouse C2C12 myotubes that express functional MuSK[10,11,13]. In each case, after the plasma incubations, we precipitated the IgG antibodies with immobilized G protein and ran the precipitates on an SDS-PAGE. We used a rabbit serum against MuSK as a positive control. Antibodies from the AChR-Ab–seronegative plasmas immunoprecipitated bands of 110 kD that were identified as MuSK by binding of a specific antibody against MuSK (Fig 1*c*). MuSK was not immunoprecipitated by healthy control plasmas, AChR-Ab–seropositive plasmas or from an AChR-Ab–seronegative plasma that had been pre-absorbed with the extracellular domain of MuSK (Ig1-4).

Currently, AChR-Ab–seronegative MG is thought to represent about 10–20% of all MG patients[3], but the true prevalence is difficult to assess because of differences in patient ascertainment and referral to specialist centers. Most AChR-Ab–seropositive MG patients present in adult life, often after the age of 50 years[14], but children with MG are frequently AChR-Ab–seronegative[3]. To establish an assay that could be used for future diagnosis and epidemiological studies, we tested sera and plasmas in an ELISA using plates coated with fragments of the extracellular domains of MuSK, expressed in secreted form from COS7 cells. We calculated a cut-off (0.08 optical density units [OD]) on the basis of the mean ± 3 s.d. of the values with healthy control plasmas or sera. Raised levels of IgG antibodies to MuSK were found in 17/24 samples from patients with AChR-Ab–seronegative generalised MG, including the 2 children who had presented before the age of 10 years. Titers were borderline or in the control range in 19 AChR-Ab–seropositive MG patients without thymoma, and in 20 with thymoma, as well as in 33 sera from patients with other neurological disorders (Fig. 2*a*). The lack of detectable MuSK antibodies in MG patients with thymoma is significant because these patients frequently have antibodies against a range of muscle proteins[15]. Detection of MuSK antibodies will provide a new and much needed tool for the diagnosis of AChR-Ab–seronegative MG in children and in adults and facilitate epidemiological studies. It will also strengthen the case for immunosuppressive therapy and provide a means of monitoring its effectiveness. Importantly, the thymus gland usually appears normal in AChR-Ab–seronegative MG (ref. 16), indicating that thymectomy (often used therapeutically in AChR-Ab–seropositive MG) may not be appropriate in those cases with MuSK antibodies. Also, the presence of MuSK antibodies should help to exclude a thymoma.

We noted a broad correlation between optical density values

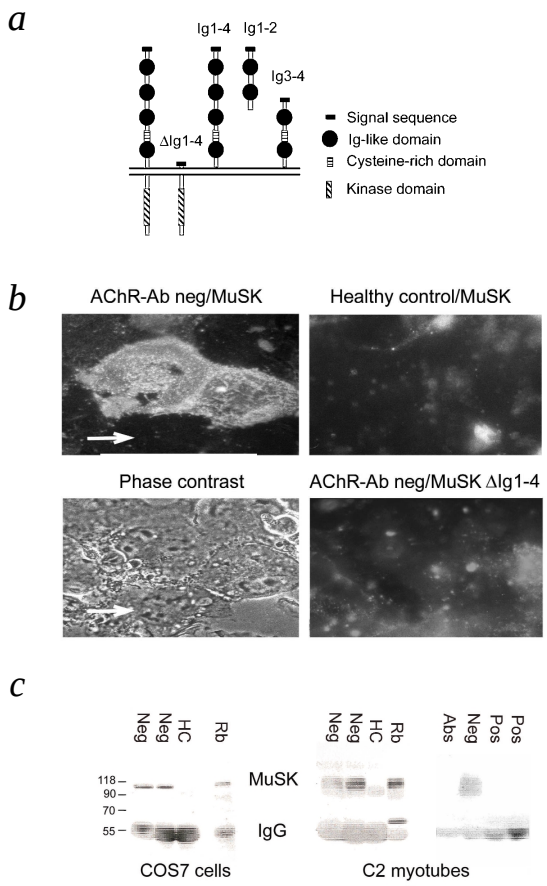

Fig. 1 Antibodies from AChR-Ab–seronegative MG patients bind to MuSK. ***a***, Domain structure of the MuSK molecule and schematic view of constructs used in Fig. 1*b* and *c* and Fig. 2. ***b***, AChR-Ab–negative MG IgGs bound to COS7 cells expressing full-length MuSK (AChR-Ab–neg/MuSK), whereas healthy control IgG did not (Healthy control/MuSK). MuSK immunoreactivity appeared as a speckled pattern, similar to that seen previously with rabbit anti-MuSK antibodies[13]. Non-transfected cells in the same field, demonstrated by phase contrast microscopy (arrows), showed non-specific binding only. No specific binding of AChR-Ab–seronegative MG IgG to cells expressing MuSK lacking the extracellular domains (AChR-Ab–neg/MuSK ΔIg1-4) was detected. ***c***, IgG from 2 AChR-Ab–seronegative MG plasmas (Neg), but not from control plasmas (HC), precipitated MuSK from detergent extracts of COS7 cells expressing MuSK, and from C2C12 (C2) myotubes. The same band was immunoprecipitated by IgG from a rabbit immunized against MuSK (Rb). MuSK was not precipitated by AChR-Ab–seropositive MG plasmas (Pos) or after absorption of AChR-Ab–seronegative plasma (Abs) with soluble MuSK Ig1-4. MuSK appears as a 110 kD band from COS-cells and as several bands representing different MuSK splice variants in the C2C12 cells[11].

for IgG binding to MuSK and IgG binding to the human TE671 cell line measured in an earlier study[8] ($r^2 = 0.72$; $P < 0.001$; $n = 12$), indicating that the previously defined, but unidentified, cell-surface antigen on TE671 cells might be MuSK. The patients who were negative for both MuSK and AChR antibodies were not clinically distinguishable from those who had MuSK antibodies, but 4 of 7 MuSK-negative samples were from patients who had received immunosuppressive treatments, compared with only 2 of 17 of the MuSK-positive samples. This indicates that the current assay may lack sensitivity for patients who are already immunosuppressed. Further analysis showed that most of the MuSK antibodies (Fig. 2*b*) were directed against the amino-terminal sequences (construct Ig1-2 in Fig. 1*a*) rather than the membrane-proximal half (construct Ig3-4 in Fig. 1*a*). Overall, there is 94% homology between rat MuSK and the published human sequence[9], and they differ by only two amino acids in the Ig1-2 construct. Nonetheless, use of human MuSK, as well as further refinement of the assay, may increase the sensitivity of the test. In addition, as we did not detect IgM antibodies to MuSK (data not shown), the target for the putative non-IgG antibodies in some patients[6], including two of the MuSK-Ab–negative patients studied here, requires further investigation.

These data indicate that the MuSK antibodies are directed against extracellular determinants and could therefore be directly involved in the pathogenesis of the disease. To determine whether the antibodies can interfere with MuSK function, we examined agrin-induced AChR clustering in myotubes derived from the mouse cell line, C2C12. In the absence of agrin (Fig. 3*a*, upper panels), very few spontaneous clusters of AChRs were found in the presence of healthy control plasma, whereas MuSK-Ab–positive MG plasma induced some AChR aggregates along the surface of the myotubes. Similar results have previously been reported for rabbit antibodies raised against purified MuSK (ref. 13). When agrin was added together with the plasmas (Fig. 3*a*, lower panels), the marked agrin-induced clustering which occurred in the presence of healthy control plasma was not seen in the presence of MuSK-Ab–positive MG plasma, indicating that the MuSK antibodies had inhibited the agrin-induced AChR clustering. A quantitative analysis of the data revealed that both the clustering (Fig. 3*b*), and the inhibitory activity (Fig. 3*c*), were found with MuSK-Ab–positive plasmas or IgG, but not with the one MuSK-Ab–seronegative preparation or the healthy controls tested. Moreover, both the clustering (Fig. 3*b*) and inhibitory (Fig. 3*c*) activity of an AChR-Ab–negative plasma were prevented by absorption with soluble MuSK, demonstrating that the MuSK antibodies were responsible for these effects. By contrast, plasmas from two patients with AChR-Ab–seropositive MG (Fig. 3*c*), and the non-IgG fractions of MuSK-Ab–positive MG patients

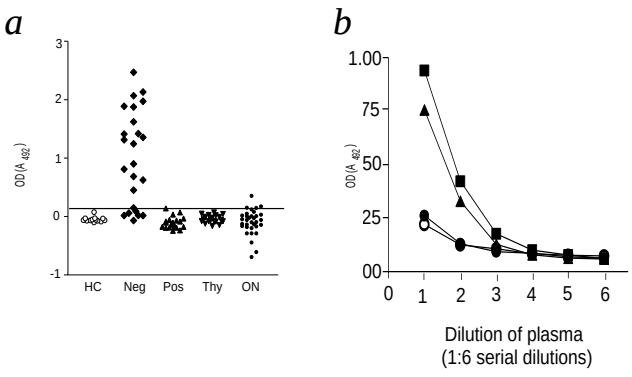

Fig. 2 MuSK antibodies can be detected by ELISA. ***a***, Antibodies to MuSK were found in 17 of 24 AChR-Ab–seronegative MG patients (Neg) compared with 13 healthy controls (HC). Negative or borderline values were found in anti-AChR–seropositive MG patients (Pos), including those with thymomas (Thy), and in patients with other neurological disorders (ON). ***b***, Titration of one AChR-Ab–seronegative MG plasma against different domains of MuSK. The antibodies bound strongly to MuSK constructs expressing the N-terminal domains, Ig1-4 (■) and Ig1-2 (▲; see Fig. 1*a*), but binding to the membrane-proximal Ig3-4 (●) domains was similar to that of a healthy control (○).

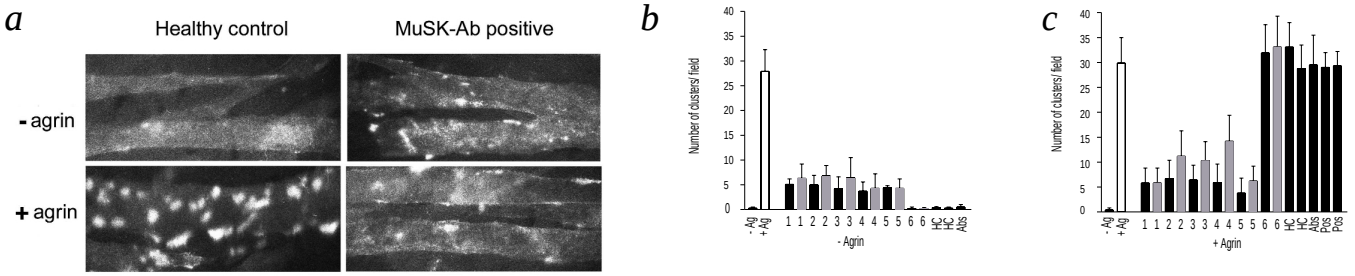

Fig. 3 MuSK IgG antibodies induce AChR clusters but inhibit agrin-induced clustering. ***a***, In the absence of agrin, a moderate number of AChR clusters, visualised by rhodamine-α-bungarotoxin, were induced in the presence of MuSK-Ab–positive MG plasma compared with that in healthy control plasma. Conversely, in the presence of agrin, large numbers of clusters were induced in healthy control plasma-treated myotubes, but not in myotubes treated with MuSK-Ab–positive MG plasma. ***b*** and ***c***, The AChR clusters without (*b*) or with (*c*) added agrin (Ag) in cultures treated with plasma (■) or IgG (■). Only the MuSK-Ab–positive plasmas and IgG preparations (1–5) affected AChR clusters; there was no effect of a MuSK-Ab–negative plasma (6), of 2 healthy control plasmas (HC), or of 1 MuSK-Ab–positive plasma after absorption with MuSK (Abs). Two AChR-Ab–seropositive samples (Pos) did not affect agrin-induced clustering. Clusters in control cultures without plasma or IgG (□).

(data not shown) did not affect agrin-induced clustering.

We show that AChR-Ab–seronegative IgG preparations containing MuSK antibodies interfere with the agrin/MuSK/AChR clustering pathway in myotubes and have the potential to alter MuSK function at the adult neuromuscular junction. As agrin does not appear to bind directly to MuSK, but via a hypothetical agrin-binding component called MASC (refs. 10,12), we speculate that the antibodies in AChR-Ab–seronegative patients bind to MuSK in such a manner as to prevent its interaction with MASC. This interaction is known to depend on the N-terminal half of the extracellular domains of MuSK (ref. 17) that we find to be the main target for the IgG antibodies in AChR-Ab–seronegative patients (Fig. 2*b*). How these antibodies cause seronegative MG is not yet clear. AChR-Ab–seronegative MG is mediated by humoral IgG antibodies that lead to a defect in neuromuscular transmission[4]. A few limited human biopsy studies[18,19] indicate that there is a reduction in AChR function at the neuromuscular junction, although total AChR numbers were variable. We propose that interference with MuSK function at the mature neuromuscular junction is one pathogenic mechanism in MuSK-Ab–seropositive MG patients, leading secondarily to reduced numbers and/or altered distribution of AChRs and other postsynaptic proteins. By analogy with AChR-Ab–positive MG (ref. 2), MuSK antibodies may not only inhibit MuSK function directly but also increase the turnover of MuSK, further reducing its activity. In addition, there could be complement-mediated damage to the AChR-containing postsynaptic membrane following IgG binding to MuSK and complement activation. Some evidence for complement deposition at the endplates of muscle from AChR-Ab–negative MG patients has been reported[20]. Elucidating the contribution of different mechanisms to the defect in neuromuscular transmission will require further *in vitro* and *in vivo* studies.

We have defined a novel specific target antigen, the receptor tyrosine kinase MuSK, for the auto-antibodies in AChR-Ab–seronegative MG. The antibodies have functional effects on agrin-induced AChR distribution in cultured myotubes, indicating that they could interfere with AChR numbers or distribution in mature muscle. The IgG antibodies were found in 70% of AChR-Ab–seronegative MG patients and not in AChR-Ab–seropositive MG patients, clearly distinguishing the two forms of the disease. Moreover, the antibodies can be detected by a simple ELISA that could be used for routine diagnosis and clinical management. Our results add a receptor tyrosine kinase (RTK) to the AChR and voltage-gated calcium and potassium channels that are already defined targets for antibody-mediated disorders at the neuromuscular junction[21]. Auto-antibodies to other RTKs have occasionally been implicated in some forms of endocrine disease[22]. Since RTKs are cell-surface molecules, regulating intracellular functions and activated by dimerization, they are attractive candidate antigens and should be considered in other antibody-mediated disorders. Moreover, as members of the RTK family are mutated in inherited diseases[23], MuSK may be involved not only in this acquired autoimmune disorder but also in congenital muscle diseases.

Methods

Patients. Samples were obtained from 24 patients (18 female, 6 male) with moderate or severe generalized MG in whom the standard radio-immunoprecipitation assay for anti-AChR antibodies[24] was negative on several occasions. All had typical fatigable muscle weakness. The diagnosis was confirmed by electromyographic evidence of a defect in neuromuscular transmission (a decrement of more than 10% in the amplitude of the compound muscle action potential on repetitive nerve stimulation at 3 Hz and/or an increase in jitter on single fiber studies), or by a positive response to anticholinesterase medication (edrophonium or pyridostigmine). The age at onset was 2–68 years (median 24) and the duration of symptoms at sampling was between 1 month and 13 years (median 1.0 year). In 18 cases, plasma was obtained during therapeutic plasmapheresis, which improved muscle strength. The remaining 6 samples were sera taken on first examination. Six of the patients had received corticosteroids for up to two months before sampling. Sera or plasmas were also obtained from healthy volunteers, from patients with AChR-Ab–positive MG, and from patients with other immune-mediated neurological disorders. IgG preparations were made using a ImmunoPure (G) IgG purification kit (Pierce, Rockford, IL)

MuSK and agrin expression constructs. Constructs encoding full-length MuSK (ref. 13) and the soluble fragment s-agrin (4/19)[25] have been described. MuSK deletion fragments comprising the entire extracellular domain (Ig1-4; aa 1–490)[9], or the first half containing two Ig-domains (Ig1-2; aa 1–231), were generated by insertion of artificial stop signals at these positions by a PCR approach. N-terminal fragments of MuSK comprising the membrane-proximal extracellular domains, including Ig-domains 3 and 4 (Ig3-4; aa 203–490), or the transmembrane region and intracellular domain (MuSK–Ig1-4, aa 491–868) were generated. The corresponding cDNA-fragments, including a newly introduced *Sph*I site, were linked to a vector containing an artificial signal sequence followed by 6 histidines and a 10-aa epitope-tag[25]. All constructs were transiently transfected into COS7 cells[11]. For the production of soluble agrin and MuSK constructs, cells were switched to serum-free medium the second day after transfection.

Conditioned media, containing MuSK or agrin fragments, were removed 24 hours later and analyzed by western blotting to confirm expression.

Immunostaining of MuSK-transfected COS7 cells. COS7 cells were plated onto chamber slides the day after transfection. Two days later, cells were fixed with 2% paraformaldehyde and stained as described[13]. Plasmas of MG patients and controls were analyzed at various dilutions (between 1:20 and 1:5000). Bound antibodies were visualized with secondary antibodies conjugated to Cy3 (anti-human IgG, Dianova, Hamburg, Germany). In all experiments, expression of transfected MuSK constructs was confirmed by staining parallel slides with rabbit antibodies against MuSK (ref. 13).

Immunoprecipitation of MuSK. Detergent extracts were prepared from MuSK-transfected COS7 cells or from C2C12 myotubes that had been fused for 5 days[11]. The immunoprecipitation was performed as described[11,13]. AChR-Ab–seronegative MG, AChR-Ab–seropositive MG plasmas and control plasmas were incubated with the extracts at 1:20. Rabbit anti-MuSK serum was used at 1:100. MuSK in the immunoprecipitates was analyzed by western blotting using affinity-purified serum antibodies directed against the MuSK cytoplasmic sequence[13]. One AChR-Ab–seronegative MG plasma was absorbed with soluble MuSK, by addition of an equal volume of conditioned medium of COS7 cells transfected with the soluble MuSK fragment Ig1-4, for 2 hours. The mixture was then diluted further before use.

ELISA detection of antibodies to MuSK. Conditioned medium from MuSK-transfected COS7 cells or from control cells mock-transfected with salmon sperm DNA, was diluted 1:1 with 100 mM $NaHCO_3$ buffer (pH 9.5) and applied overnight to ELISA plates. Plasmas were first tested at 1:5 in triplicates and subsequently at 1:10 in duplicates. Bound antibodies were detected by horseradish peroxidase-protein A (Amersham, Braunschweig, Germany) followed by o-phenylenediamine and measuring A_{492}. For each sample, nonspecific immunoreactivity, determined by incubation of plates coated with conditioned medium from mock-transfected COS7 cells, was subtracted. The efficient immobilization of deletion fragments of MuSK was confirmed by ELISA with an antibody directed against the HA-epitope.

AChR aggregation assay. The mouse muscle cell line, C2C12, was used to determine functional effects of antibodies. Cells were plated onto chamber slides, fused and treated with or without agrin and/or plasmas or IgGs for 5 hours[13]. After fixation, AChRs were visualised with rhodamine-α-bungarotoxin and the number of aggregates from more than 20 microscopic fields, and at least 2 independent cultures, were measured as described[25]. Control experiments showed that the MuSK fragment, used for preincubation of some plasmas, did not inhibit AChR aggregation in the final concentration present in the assay. Results are expressed as mean ± s.e.m.

Acknowledgments

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