厚生労働科学研究費補助金
こころの健康科学研究事業

重症筋無力症の病態解明と診断法および治療法の開発

平成20年度　総括研究報告書

主任研究者　重本和宏

平成21（2009）年　4月

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主任研究者　　重本和宏

財団法人東京都高齢者研究・福祉振興財団　東京都老人総合研究所　研究部長

研究要旨

我々は、精製したMuSK蛋白をウサギに免疫することにより、抗MuSK自己抗体により重症筋無力症を発症することを世界で初めて示すことを報告した(*JCI*. 2006)．　これにより抗MuSK抗体が重症筋無力症の原因となることを証明して、神経筋疾患の新しい疾患概念を確立することに貢献した．　また、MuSK抗体測定法を開発して(*Neurology*. 2004, 2005)、MuSK抗体MG患者の調査研究を宇多野病院、神戸薬科大学と共同で行い昨年報告した(*Eur J Neurology*. 2007、*J Neurol Scineces*. 2007)．そして抗MuSK抗体陽性重症筋無力症患者の病態研究から，発症メカニズム、診断、治療法に関する解決目標を明らかにしている．しかし患者を対象とした研究は倫理面の制約があり、患者と同じ病態の疾患モデル動物が必要である．当該研究では世界に先駆けてその作成に成功したウサギ疾患モデルとマウスを使い、抗MuSK抗体陽性重症筋無力症の病態を明らかにして、さらに治療法の開発を目的として研究を進めている．　今年度は、ウサギ疾患モデルの神経筋シナプスの形態変化を共焦点顕微鏡と透過型電子顕微鏡で詳細に明らかにしたので報告する．発症したウサギの神経筋シナプス全体の形態変化が観察され、自己抗体がMuSKによるシナプスの維持機構を阻害することで発症することが明らかとなった．　抗MuSK抗体陽性の重症筋無力症と抗AChR抗体陽性重症筋無力症との病態像の違いはそのメカニズムによるものと考えられる．またウサギモデルの血清に存在する抗MuSK抗体は、一価の抗原結合部位しかないにも関わらずMuSKの機能を抑制する．発症したウサギのシナプス後膜に補体による破壊像が観察されない結果とあわせて、重症筋無力症が補体の関与がなくても発症することが明らかとなった．

A. 研究目的

高齢社会を背景に重症筋無力症 (myasthenia gravis:MG) の患者数が我が国でも増加していることが、2006年に実施された厚生労働省の免疫性神経疾患に関する調査で明らかになった．18年前の全国調査に比べ総数で2.5倍 (いずれも推定で6000人から1万5,100人へ)、10万人当たりの有病率も5.1人から11.8人へと増えている．欧米では1990年代になってから、50才以上の年代で予想されたよりも多くの患

などでも同様の報告が発表された．そして、2007 年に開催された 11th International Conference "Myasthenia Gravis and Related Disorders" (New York Academy of Science 主催) でも、高齢者 MG の増加とその臨床的特徴が注目された． 高齢者の MG 診断では、眼瞼下垂、複視、構音障害、嚥下困難を含む筋力低下などの MG に特徴的な症状が、若年者に比べ見過ごされがちになる．

抗 MuSK 抗体陽性重症筋無力症はしばしば急激に症状が悪化し、球筋や呼吸筋力の低下による重症化するために早急に治療の方針計画を立てる必要がある．しかも治療に反応せず急速に悪化する症例もある． 抗アセチルコリンエステラーゼ薬は抗 AChR 抗体陽性重症筋無力症のほとんどに対して有効であるが、抗MuSK抗体陽性重症筋無力症患者に対しては効果が薄いばかりでなく，むしろ抗コリン作動性クリーゼなど過敏性を示す症例も多いことが明らかとなっている． 胸腺摘出は抗 MuSK 抗体陽性重症筋無力症に対する有効性は我々の胸腺摘出の有効性に関して、我々や他の報告からも否定的であるのが現状である．

我々は、抗MuSK抗体の発見された2001年からMuSK抗体測定の診断システムを神戸薬科大学(太田教授)との共同研究で開発し実用化している． 臨床現場からの依頼に対して、宇多野病院にて無償で検査を受け 1-2日で診断結果を伝え迅速な医療方針の確立に貢献している．MuSK-MGの病態はAChR-MGとは異なることを明らかにしている．本邦の症例でも欧米と同じく重症例が多く、眼症状，構音や嚥下障害などの球麻痺，および呼吸筋麻痺が抗AChR抗体陽性重症筋無力症と比較して多く症状も重症であるが、四肢の筋力低下はあまり顕著でないケースもあることを昨年度報告している． しかし抗AChR抗体陽性重症筋無力症とは異なる病態が生じるメカニズムは未だ解明されていない．そして従来の治療法に対して難治性である抗MuSK抗体陽性重症筋無力症の発症メカニズムを明らかにするためには、疾患動物モデルを使った研究は重要な鍵を握っている．

我々はウサギを使った動物実験によりMuSK抗体でMGが発症することを世界で最初に報告している．筋電図所見や病理組織像は重症筋無力症と合致していることから、この疾患モデル動物を使って、神経筋シナプスの病態変化を解析することが可能となった．また発症したウサギのシナプスではAChR凝集が抑制していることを明らかにしているが、そのメカニズムをウサギの抗MuSK抗体を使って解析を行った．

B. 研究方法

抗 MuSK 抗体による動物発症モデルを作成して、重症筋無力症の発症メカニズムを明らかにするためにシナプスの形態変化を指標にして解析を行った．また、抗 MuSK 抗体による AChR 凝集抑制のメカニズムをリコンビナント agrin で誘導される C2C12 培養筋管細胞の AChR 凝集アッセイシステムを使い検討した．

1. 抗 MuSK 抗体で発症ウサギモデルのシナプス病理学的形態変化の解析

動物実験計画は実験施設 (愛媛大学、東京都老人総合研究所) で承認された方法に従って行った． マウスの筋肉から精製した RNA から MuSK の cDNA を PCR 法でクローニングした．MuSK はリセプター型タイロシンカイネースであるが、 その細胞外ドメインの発現ベクターを作成して Cos7 細胞にトランスフェクション

の後、分泌リコンビナント蛋白として精製した. リコンビナント蛋白作成にあたって動物モデル作成、メカニズム解明、さらには抗体価を測定するための臨床検査システムを目的として、アルカリフォスファターゼ(AP), ヒト免疫グロブリン IgG, His-tag とそれぞれ融合させた MuSK 蛋白を作成した. ウサギに対して1回に 100-400 μg の精製した MuSK-Fc、-His-tag リコンビナント蛋白を 3 回以上免疫した. 免疫したウサギは少なくとも3ヶ月以上観察し発症するかどうか観察した. 発症したウサギからは、採血ののち血清を分離した. また、4%パラフォルムアルデヒド(0.1M リン酸バッファー, pH7.4)で環流固定の後、筋を採取して同じ固定液で 4 度にて1日インキュベーションした. 筋を 10%, 20%, 30%ショ糖-PBS の溶液中に段階的に浸してインキュベーションしたのち、O.C.T. Compound と一緒にドライアイスで凍結した. 凍結した筋組織を筋線維に対して平行に 30μ 厚の凍結切片を作成してスライドガラスに固定した. α-bungarotoxin-rhodamin と一次抗体(抗ニューロフィラメント抗体と抗シナプトフィジン抗体の 1 : 1混合)で4度にて反応させ洗浄したのち、二次抗体(Alexa488-抗ヒツジ抗体)で蛍光染色した. 共焦点顕微鏡(Nikon)で神経筋シナプスの形態変化を観察記録した.

透過型電子顕微鏡でウサギの神経筋シナプスを観察するために、4%パラフォルムアルデヒド(0.1M リン酸バッファー, pH7.4)で環流固定した筋をグルタール溶液で固定して透過型電子顕微鏡の観察用切片を作成した.

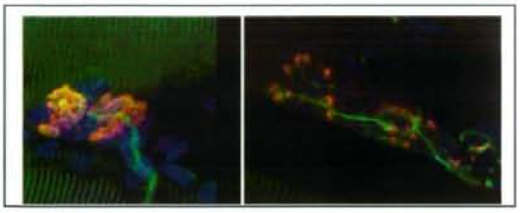
2. 抗 MuSK 抗体の機能解析

MuSK 蛋白を免疫して発症したウサギ血清に含まれる抗 MuSK 抗体がどのような機序により神経筋シナプスの AChR 凝集を抑制するか培養 C2C12 筋細胞を使って検討した. C2C12 細胞は筋芽細胞から筋管細胞へ分化誘導することができる. さらにリコンビナント agrin を培養細胞に添加すると30分以内に MuSK のタイロシンリン酸化が誘導するとともに、数時間以内に細胞表面の AChR 凝集を誘導する. この解析システムを使うことにより agrin-MuSK を介した AChR 凝集シグナル伝達の生化学的解析と機能解析を統合的に行うことが可能である. 我々はこのアッセイシステムを使って、agrin と抗 MuSK 抗体を同時に C2C12 に添加して AChR 凝集抑制に伴う MuSK 活性化シグナルの変化を解析した. 血清中の抗 MuSK 抗体の IgG サブクラスをプロテイン A で精製して解析に用いた. さらに精製した抗 MuSK 抗体 IgG をパパイン処理して Fab 分画を精製して同様に抑制実験に用いた.

MuSK のリン酸化は agrin を分化した C2C12 培養筋細胞に添加して 30 分後に作成したライゼートに対して、抗リン酸化抗体(4G10 と PY20 抗リン酸化モノクローナル抗体)によるウエスターン解析により検討した. C2C12 培養筋細胞のライゼートに対して抗 MuSK 抗で免疫沈降サンプルを泳動し PVDF メンブレンに転写した. 二次抗体は抗マウス peroxidase を使って発色した. 同じメンブレンを洗浄し、ウサギの抗 MuSK 抗体で MuSK 蛋白を検出した. AChR のリン酸化検出はライゼートをビオチン化した α-bungarotoxin とアビジン agarose で沈降して泳動し PVDF メンブレンに転写したのち抗リン酸化抗体(4G10 と PY20 抗リン酸化モノクローナル抗体)と抗 AChR -beta モノクローナル抗体によるウエスターン解析により検討した.

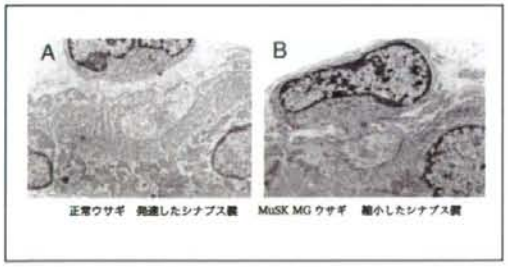
C.　研究結果

1. 発症したウサギのシナプスの形態変化

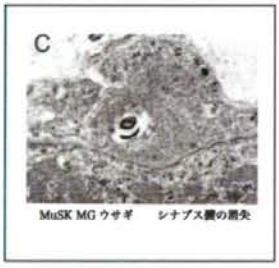


我々はウサギ MG 発症動物の神経筋シナプスの解析から、MuSK 抗体はシナプスの後膜だけにとどまらず全体構造が縮退することを発見した(未発表). (上図、左：正常ウサギ、右：発症したウサギ、緑:運動神経とシナプス前膜、赤 AChR：シナプス後膜) .

また透過電顕解析から正常マウスに比べ、発症したウサギのシナプス後膜襞(convolution と complexity)の顕著な減少が観察された(右上図、A:正常マウスのシナプス

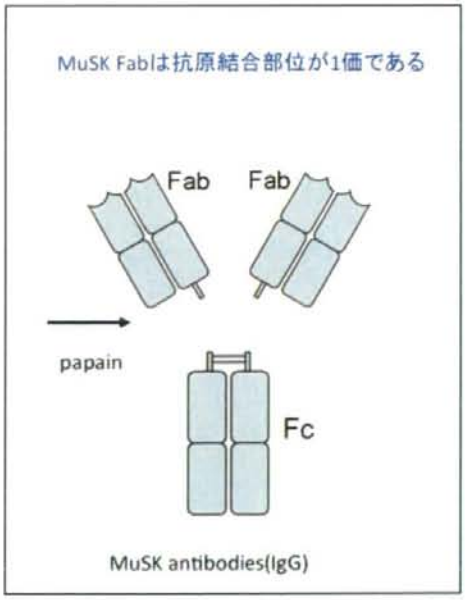


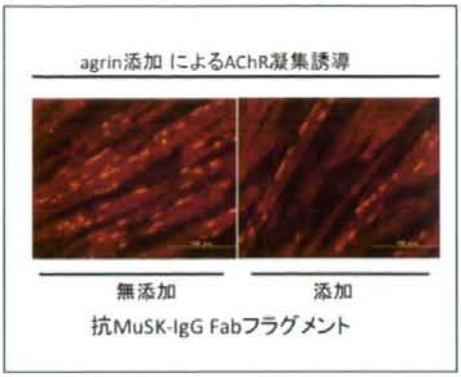
後膜襞、B と C:発症したウサギのシナプス後膜襞.)

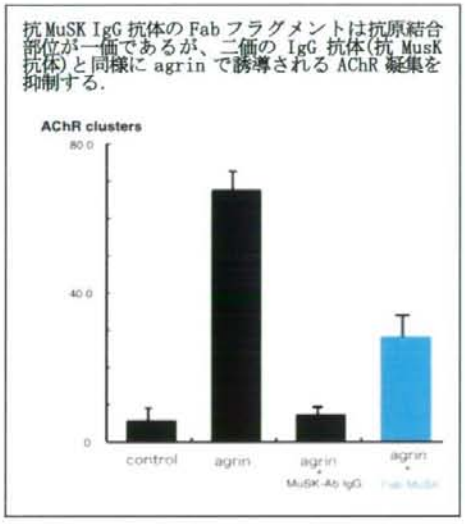


2. 発症したウサギの抗 MuSK-IgG の Fab フラグメントは AChR 凝集を抑制する.

抗 MuSK 抗体はウサギの神経筋シナプスの AChR を減少させ筋麻痺を発症させるにもかかわらず、MuSK 蛋白のタイロシンリン酸化活性をむしろ誘導することを明らかにしている. そして抗 MuSK 抗体が、培養 C2C12 細胞の AChR 凝集に抑制効果があるかどうか検討したところ, 抗 MuSK 抗体は agrin による AChR 凝集を強く抑制することが明らかにしている (Shigemoto et al. JCI 2006). これは、生体内で抗 MuSK 抗体が MuSK の機能を直接阻害してシナプス後膜の AChR 凝集抑制を誘導することを示唆していた. そこで、この抗 MuSK 抗体の IgG をパパインで処理して抗原結合部位が一価のみ有する Fab フラグメントを作成して、もとの二価の IgG と同様に agrin による AChR 凝集を抑制するかどうか検討した. その結果、二価の抗 MuSK 抗体と同様に agrin による AChR 凝集を強く抑制することを明らかにした.







次に抗原結合部位が一価しかない Fab フラグメントが、抗 MuSK-IgG 抗体と同様に agrin が無くても C2C12 細胞の MuSK を活性化するかどうか検討した. その結果下図のように Fab フラグメントは MuSK のリン酸化を誘導しないことが明らかとなった.

D. 考察

我々は、精製した MuSK 蛋白をウサギに免疫することにより、抗 MuSK 自己抗体により重症筋無力症を発症させその病態を解析している. 発症したウサギの神経筋シナプスでは AChR の凝集が減少していることを既に報告しているが、今回シナプスの形態変化を解析したところシナプス後膜の変化だけでなく、その病態はシナプス前膜まで及んでいることが明らかとなった. 抗 MuSK 抗体は MuSK によるシナプス後膜の AChR 凝集だけでなく、おそらく MuSK を介したシナプス前膜への逆行性の維持シグナルも抑制することが考えられる. また透過型電子顕微鏡で、発症したウサギのシナプス後膜を観察したところシナプス襞の単純化や減少、さらには消失も観察された. 一方で、抗 AChR 抗体発症する重症筋無力症のシナプス後膜で観察される補体による膜破壊像は全く観察されなかった. これらの結果から、我々が提唱している発症メカニズムの仮説をさらに裏付けることができた. その仮説とは、抗 MuSK 抗体が神経筋シナプスに発現する MuSK と結合して (a) MuSK の機能を直接阻害する、 (b) MuSK 蛋白の発現減少 (antigenic modulation) の結果 MuSK の機能を抑制する. おそらく(a)と(b)の両者が作用していると考える.

抗 MuSK 抗体陽性の重症筋無力症患者血清に存在する抗 MuSK 抗体 IgG のサブクラスは圧倒的に IgG4 である (一部 IgG2 も検出することができるが大変少ない. ヒト IgG4 には補体活性化能はないことから、抗 MuSK 抗体による病態機序には補体による後シナプス膜破壊の関与はほとんどないことが示されており、我々の疾患モデル動物はヒトの病態に近いことがわかった. ウサギだけでなくマウスを使った発症動物モデルを作成することに成功している. 抗 MuSK 抗体による重症筋無力症発症のメカニズム、有効な治療法や予防法の開発には動物モデルが必要であるが、我々の作成した疾患動物モデルは重症筋無力症の病態を良く反映していることが明らかとなった.

E. 結論

(1)抗 MuSK 抗体で重症筋無力症を発症する疾患動物モデル(ウサギ)を使いその病態を解析した. 発症したウサギのシナプスでは、抗MuSK抗体により MuSK の機能を抑制することで、シナプス後膜だけでなく前膜を含むシナプス全体の維持機構を障害していることが明らかとなった. またシナプス後膜では AChR 凝集だけでなく、シナプス襞の構造にも異常が見られた. それらの原因により、シナプスの伝達が障害され疾患が発症すると考えられる.

(2)抗MuSK 抗体はagrin と同様にMuSK のタイロシンリン酸化を活性化するが、一価の抗原結合部位しか持たないFab 分画はMuSK の活性能は無い. しかし二価の抗体と同様に agrin による AChR 凝集を抑制することから、抗体がMuSK 細胞外ドメインに結合することで機能を抑制していることが強く示唆された.

(3)抗 MuSK 抗体は重症筋無力症患者の神経筋シナプス後膜の AChR 凝集維持だけでなく、シナプス構造全体の維持にも必要で自己抗体はその機能を阻害することで発症すると考えられる. 我々の疾患動物モデルは抗 MuSK 抗体陽性重症筋無力症の病態解明や治療法の開発に有用であることが示された. また実際に新しい治療法に関する知見を得ており、詳細に検討を進めている.

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

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H. 知的財産権の出願・登録状況

なし.

研究成果の刊行に関する一覧表

雑誌

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研究成果の刊行物・別刷

Myasthenia Gravis Experimentally Induced with Muscle-specific Kinase

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Here we present the first evidence that muscle-specific kinase (MuSK) antigen can cause myasthenia in animals. MuSK is expressed at the postsynaptic membranes of neuromuscular junctions (NMJ) and forms complexes with acetylcholine receptors (AChR) and rapsyn. MuSK is activated by agrin, which is released from motoneurons, and induces AChR clustering and subsequent formation of NMJ in embryos. Notably, autoantibodies against MuSK were found in a proportion of patients with generalized myasthenia gravis (MG) but without the characteristic AChR autoantibodies. However, MuSK autoantibodies had no known pathogenic potential, and animals immunized with purified MuSK proteins did not develop MG in former studies. In contrast, we have now injected rabbits with MuSK ectodomain protein *in vivo* and evoked a MG-like muscle weakness with a reduction of AChR clustering at the NMJ. Our results showed that MuSK is required for maintenance of synapses and that interference with that function by MuSK antibodies causes myasthenic weakness. *In vitro*, AChR clustering in myotubes is induced by agrin and agrin-independent inducers, which do not activate MuSK. Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty, but MuSK autoantibodies in myasthenic animals inhibited both agrin and agrin-independent AChR clustering. MuSK plays multiple roles in pre-patterning of the postsynaptic membrane before innervation and formation of NMJ in embryos. Some of these mechanisms may also participate in the maintenance of mature NMJ. This model system would provide new knowledge about the molecular pathogenesis of MG and MuSK functions in mature NMJ.

Key words: **myasthenia gravis (MG); experimental autoimmune MG (EAMG); muscle-specific kinase (MuSK); acetylcholine receptor (AChR); neuromuscular junction (NMJ); congenital myasthenic syndromes (CMS)**

Introduction

Although autoantibodies against muscle-specific kinase (MuSK) have been found in patients with myasthenia gravis (MG),[1] any pathogenic contribution of MuSK antibodies to the muscle weakness that typifies MG has remained in dispute. That is, until now, MuSK

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antibodies have not produced experimental autoimmune MG (EAMG).[2,3] Here we describe the recent progress toward understanding this phenomenon.

Autoantibodies against MuSK

About 80% of patients with MG have autoantibodies against acetylcholine receptor (AChR). A seminal experiment by Patrick and Lindstrom demonstrated the pathogenicity of autoantibodies to AChR about 30 years ago.[4] Although a number of studies have documented that AChR antibodies cause structural and functional damage to the neuromuscular junction (NMJ), autoantigens, in the nearly 20% of MG patients without such antibodies, remained obscure.[1] Then, in 2001, Hoch *et al.* identified antibodies against MuSK in a proportion of patients with generalized MG.[1] MuSK is required for clustering of AChR during the formation of NMJ and is expressed predominantly at the postsynaptic membrane in mature NMJ.[5,6] In MuSK knockout mice, AChRs fail to cluster opposite to growing motoneuron terminals on the surfaces of myotubes.[7] Additionally, a case of heteroalleric MuSK mutations that caused the reduction of MuSK expression has been associated with congenital myasthenic syndrome (CMS).[8] Further, the reduction of MuSK expression in rat muscles *in vivo* upon RNA interference induced disassembly of synapses.[9] Even though the function of MuSK in mature NMJ is still uncertain, a causal relationship between MuSK autoantibodies and MG has been proposed.[1,10,11]

Recent studies by Vincent and others showed that the frequency of MuSK antibodies in MG patients who were AChR seronegative (lacked autoantibodies to AChR) varied from 4 to 50%.[11–17] We detected MuSK antibodies in 29% of seronegative MG patients but not in any MG patients with AChR antibodies (seropositive MG) or with other autoimmune diseases.[16] Previously, we identified antibodies against a recombinant MuSK fusion protein with human alkaline phosphatase (AP) in seropositive MG patients[15] and later revealed that 8.8% of seropositive MG patients had autoantibodies to AP but not to MuSK.[16] We are currently studying the clinical significance of the autoantibodies to AP in seropositive MG.

Clinical features of patients with MG and MuSK antibodies are distinctive. Such patients often have severe bulbar dysfunctions that can be difficult to treat effectively with immunosuppressive and immunomodulatory strategies, and atrophy of facial and tongue muscles is common.[12,13,18,19] After the identification of MuSK antibodies in MG patients, laboratory quantification of these antibodies is now required to confirm the diagnosis of MG, the appropriate clinical treatment, as well as the presence of AChR antibodies.[18,20,21]

Experimental Autoimmune MG

Although MuSK antibodies are present in some seronegative MG patients and the clinical features are distinctive, proving the pathogenicity of MuSK antibodies has been difficult because these antibodies did not induce myasthenia in experimental animals. Formerly, the pathogenicity of AChR antibodies was shown when rabbits injected with AChR protein purified from electric eels developed muscle weakness and paralysis.[4] Injection of eel AChR protein stimulates the production of antibodies that cross-react with rabbit AChR at the NMJ. Electrophysiological studies confirmed that the flaccid paralysis in this animal model resembled that in MG patients. Similarly, EAMG appeared in other species after immunization with purified AChR protein. In addition, the antibodies to AChR in human MG patients could passively transfer disease to mice.[22] Therefore, creating an EAMG model induced by MuSK antibodies was indispensible for proving the pathogenicity of MuSK antibodies and investigating their pathogenic mechanisms in MG.[10,20,21]

To pursue this objective, we recently immunized rabbits with MuSK ectodomain, which caused myasthenic weakness and produced electromyographic findings that were compatible with a diagnosis of MG, as shown by Patrick and Lindstrom.[23] The extracellular segment of MuSK comprises five distinct domains, i.e., four immunoglobulin-like domains and one cysteine-rich region.[5,6] The fusion protein expression constructs we generated consisted of mouse MuSK ectodomain with the Fc region of human IgG1 or His-tag and were used to transfect COS-7 cells.[23] The recombinant MuSK-Fc and MuSK-His proteins secreted were purified by using protein-A Sepharose and histidine affinity columns, respectively (FIG. 1). New Zealand white rabbits were then immunized with 100–400 mg of this purified MuSK recombinant protein. After three to four injections of MuSK protein, all six treated rabbits manifested flaccid paralysis (FIG. 2). Sera from the paretic rabbits contained high titers of MuSK antibodies that reacted specifically with MuSK molecules as observed by testing sera from MG patients with MuSK antibodies.[1,24] The paretic rabbits developed severe muscular exhaustion revealed by histological studies showing alterations in muscle fibers

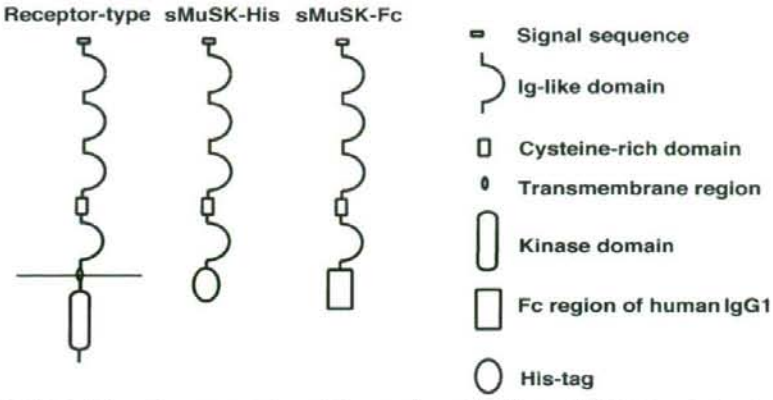

FIGURE 1. Schematic representation of the muscle-specific kinase (MuSK) domain structure and expression of secretory MuSK proteins in COS-7 cells. The domain structures of recombinant secretory MuSK protein (MuSK-His and MuSK-Fc) and receptor-type MuSK are shown. The whole coding region of the MuSK extracellular domain was fused with the His-tag or Fc region of human IgG1 as shown.

ranging from subtle to angular atrophy intermingled with normal muscle. Atrophic changes of this type can result from MG, reduced mechanical activity of muscles, or cachexia. Repetitive electromyograms of a paretic rabbit were then done to measure the result of stimulating the retroauricular branch at 20 Hz and recording responses from the retroauricular muscle. The compound muscle action potential (CMAP) showed a decremental pattern, consistent with MG.[23] However, the injection of ACh esterase inhibitor did not significantly offset the CMAP decrement or decrease the symptoms. Importantly, the induction of EAMG by MuSK antibodies is not limited to rabbits, i.e., we and others have also produced EAMG in mice by injecting MuSK protein (FIG. 2).[25]

AChR Clustering and Structure of NMJ in Rabbits with EAMG and MuSK Antibodies

The clustering of AChR necessary for NMJ formation is completely abolished in MuSK knockout mice,[7] and AChR clustering at the NMJ is reduced in subjects with CMS and MuSK mutations.[8] In a previous RNA interference experiment, injection of double-stranded RNA (dsRNA) targeting MuSK diminishes the expression of MuSK protein and AChR clusters in rat muscle fibers *in vivo*, whereas dsRNA targeting nonessential proteins does not have any effect (RNA-interference experiment).[9] Therefore, we examined the expression of AChR at NMJ in soleus muscles of paretic and normal rabbits by fluorescence microscopy after applying a rhodamine-conjugated AChR agonist, a-bungarotoxin. Images of AChR clustering were then recorded by using a digital camera.[23] The sizes and optical densities were measured using National Institute of Health (NIH) image analysis software with unprocessed digitized NIH images (version 1-62; http://rsb.info.nih.gov/nih-image). The results unequivocally pictured a significantly reduced area and intensity of AChR fluorescence in the paretic rabbits compared with their normal counterparts. In addition, a structural examination showed that the size and branching of the NMJ were significantly diminished in paretic rabbits. Similar changes of NMJ structure were observed in rats with reduced expression of MuSK evident by RNA interference,[9] in a patient with CMS and MuSK mutations, and in mice expressing the missense mutation by electroporation experiments.[8] Our results demonstrated that MuSK antibodies also elicited synaptic changes in EAMG, including the reduced expression of surface AChR at postsynaptic membranes of NMJ. Further examination of MuSK knockout mice disclosed presynaptic defects in addition to postsynaptic ones,[7] indicating that MuSK is also required for presently unidentified retrograde signals to maintain the presynaptic structure in mature NMJ.

Pathogenic Mechanisms of MuSK Antibodies in AChR Clustering at NMJ

MuSK plays multiple roles in clustering AChR during development of the postsynaptic membrane of NMJ. Contact of the motor-nerve growth cone with

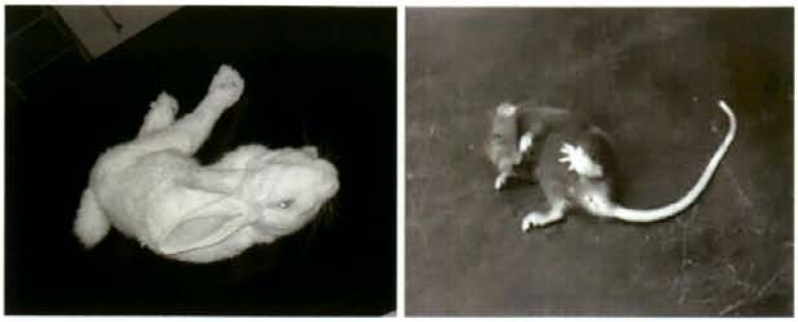

FIGURE 2. Manifestation of muscle weakness after injection of purified MuSK proteins in experimental animals (*left*, a paretic rabbit; *right*, a paretic mouse).

the muscle induces a narrow, distinct, endplate zone in the mid-muscle that is marked by a high density of AChR clustering.[26–29] In this step, agrin released from motoneurons activates MuSK and redistributes AChR clusters to synaptic sites. However, a direct physical interaction between MuSK and agrin has so far not been demonstrated, despite many efforts to do so.[27] Thus, the mechanisms of MuSK activation and the following events remain obscure, although a co-receptor of MuSK, co-ligand of agrin or either post-translational modification of agrin or MuSK have been postulated. Intriguingly, MuSK is also required for organizing a primary synaptic scaffold to establish the post-synaptic membrane.[30,31] Preceding muscle innervations, AChR clusters form at the central regions of muscle fibers, creating an endplate zone that is somewhat broader than that in innervated muscle. Thus, MuSK is required for pre-patterning of AChR clustering in the absence of motor innervation. The scenario of MuSK's roles in the process is somewhat complicated; possibly an element other than agrin achieves activation of MuSK and triggers postsynaptic specialization at the NMJ, and/or MuSK acts as a primary scaffold molecule without activation. The listed pleiotropic roles of MuSK in AChR clustering at NMJ development could also require the maintenance of mature NMJ. Studies performed *in vivo* have shown that synaptic AChRs intermingle completely over a period of approximately 4 days and that many extrasynaptic AChRs are incorporated into the synapse at the mature NMJ, although the synaptic membrane in adult muscle appears to be macroscopically stable.[32,33] Therefore, the mechanisms at play in AChR clustering during NMJ development are also required in mature NMJ when postsynaptic complexes, including AChR and MuSK, are dynamically turning over for maintenance.

To elucidate the mechanisms of AChR clustering at NMJ, a number of studies were performed using cultured C2C12 myotubes. Agrin induces clustering of AChR in C2C12 myotubes following MuSK autophosphorylation.[26,27,29] This event *in vitro* represents a major cascade of AChR clustering at the NMJ after innervation by motoneurons.[27,34–36] Laminin-1 and the *N*-acetylgalactosamine (GalNAc)-specific lectin *Vicia villosa* agglutinin (VVA-B4) can induce AChR clustering in C2C12 myotubes without activation of MuSK.[34,37–40] Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty. However, these mechanisms may also play important roles in the maintenance of NMJs via agrin-independent pathways and in their formation, as shown by genetic studies.[30,31]

In their previous study, Hoch *et al.* observed that the MuSK antibodies of MG patients inhibited agrin-induced AChR clustering in C2C12 myotubes.[1] We also found that agrin-induced clustering of AChR was strongly blocked in the presence of MuSK antibodies, whereas absorption of the antibodies with purified MuSK products prevented this blocking effect.[23] Thus, MuSK antibodies were responsible for inhibiting the formation of agrin-induced AChR clustering. We also perceived that MuSK-specific antibodies strongly inhibited AChR clustering induced by all known agrin-independent pathways as well as by agrin itself.

Conclusions

In our experimental model of myasthenia, MuSK antibodies routinely mediated pathogenesis in rabbits and mice.[23,25] Consequently, we now believe that MuSK antibodies cause MG in patients. However,

the pathogenic mechanisms of these antibodies entail multiple events in which MuSK acts as a multifunctional platform from which to regulate synapse formation and maintenance. These are reflected in a diversity of clinical features ranging from typical MG to a multitude of variants.[12,13,18,19]

AChR antibodies have been shown to affect neuromuscular transmission by three main mechanisms: (a) binding and activation of complement at the NMJ; (b) accelerated degradation of AChR molecules cross-linked by antibodies (antigenic modulation); and (c) functional AChR block.[20,21] Intriguingly, MuSK antibodies in MG patients are mainly of the IgG4 subclass, which does not activate complement.[41] Electron microscopic observations of NMJ in the EAMG rabbits demonstrated a significant reduction of synaptic folds but no destruction, thus our EAMG model resembles the phenotypes of MG with MuSK antibodies. MuSK antibodies against compound antigenic determinants on the extracellular domain may elicit pathogenic effects through antigenic modulation and/or restraint of MuSK functions,[41] and the consequences of these effects range from a partial to entire loss of MuSK functions.

Recently, a new MuSK-interacting cytoplasmic protein, called Dok-7, has been discovered.[42] Dok-7 knockout mice underwent a marked disruption of neuromuscular synaptogenesis that was indistinguishable from the features found in MuSK-deficient mice. Mutations in Dok-7 caused a genetic form of limb-girdle myasthenia (CMS).[43,44] Some clinical features of these patients resemble the severe type of MG with MuSK antibodies[44]; therefore, the EAMG model with MuSK antibodies presented here promises to facilitate resolution of the pathogenic basis of MG and CMS at the molecular level and identification of beneficial treatment strategies against them.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Myasthenia gravis induced by autoantibodies against MuSK

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Myasthenia with MuSK antibodies

Abstract

Myasthenia gravis (MG) is caused by the failure of neuromuscular transmission mediated by autoantibodies. That is, the binding of autoantibodies to postsynaptic membranes in neuromuscular junctions (NMJ) results in weakening of the ocular, bulbar and limb muscles and produces the characteristic syndrome of MG. This relatively rare disease serves as a model not only for study of the pathogenesis and treatment of all autoimmune disorders but also for understanding the basic mechanisms of neuromuscular transmission at the NMJ. About 80 to 85% of patients with MG have autoantibodies against acetylcholine receptors (AChR). Although a number of studies have shown the possible existence of other autoantibodies in the remaining ~20% of MG patients, the responsible autoantigens remain elusive. However, antibodies against muscle-specific kinase (MuSK) have been found in 30% of MG patients without AChR antibodies. MuSK, a tyrosine kinase receptor, is required for the development of NMJ's postsynaptic membranes. Still, the pathogenicity of MuSK antibodies as a cause of muscle weakness in patients with MG remains a matter of dispute, because the experimental autoimmune MG caused by MuSK antibodies in animals was absent. Here we describe recent progress toward understanding the pathogenic role of MuSK antibodies in the decline of muscle strength that typifies MG.

Key words: myasthenia gravis (MG); experimental autoimmune MG (EAMG); muscle-specific kinase (MuSK)

Myathenia gravis caused by antibodies to AChR

Myasthenia gravis (MG) is a rare neuromuscular disease, but a well-recognized disorder because of such characteristic clinical features as ptosis with fluctuating general fatigue and muscle weakness that worsens with repeated activity (1, 2)but tends to improve with rest. Ptosis and diplogia occur early in the majority of these patients. With passing time, when the weakness of bulbar and respiratory muscle becomes worsen, it makes the disease becomes life-threatening so that intubation with mechanical ventilation is required. About 80% of patients with MG have autoantibodies against acetylcholine receptors (AChR) (1, 2). Patrick and Lindstrom provided the first piece of evidence indicating the pathogenicity of AChR antibodies by experimentally induced myasthenia gravis in 1973(3). While a number of studies showed the pathogenic (4-8)roles of AChR antibodies in causing structural and functional damage of the neuromuscular junction (NMJ), autoantigens of the remaining MG patients (~20%) were elusive (5). Although these patients do not have AChR antibodies, they respond to immunotherapies, and their serum antibodies can transfer a defect in neuromuscular transmission to mice, indicating that the muscle weakness is also induced by autoantibodies against NMJ.

MuSK antibodies in MG patients

For the last three decades, causative autoantibodies other than those to AChR have been sought in MG patients but have eluded identification in spite of extensive research efforts (1, 2). In 2001, Hoch et al. found autoantibodies against muscle-specific kinase (MuSK) in a proportion of patients with generalized MG (5). MuSK is essential during the development of NMJ, when it organizes fetal AChR clustering at the postsynaptic membrane. Subsequently, in mature NMJ, MuSK is expressed predominantly at the postsynaptic membrane. Studies by Vincent and others showed that the frequency of MuSK antibodies in "seronegative MG patients," i.e., those who lack autoantibodies to AChR, varied from 4 to 50% (4-8). Ohta et al. detected MuSK antibodies in about 30% of seronegative MG patients but not in any MG patients with AChR antibodies (seropositive MG) or other autoimmune diseases (9-11). The clinical features of MG with MuSK antibodies are distinctive. These individuals often suffer from a severe bulbar dysfunction that is difficult to resolve with immunosuppressive and immunomodulatory treatments, and muscular atrophy of facial and tongue muscles is