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ORIGINAL COMMUNICATION

Anti-LRP4 autoantibodies in AChR- and MuSK-antibody-negative myasthenia gravis

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Abstract Myasthenia gravis (MG) is an autoimmune disorder characterized by a defect in synaptic transmission at the neuromuscular junction causing fluctuating muscle weakness with a decremental response to repetitive nerve stimulation or altered jitter in single-fiber electromyography (EMG). Approximately 80% of all myasthenia gravis patients have autoantibodies against the nicotinic acetylcholine receptor in their serum. Autoantibodies against the tyrosine kinase muscle-specific kinase (MuSK) are responsible for 5–10% of all myasthenia gravis cases. The autoimmune target in the remaining cases is unknown. Recently, low-density lipoprotein receptor-related protein 4 (LRP4) has been identified as the agrin receptor. LRP4 interacts with agrin, and the binding of agrin activates MuSK, which leads to the formation of most if not all postsynaptic specializations, including aggregates containing acetylcholine receptors (AChRs) in the junctional plasma membrane. In the present study we tested if autoantibodies against LRP4 are detectable in patients with myasthenia gravis. To this end we analyzed 13 sera from patients with generalized myasthenia gravis but without antibodies against AChR or MuSK. The results showed that 12 out of 13 antisera from double-seronegative MG patients bound to proteins concentrated at the neuromuscular junction of adult mouse skeletal muscle and that approximately 50% of the tested sera specifically bound to HEK293 cells transfected with human LRP4. Moreover, 4 out of these 13 sera inhibited agrin-induced aggregation of AChRs in cultured myotubes by more than 50%, suggesting a pathogenic role regarding the dysfunction of the neuromuscular endplate. These results indicate that LRP4 is a novel target for autoantibodies and is a diagnostic marker in seronegative MG patients.

Keywords Myasthenia gravis · Low-density lipoprotein receptor-related protein · LRP4 · Autoimmune disorder

Introduction

Myasthenia gravis (MG) is an autoimmune disorder characterized by use-dependent muscle weakness due to compromised synaptic transmission at the neuromuscular junction [1–3]. In approximately 80% of myasthenia gravis patients, the disease is mediated by antibodies against the nicotinic acetylcholine receptor (AChR) [4]. These antibodies reduce the number of functional AChRs at the muscle fiber endplate by increasing their degradation and turnover [5], induce complement-mediated damage to the muscle fiber [6], and block acetylcholine binding to the receptor [7]. In up to 50% of MG patients without autoantibodies to AChR, autoantibodies against the muscle-specific tyrosine kinase MuSK have been detected [8–11]. MuSK is a 110-kDa protein initially isolated from rat skeletal muscle. In skeletal muscle, MuSK is concentrated

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at the neuromuscular junction and plays a key role in the agrin-mediated formation, maintenance, and regeneration of postsynaptic specializations, including aggregates containing AChR and many other proteins [12–14]. The spectrum of clinical symptoms of MG with autoantibodies to AChR is comparable but not identical to those with autoantibodies to MuSK [11, 15, 16]. Moreover, autoantibodies against MuSK from MG patients inhibit agrin-mediated aggregation of AChRs and reduce AChR expression in C2C12 cells in vitro, and upregulate muscle RING finger protein 1 (MuRF-1) in C2C12 cells in vitro as well as in muscle tissue in vivo and atrogin expression in TE671 cells in vitro, indicating muscle atrophy [8, 17–19]. These results suggest that anti-MuSK antibodies may alter AChR clustering and in addition have downstream effects on expression of AChR and potentially other proteins concentrated at the neuromuscular junction. Collectively, these findings might explain the generalized muscle weakness observed in MuSK-seropositive MG.

In approximately 10% of patients with generalized MG, no autoantibodies against MuSK or against AChRs could be detected by routine methods [3, 20]. Up to 66% of these patients, however, have low titer of low-affinity antibodies against AChR that were only detectable in a cellular assay using HEK cells cotransfected with human AChR subunits and rapsyn to induce AChR clustering on the cell surface [21]. Onset and progression of the disease in these double-seronegative MG cases is very similar to AChR- or MuSK-seropositive MG, although subtle differences exist in age at onset, involvement of thymus, and maximum severity and regional distribution of myasthenic weakness [20, 22, 23]. Several lines of evidence have indicated that, in these double-seronegative patients, the muscle weakness is caused by pathogenic autoantibodies; for example, exchange of plasma or immunosuppressive therapy resulted in marked but transient improvement of myasthenic weakness [23], and transfer of immunoglobulin G (IgG) from these patients induced defects in neuromuscular transmission in mice [7, 24].

Recently, low-density lipoprotein receptor-related protein 4 (LRP4) was identified as the agrin receptor in skeletal muscle [25, 26]. Since LRP4 is concentrated at the neuromuscular junction, binds to agrin, interacts with MuSK, and is essential for the formation of synaptic specializations at the neuromuscular junction, including aggregates of AChR, it is a candidate for autoantibodies in double-seronegative MG. In agreement with this hypothesis, antibodies against the extracellular portion of LRP4 have very recently been detected in 9 out of 300 AChR- and MuSK-seronegative MG patients, but further details of neuromuscular symptoms or neurophysiological findings of the patients were not reported [27]. In this study we analyzed the presence of anti-LRP4 antibodies in sera from AChR- and MuSK-seronegative MG patients. We report that approximately 50% of the sera from double-seronegative MG patients contain antibodies that bind to LRP4. Moreover, we show that some of these sera inhibit agrin-mediated AChR aggregation in cultured myotubes in a dose-dependent manner. These data confirm the neuromuscular junction as a target for autoantibodies in some double-seronegative MG cases and indicate LRP4 as a novel autoantigen in myasthenia gravis patients. Moreover, our results suggest that autoantibodies against LRP4 are pathogenic and lead to muscle weakness by interfering with the agrin:LRP4:MuSK complex.

Materials and methods

Sera from AChR- and MuSK-seronegative MG patients

Sera were obtained from outpatients of the Friedrich-Baur-Institute, Munich, from the Department of Neurology of the University Medical Center Tübingen, and from the Department of Neurology at the University Clinic Regensburg. Sera were considered seronegative for AChR and MuSK on the basis of standard assay procedures (radioimmunoprecipitation using ^{125}I α-bungarotoxin:AChR complexes or ^{125}I-labeled recombinant human MuSK protein, respectively). We initially screened sera from a total of 13 double-seronegative MG patients (Table 1). Additionally, one serum from a MuSK-seropositive patient (index case 2) and one from a patient with genetically confirmed late-onset Pompe disease (index case 14) were included for control purposes in addition to four sera form healthy controls. Moreover, 11 sera from confirmed MuSK-seropositive MG patients were screened for coexistence of anti-LRP4 immunoreactivity. During the course of the study we obtained 25 additional double-seronegative antisera which were, however, only analyzed for their reactivity with human LRP4 (huLRP4). All samples were obtained with the full consent of the patients in accordance with the 1964 Declaration of Helsinki. The work has been approved by the Ethical Committee of the Ludwig-Maximilians-University (LMU) of Munich. Sera were anonymized, and the experiments were performed double-blind to comply with the regulations of the Ethics Committee of the LMU Munich.

MG in these patients has been diagnosed on the basis of clinical features and neurophysiological investigations. Clinical information collected included demographic data, symptoms, and signs. Laboratory data included information on edrophonium testing and repetitive nerve stimulation (RNS). Treatment and efficacy, including response to acetylcholine esterase (AChE) inhibitors, were also recorded. Nonresponsiveness to AChE inhibitors comprised

Table 1 Summary of the reactivity of the sera from AChR- and MuSK-seronegative MG patients

Index case	Sex	Age at onset (years)	Facial, bulbar, neck weakness	Limb weakness	Decrement (EMG)	Response to AChE inhibitors	NMJ staining	huLRP4 staining	AChR aggregation as % of control
1	F	32	+	+	+	+	−	+	67
2 (MuSK seropositive)	M	42	+	+	+	+	++	+	37
3	M	39	+	+	+	+	++	+	73
4	F	54	+	−	+	+	++	−	77
5	F	28	+	+	+	+	+	+	60
6	M	36	+	+	+	−	++	+	16
7	F	17	+	+	+	+	+	−	69
8	F	46	−	−	+	+	+	+	26
9	M	41	+	+	+	+	+	+	64
10	F	62	+	+	+	+	+	−	27
11	F	60	+	+	+	+	+	−	63
12	F	57	+	−	+	+	++	−	75
13	F	79	+	−	+	+	+	−	77
14 (Pompe dis.)	F	56	−	+	−	+	−	−	97
15	F	39	–	+	+	+	+	+	18

Staining of the neuromuscular junction (NMJ) was scored as strongly positive (++), weakly positive (+), or negative (–) according to the criteria detailed in the "Results" section and in Fig. 1

hypersensitivity or worsening of MG symptoms, intolerance of side-effects, or lack of clinical response.

Transfection and staining of HEK293 cells

Full-length complementary DNA (cDNA) coding for human LRP4 (huLRP4) was obtained from Imagenes GmbH (Berlin, Germany) and subcloned into the pMES vector [28], which allows identification of transfected cells by simultaneous expression of enhanced green fluorescent protein (EGFP). Successful cloning and surface expression of LRP4 was confirmed by Western blotting and immunocytochemistry, respectively.

HEK293 cells were transfected using Superfect transfection reagent (Invitrogen) and analyzed 48 h after transfection. Either cells were stained after fixation in 4% paraformaldehyde or unfixed cells were live-stained with the primary antibody and fixed thereafter for 10 min in 4% formalin. Live-staining appeared to result in lower background and improved signal-to-noise ratio, but the principal findings were identical with both methods. Excess fixative was removed by washing the cultures with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cells were permeabilized using 0.2% Triton X-100 and subsequently stained by incubation with the primary or with secondary antibody (Alexa594-conjugated anti-human IgG antibody; Molecular Probes; 24 µg/ml final concentration). Primary antibodies were the sera from MG patients, from healthy control persons (dilution 1:2,000), or a commercially available rabbit anti-LRP4 antiserum (Atlas Antibodies, Stockholm, Sweden; dilution 1:500); this antibody is specific for LRP4 and does not cross-react with other members of the LRP protein family. Nuclear staining was obtained using 4′,6-diamidino-2-phenylindole (DAPI). Cells were analyzed using a Zeiss LSM 710 laser scanning confocal microscope.

Immunohistochemistry

Longitudinal cryostat sections of adult mouse skeletal muscle were obtained from 4% paraformaldehyde immersion-fixed mouse leg muscles. Sections of 12 µm thickness were stained as detailed in [29] using the appropriate Alexa488-conjugated goat anti-rabbit or goat anti-human secondary antibody (Molecular Probes; 24 µg/ml final concentration). Rabbit antibodies against agrin served as positive control [30]. Neuromuscular junctions were identified by costaining with Alexa594-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR). Specimens were analyzed using a photomicroscope (Olympus BX61) equipped with epifluorescence optics using fluorescence filters of the appropriate wavelength.

Myotube cultures and AChR aggregation assay

Chick myotube cultures were prepared as described in [31] with the modifications from Gesemann et al. [32]. After 5 days in culture, 75 µl serum was added to 1.5 ml culture medium simultaneously with 8 U chick agrin (C125 isoform A4B8). We found no evidence for myotoxicity at this

concentration. AChR aggregates were analyzed 16 h later by staining with Alexa594-conjugated α-bungarotoxin as described [31]. Aggregation activity was quantified by counting the number of AChR aggregates per myotube segment. At least 10 segments per dish and at least three dishes were scored in each of three independent experiments. Results were expressed as percent of control (which was set to 100%) after subtraction of the background number of spontaneous AChR aggregates.

Results

Clinical presentation and neurophysiological examination summary

The initial study started with sera from 13 double-seronegative MG patients, which were numbered consecutively (Table 1). All but one patient were Caucasian; one patient came from Ghana. There was marked female predominance of over 80%. Age at onset was 17–79 years (mean 46.5 years).

Clinical details

Duration from first symptoms to clinical diagnosis of MG ranged from 2 months to 20 years. Symptoms reported included facial and bulbar weakness in 12 (86%) patients, neck weakness in 12 (86%), limb weakness in 10 (71%), ocular weakness in 7 (50%), and breathing difficulty in 4 cases (29%). One patient experienced a myasthenic crisis. Duration of the disease ranged between 2 and 20 years (mean 7.5 years).

Diagnostic testing

Edrophonium testing was positive in 13 out of 13 tested cases (100%). Repetitive nerve stimulation at 3 Hz (RNS) was abnormal in 13 of 13 (100%). RNS testing of muscles innervated by the facial nerve was abnormal in 13 of 13 tested patients (100%), while accessory RNS testing was abnormal in only 7 of 13 patients (54%). RNS testing of muscles innervated by the ulnar nerve was abnormal in 3 of 13 (23%) cases.

Treatment response

With the exception of one, all patients responded to AChE inhibitors. Twelve patients (86%) were treated with pyridostigmine, prednisone, and immunosuppressive agents (usually azathioprine) at varying times. Nonresponsiveness to AChE inhibitors was observed in one patient, and hypersensitivity or worsening of symptoms by AChE inhibitors in two patients. Only three patients improved on AChE therapy without additional use of immunosuppressants. Twelve patients received two forms of immunotherapy. Improved status was seen in 50% of patients on corticosteroids and azathioprine. Intravenous immunoglobulins were given in a minority of three cases with good results. Two patients were on corticosteroids alone. Thymectomy was performed in one patient (index case 5) at age of 22 years. A written histopathology report stated that no thymoma or thymic hyperplasia was found.

Follow-up ranged from 2 months to 12 years (mean 6.1 years). Long-term outcome was assessed in 10 patients who were followed for at least 4 years, 2 of whom (20%) achieved complete stable remission, pharmacological remission, or minimal manifestation status. Improved status was achieved in all 10 patients. These data collectively show that double-seronegative MG patients are clinically similar to MG patients with autoantibodies to AChR.

Sera from seronegative MG patients label adult mouse neuromuscular junctions

To test if the sera from the double-seronegative MG patients contained antibodies against proteins concentrated at the neuromuscular junction, we stained cryostat sections of adult mouse skeletal muscle. Neuromuscular junctions were identified by their high concentration of AChR, labeled by Alexa594-conjugated α-bungarotoxin. Figure 1 shows representative examples of the three different staining patterns that were observed. Two sera (index cases 1 and 14) did not specifically label the neuromuscular junction (Fig. 1b, b′; scored as "−" in Table 1). Likewise, none of the healthy control sera stained the neuromuscular junction (data not shown). Eight sera weakly but specifically labeled the neuromuscular junction (scored as "+" in Table 1). However, the labeling did not always precisely align with the extent of the pretzel-like pattern of the AChR (index cases 5, 7–11, 13, and 15; Fig. 1c, c′, d, d′). Five sera strongly labeled the adult mouse neuromuscular junctions, and the staining overlapped with the AChR aggregates along the entire extent of the neuromuscular junction (sera 2–4, 6, and 12; Fig. 1e, e′; scored as "++" in Table 1). These results show that 12 out of the 13 double-seronegative sera cross-react with mouse tissue and labeled proteins concentrated at the neuromuscular junction, indicating that sera from AChR- and MuSK-antibody-negative MG patients have antibodies against other proteins concentrated at the neuromuscular junction. The results also show the expected absence of immunoreactivity in index case 14 (diagnosed with Pompe disease) and confirm the reactivity of the MuSK-seropositive patient (index case 2) with the neuromuscular junction.

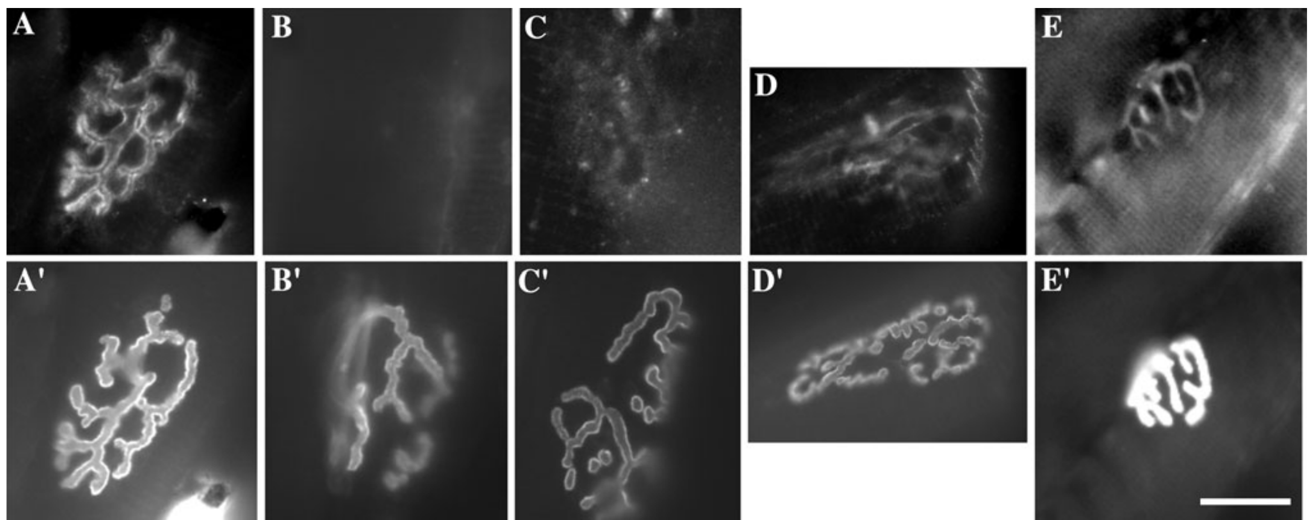

Fig. 1 Staining of adult NMJs with antisera from double-seronegative MG patients. Representative examples for the different patterns obtained by staining adult mouse neuromuscular junctions with the sera from double-seronegative MG patients (**a**–**e**) and with Alexa594-conjugated α-bungarotoxin (**a**′–**e**′) in the same section are shown. While anti-agrin immunoreactivity (**a**, **a**′) was concentrated at the neuromuscular junction, serum from index case 1 did not label the NMJ (**b**, **b**′). Sera 5 (**c**, **c**′) and 8 (**d**, **d**′) stained the NMJ. However, in the latter cases the staining did not precisely align with the Alexa594-conjugated α-bungarotoxin staining. In contrast, serum 12 (**e**, **e**′) strongly stained the neuromuscular junction, and the staining precisely overlapped with the AChR labeling. *Scale bar* 30 µm

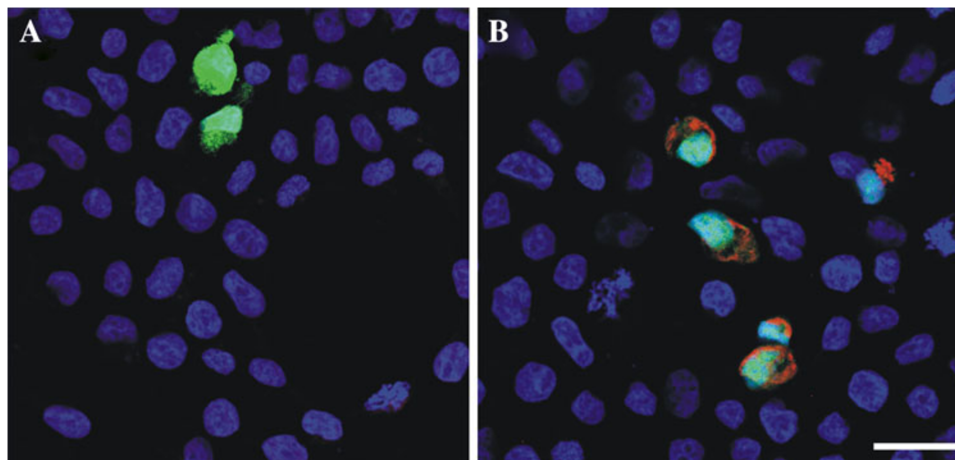

Fig. 2 Staining of HEK293 cells transfected with huLRP4 by double-seronegative antisera. Confocal images are shown of HEK293 cells transiently transfected with human LRP4 cDNA. EGFP expression (*green channel*) indicates transfected cells; *blue color* shows DAPI nuclear staining. While some sera did not label the transfected cells (serum 4, **a**), others strongly reacted with LRP4-expressing cells (serum 6, **b**), indicated by *red fluorescence*. Both sera were used at dilution of 1:2,000. In the cases shown, cells were stained after fixation and permeabilization. *Scale bar* 20 µm

Sera from seronegative MG patients react with LRP4-expressing cells

To test if any of the sera contained antibodies that bind to LRP4, we stained HEK293 cells transiently transfected with the full-length human LRP4 cDNA. Figure 2 shows representative examples of sera reacting with LRP4-expressing HEK293 cells (index case 6 in Fig. 2b) and of a serum not reacting with LRP4 (index case 4 in Fig. 2a). We found no anti-LRP4 immunoreactivity with serum from index case 14 diagnosed with late-onset Pompe disease and with sera from healthy controls. We did however detect anti-LRP4 antibodies in the MuSK-seropositive serum (index case 2). Analysis of the sera showed that 7 of the 13 double-seronegative antisera (corresponding to approximately 50%) strongly reacted with LRP4-transfected cells (Table 1). None of the sera reacted with cells transfected with the empty pMES vector (data not shown).

Since a previous study had shown antibodies against LRP4 in sera from MuSK-seropositive MG in 3 out of 28

cases [27], and since we detected anti-LRP4 autoantibodies in a MuSK-seropositive serum (index case 2), we analyzed 10 additional sera from confirmed MuSK-seropositive patients. We found no immunoreactivity on cells transfected with the huLRP4 cDNA with any of these sera, indicating that index case 2 is likely to be a rare exception (data not shown).

To more quantitatively analyze the percentage of LRP4-seropositive MG patients within the cohort of AChR- and MuSK-seronegative patients, we tested 25 additional sera from double-seronegative patients. We observed anti-LRP4 immunoreactivity in 12 of these patients, further confirming that anti-LRP4 antibodies can be detected in approximately 50% of double-seronegative patients (data not shown). Collectively, these results indicate that approximately half of the AChR- and MuSK-seronegative MG patients produce antibodies that bind to native LRP4, suggesting that autoantibodies against LRP4 are pathogenic in double-seronegative MG.

Sera from seronegative MG patients inhibit agrin-induced AChR aggregation

To investigate if the antisera from seronegative MG patients interfered with agrin-induced AChR aggregation, AChR aggregates were induced by agrin in chick primary myotube cultures in the presence or absence of the MG sera. Several sera strongly inhibited agrin-induced AChR aggregation by more than 50%, including the sera from index cases 2, 6, 8, and 15 (Fig. 3). Other sera inhibited AChR aggregation by only 20–40%. The inhibition of AChR aggregation was dose dependent (data not shown). The control serum from the genetically confirmed late-onset Pompe disease patient (index caseonly 14) did not significantly inhibit agrin-induced AChR aggregation, indicating absence of general anti-AChR aggregation activity in human serum (Fig. 4).

Antibody-mediated dimerization and, thus, activation of MuSK tyrosine kinase activity has been shown to induce AChR aggregation on cultured myotubes even in the absence of agrin [33]. To rule out that the sera from seronegative MG patients contain AChR aggregation activity, we incubated chick myotube cultures with antisera in the absence of agrin. None of the sera induced AChR aggregates (data not shown), indicating that the inhibition of AChR aggregation is not compromised by endogenous AChR aggregation activity present in the sera from double-seronegative patients.

Discussion

Previous studies identified autoantibodies against intracellular antigens concentrated at the neuromuscular junction specifically in double-seronegative MG, including antibodies against filamin and vinculin [34, 35], but the cause of muscle weakness in these cases remained unclear. Identification of LRP4 as the MuSK-binding agrin receptor

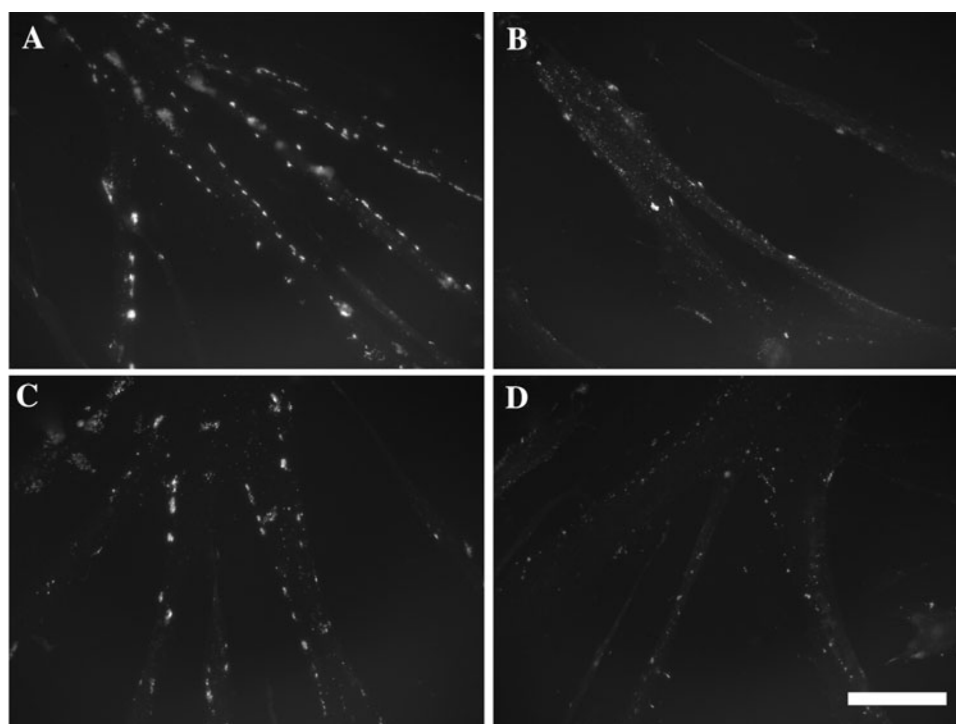

Fig. 3 Inhibition of agrin-induced AChR aggregation by MG sera. Examples of fluorescent micrographs from 6-day-old cultures of chick myotubes that were incubated overnight with approximately 8 U agrin in the presence of 75 μl serum from MG patients are shown. The myotubes were stained with Alexa594-conjugated α-bungarotoxin. While agrin induced numerous AChR aggregates in the presence of control serum (index case 14; **a**), the aggregation activity of agrin was severely inhibited by several sera, including sera 15 (**b**) and 6 (**d**). Other sera, including serum 4 (**c**), only slightly reduced the number of AChR aggregates. *Scale bar* 50 μm

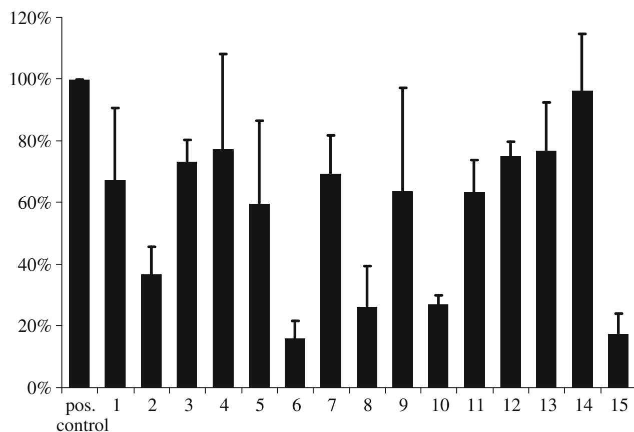

Fig. 4 Quantification of AChR aggregation inhibition by seronegative sera. Inhibition of AChR aggregation was determined by counting the number of AChR aggregates per myotube segment in the presence of the indicated serum. The number of aggregates in the absence of any serum served as a positive control and was set to 100%. The number of spontaneous aggregates was subtracted from all values. Several sera, including those from index cases 6, 8, 10, and 15, strongly inhibited agrin's AChR aggregation activity, while others only slightly reduced the number of AChR aggregates. *Bars* show mean ± standard error of the mean (SEM) with $N = 3$ in all cases

in skeletal muscle tissue suggested the possibility that autoantibodies against this membrane protein might cause myasthenia gravis [25, 26]. In agreement with this hypothesis, antibodies against LRP4 have been detected in 9 out of 300 double-seronegative MG patients [27].

LRP4 is a member of the low-density lipoprotein receptor-related protein family of transmembrane proteins. It is expressed in multiple tissues in mouse and has important functions during development and morphogenesis of limbs, ectodermal organs, lung, and kidney [36–39]. In adult skeletal muscle, LRP4 is specifically expressed by subsynaptic myonuclei and the protein is concentrated at the NMJ [40], where it binds the extracellular matrix proteoglycan agrin and subsequently activates the tyrosine kinase MuSK. This interaction activates an intracellular signaling cascade leading to the formation of most if not all postsynaptic specializations, including aggregates containing AChRs and other molecules [41]. The phenotype of mice with complete deletion of MuSK or LRP4 are very similar. Both die at birth with defects in presynaptic and postsynaptic differentiation, a lack of aggregates containing AChRs and other postsynaptic proteins, as well as a lack of synapse-specific gene expression [40, 42]. In contrast, LRP4 point mutations in humans affect plasma membrane localization of the protein and cause bone malformations including syndactyly, altered Wnt/β-catenin signaling, and renal malformations, and result in Cenani–Lenz syndrome [43]. Interestingly, no neuromuscular phenotype was detected in these patients, possibly due to the low amount of residual LRP4 protein expression. Conversely, bone malformations were not observed in the MG patients in our study, suggesting that the main immunogenic regions of the autoantibodies do not interfere sufficiently with LRP4 function in bone metabolism. In addition, the late onset of MG might allow activation of compensatory mechanisms or might not have the severe consequences observed in Cenani–Lenz syndrome or in corresponding mouse models.

In the present study, several lines of evidence (summarized in Table 1) suggest that LRP4 is a novel target for autoantibodies in patients with double-seronegative myasthenia gravis. Specifically, our results indicate autoantibodies against LRP4 in approximately 50% of double-seronegative MG cases, considerably higher than the 3% reported previously [27]. The reasons for this difference are unknown, but explanations might be the Asian versus Caucasian origin of the patients analyzed or the screening method used. In any case, it appears likely that targets for autoantibodies in addition to AChR, MuSK, and LRP4 exist in rare cases of MG.

The cohort of patients that was analyzed in the present study corroborates some cardinal features of LPR4-seropositive MG, including marked female predominance, similar to what has been described for MuSK-antibody-positive MG [11, 20]. Mean age at disease onset was 46.5 years in our pilot cohort, thus the anti-LRP4 antibody-mediated MG onset seems to be later than for other MG groups. Our study shows that 86% of LPR4 MG patients have moderate to severe weakness at onset. Thus, major clinical feature in LRP4-antibody-positive patients seems to be rather indistinguishable from AChR-antibody-positive patients. All patients received AChE inhibitor therapy. Nonresponsiveness to AChE was observed in one patient; hypersensitivity or worsening of symptoms was observed in two patients. All patients improved on a combination of AChE inhibitors and two or more forms of immunotherapy. All 13 patients achieved complete stable remission, pharmacologic remission, or minimal manifestation status. There was no MG-related death in this cohort.

A recent report indicated that more than 60% of AChR- and MuSK-antibody-negative MG patients have low-affinity autoantibodies to AChR [20]. These antibodies were of low affinity and could not be detected unless human muscle AChR was aggregated on the cell surface by rapsyn coexpression [21]. This indicates that at least some double-seronegative MG patients have antibodies to AChR which are not detected by the routine immunoprecipitation assay. In agreement with the occasional coexistence of distinct autoantibodies, 3 out of 28 MuSK-seropositive MG patients were positive for LRP4 [27], suggesting the presence of multiple targets for autoantibodies also in double-seronegative MG patients [44]. One of the sera analyzed in this study was MuSK-antibody-positive and also stained

LRP4-transfected cells (index case 2). This suggests the rare coexistence of multiple antibodies against several neuromuscular junction-associated proteins in LRP4-antibody-positive MG patients. However, testing 11 additional sera, we failed to detect anti-LRP4 antibodies in MuSK-seropositive MG cases. Although we cannot rule out that the sensitivity of our immunocytochemical assay was not high enough to detect low concentrations of anti-LRP4 antibodies, our results as well as those from Higuchi et al. [27] suggest that MG cases with antibodies against MuSK and LRP4 are rare. It will be interesting to analyze the entire spectrum of MG-associated autoantibodies, including LRP4, MuSK, titin or the ryanodine receptor, in a more systematic manner, in an assay system with higher sensitivity, and in a larger cohort of patients.

While antibodies against MuSK as well as sera from MuSK-antibody-positive MG patients dimerize MuSK and induce MuSK autophosphorylation as well as aggregation of AChRs on cultured C2C12 muscle cells [33], antisera against LRP4 did not appear to have such activity. This is surprising, since MuSK and LRP4 have been shown to interact independently of agrin binding to LRP4 [26]. This suggests that dimerization of LRP4 is not sufficient for MuSK activation and initiation of the intracellular signaling cascade leading to aggregation of AChRs.

While there is convincing evidence for a role of LRP4 during the formation of synaptic specializations at the developing neuromuscular junction [25, 26, 40], little is known about its role in mature skeletal muscle, where the neuromuscular junction is rather stable. It therefore remains to be determined how antibodies against LRP4 could cause the muscle weakness and the deficits in neuromuscular transmission in the MG patients. One possibility is that they bind complement and cause a complement-mediated destruction of the motor endplate, similar to that in AChR-seropositive MG [6]. An alternative possibility is that the antibody binding to LRP4 causes increased turnover of the membrane protein, as has been shown for AChR-seropositive myasthenia gravis. Finally, it is possible that the sera reverse AChR aggregation or cause dispersal of AChRs at adult neuromuscular junctions by either interfering with agrin binding to LRP4 or with the LRP4 interaction with MuSK. In agreement with a role for the agrin–LRP4 interaction in the pathogenesis of MG, the IgG fraction from 3 out of 300 double-seronegative MG patients interfered with the binding of agrin to LRP4 in vitro [27]. This suggests the possibility that, in these specific cases, the muscle weakness is due to inhibition of the agrin–LRP4 interaction at the neuromuscular junction. Our results showing inhibition of agrin-mediated AChR aggregation in cultured myotubes are in agreement with the interference of the antibodies with the agrin–LRP4–MuSK interaction. Although we cannot distinguish if this inhibition of AChR aggregation is due to an interference with the agrin–LRP4 or of the LRP4–MuSK interaction, in both cases the AChR concentration at neuromuscular junctions would subsequently be reduced. This suggests a mechanism that might lead to the muscle weakness observed in MG patients. These results also indicate that continuous activity of LRP4 is required to maintain the synaptic specializations at the adult neuromuscular junction.

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Conflict of interest The authors declare that they have no conflict of interest.

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