方)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、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.　これは、走査型電子顕微鏡疾患による疾患モデルマウスのシナプス形態変化の観察からも明らかにすることができた.そして、我々が提唱している発症メカニズムの仮説を裏付けることができた.　その仮説とは、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 (図29).

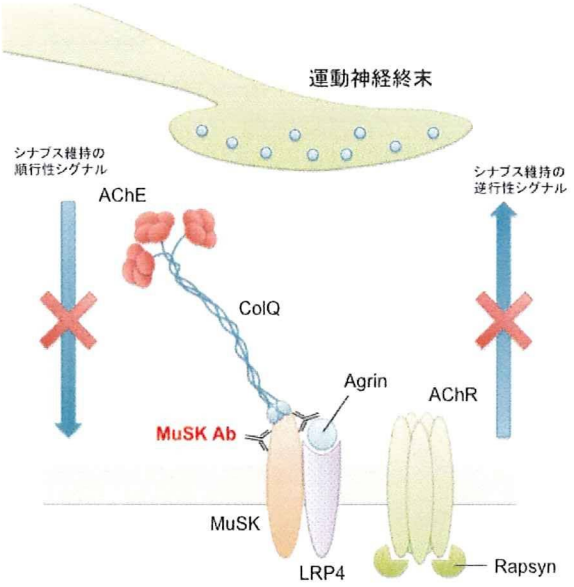

図29　MuSK抗体MG発症のメカニズム

④　我々の開発した疾患動物は、MuSK-MG 患者と同じようにAChE 阻害剤に対して過敏性を示すことを発見した.　その機序としてAChE はコラーゲンQ を介してMuSK に結合しておりMuSK の機能あるいは発現が抑制されることによりAChE の発現と機能が減少するため、阻害剤に対する過敏性が出現すると考えることができる (図29).

⑤　同調して100%発症する疾患モデル動物は真に有効な治療法、とくに薬物のスクリーニングに大変有用である.我々は、3,4-DAP がMuSK-MG に対して有効であることを示した.この機序を明らかにすることで、疾患の発病メカニズムとあわせて新しい治療方針の確立が可能となった.

⑥　今回使用したマウスの系統には、免疫学的に自己免疫疾患を発症させる遺伝子変異を有していることがわかった.　この遺伝子型を明らかにすることで、自己免疫疾患の発病メカニズムを明らかにすることができるであろう.

MuSK-MG の臨床研究においても成果を上げることができた.独自に開発した RIA 法による極めて高感度のMuSK 抗体測定系を使い重症筋無力症患者のMuSK 抗体を測定したところ,SNMG の 27 %が陽性であり, しかもすべて極めて高い力価を示した.しかもSPMG やMG 以外の神経筋疾患・自己免疫疾患患者および健常者では検出されなかったことより, MuSK 抗体測定はSNMG と診断された患者を診断する上で極めて有用な血清診断学的マーカーとなることが示された.

MuSK 抗体陽性患者は女性に多く, 球麻痺, および呼吸筋麻痺がSPMG と比較して多くみられ, クリーゼになりやすく症状も重症であった.一方, 四肢筋力低下は, SPMG と比較して少なかった.MuSK 抗体が顔面筋により選択的に作用し, 障害を引き起こしていると考えられる.

MuSK 抗体陽性 MG では胸腺腫を合併せず，胸腺過形成も少なかった.

治療に関して、ステロイド療法を行った患者では，全例で MuSK 抗体価の減少およびそれに関連した臨床的症状の改善がみられた. 血漿交換療法を行った患者では，劇的な MuSK 抗体価の減少および臨床的症状の改善がみられた. 一方，胸腺摘出後，MuSK 抗体価の減少および臨床的症状の改善はみられなかった. 1 例では胸腺摘出後，むしろ MuSK 抗体価の増加がみられ，臨床的症状が悪化した. このように MuSK 抗体の測定は，MG の治療方針や予後の推定に有用であることを示した.

AChR 抗体陽性で発見した新規 AP 抗体が検出されるケースは重症の全身型 MG 症例が多く，中でも呼吸筋障害例が多くみられたことから，本抗体の測定は，臨床的重症化の指標となる可能性を示した.

E. 結論

当該研究により、MuSK 抗体で発症する重症筋無力症は進行性の筋萎縮に至る重症例が多く従来の治療法に対して難治性である等の臨床像と解決目標(問題点)を明らかにした. その調査結果を論文と学会、医学専門雑誌を通して臨床現場へ提供し成果を普及させ、我が国の医療技術の向上に貢献している. また、MuSK 抗体測定法を開発して国内外の病院(大学病院ほか多数)から依頼を受け迅速に結果を医療現場へ無償で提供することができている. これまで原因不明であった重症筋無力症患者の確定・除外診断や治療効果判定に使われ国民医療の水準向上に対して貢献している. 一方で、MuSK 抗体 MG 総患者数(推定 1000 人)のまだ約 10%(約 100 人) しか確定診断されていない. 特に我が国の治療指針で MG 患者全般に対して AChE 阻害剤が示されていることから、その改定と抗 MusK 抗体陽性 MG の診断技術を普及させる必要がある.

当該研究は，MuSK 抗体で発症する MG の発症メカニズムを解明して、科学的根拠に基づいた有効な診断と治療法(治療指針への提言)を行うことを目的としてスタートしたが、我々は従来の MG の治療薬である AChE 阻害剤が MuSK 抗体 MG では使用すべきでないことを、患者研究と基礎研究から明らかにしている. 分担研究者の宇多野病院　小西哲郎院長が中心となり、その治療方針に対する提言を行っている.

我々は MuSK-MG をマウスに 100%の頻度で同調させて発症させることに世界で初めて成功した. この疾患モデルを使うことで、補体が関与しない MuSK-MG の発症メカニズムの解明、MuSK によるシナプスの維持機構、AChE 阻害薬による過敏症の機序，真に有効な薬物のスクリーニングが可能となった. また我々は従来の MG の治療薬である AChE 阻害剤が抗 MuSK 抗体 MG では使用すべきでないことを、患者研究と基礎研究から明らかにすることができた. 平成 19 年度分担研究者の宇多野病院　小西哲郎院長が中心となり、その治療方針に対する提言を行っている.-

F. 健康危険情報

なし

G. 研究発表

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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

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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重本和宏、久保幸穂、丸山直記	神経筋接合部位の異常と筋力低下	日本老年医学会雑誌.	46(2)	106-113	2009
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書籍

Shigemoto,K. Kubo,S. Mori.S. Yamada,S. Miyazaki,T. Akiyoshi,T and Maruyama,S	The Immunopathogenesis of Experimental Autoimmune Myasthenia Gravis Induced by Autoantibodies against Muscle-specific kinase.	*Book title: Myasthenia gravis Diseas Mechanisms and Immune Intervention. Linus Publication, Inc.*	Chapter 17	304-323	2009

研究成果の刊行物・別刷

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Clinical and experimental features of MuSK antibody positive MG in Japan

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clinical features, domain, IgG subclass, muscle specific tyrosine kinase, MuSK antibody, myasthenia gravis, seronegative myasthenia gravis

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We investigated the presence of antibodies (Abs) against muscle-specific tyrosine kinase (MuSK) in Japanese myasthenia gravis (MG) patients. MuSK Abs were found in 23 (27%) of 85 generalized seronegative MG (SNMG) patients but not in any of the ocular MG patients. MuSK Ab-positive patients were characterized as having female dominance (M:F, 5:18), age range at onset 18 to 72 (median 45) years old, and prominent oculobulbar symptoms (100%) with neck (57%) or respiratory (35%) muscle weakness. Limb muscle weakness was comparatively less severe (52%), thymoma absent. Most patients had good responses to simple plasma exchange and steroid therapy. MuSK IgG from all 18 patients was exclusively the IgG 4 subclass and bound mainly with the MuSK Ig 1–2 domain. Serial studies of 12 individuals showed a close correlation between the variation in MuSK Ab titers and MG clinical severity ($P = 0.01$ by Kruskal–Wallis). MuSK Ab titers were sharply decreased in patients who had a good response to early steroid therapy or simple plasma exchange, but there was no change, or a rapid increase on exacerbation after thymectomy. Measurement of MuSK Ab titers aids in the diagnosis of MG and the monitoring of clinical courses after treatment.

Introduction

Muscular weakness in most patients with myasthenia gravis (MG) is caused by an antibody (Ab)-mediated autoimmune response to muscle nicotinic acetylcholine receptors (AChRs), but there is no correlation between the AChR Ab level and degree of muscle weakness. This may be because of AChR Abs heterogeneity and epitope specificity or the presence of Abs against other functionally important muscle antigens. Fifteen percent of patients with generalized MG who have no detectable circulating Abs to AChR are termed seronegative MG (SNMG). Autoantibodies against muscle-specific tyrosine kinase (MuSK) have been identified in that population [1]. The positivity for MuSK Ab in SNMG patients varied from 3.8% to 71% by studies [1–11], which may be due to geographical or ethnic differences. Immunoglobulin allotypes in Caucasian and Chinese MG patients differ from those in Japanese patients [12]. We performed a MuSK Ab survey of a large number of Japanese MG patients and characterized the clinical features of those who were MuSK Ab positive. Furthermore, we investigated the correlation between MuSK Ab titer and disease severity, epitope specificity, and the IgG subclass of MuSK IgG.

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Patients and methods

Patients

We studied 85 patients (27 men, 58 women, mean age 56 years old, range 18–76 years) who had generalized SNMG and were consistently negative for serum AChR Abs, as well as 272 AChR Ab-positive MG (SPMG) patients (87 men, 185 women, mean age 54 years old; age range 32–74 years); 50 with and 222 without thymoma. The control populations comprised 70 healthy participants (29 men, 41 women; mean age 50 years old, range 27–74 years) and 91 patients (37 men, 54 women; mean age 50 years old, range 32–74 years) with other neurological or immunological diseases (five Lambert-Eaton myasthenic syndrome, six polymyositis, 10 muscular dystrophy, 15 thyroiditis, 10 type 1 diabetes mellitus, five rheumatoid arthritis, 10 multiple sclerosis, five spinal progressive muscular atrophy, five chronic inflammatory demyelinating polyneuropathy, 10 amyotrophic lateral sclerosis, and 10 epilepsy). The study was approved by the ethics committee of Utano National Hospital. All persons gave their informed consent prior to their inclusion in the study.

Preparation of recombinant human MuSK protein

To produce his-tag human MuSK protein, the entire extracellular domain (MuSK 1–4; nucleotides 107–1526,

GenBank/EMBL accession number AF006464) of human MuSK, and MuSK fragments comprised of the first half bearing two Ig-domains (MuSK 1–2; nucleotides 107–700) were linked to the PCR3.1/Myc-His vector (Invitrogen Corporation, Carlsbad, CA, USA) [13]. Membrane-proximal extracellular domains, including Ig-domains 3 and 4 (MuSK 3–4; nucleotides 701–1526), were linked to the pSecTag-His vector (Invitrogen) carrying the ER signal sequence of the mouse Igκ gene. All constructs were transiently transfected to COS7 cells [14]. The recombinant his-tag MuSK secreted was purified in a histidine-affinity column (Clonetech Laboratories, Palo Alto, CA, USA). Recombinant protein purity was determined by SDS-PAGE with silver staining. Recombinant protein concentrations were obtained with a BCA Protein assay kit (Pierce, Biotechnology, Inc., Rockfold, IL, USA) with bovine serum albumin as the standard. The MuSK extracellular domain and MuSK fragments then were labeled with ^{125}I [15].

Detection of MuSK Ab by radioimmunoprecipitation assay

All the sera underwent a radioimmunoassay (RIA) to determine the presence of MuSK Ab. In brief, 5 μl of each sample was incubated overnight at 4°C with 50 μl of ^{125}I-his-tag MuSK (40 000 cpm), after which 50 μl of anti-human IgG was added, and the sample incubated for another 2 h at room temperature. Radioactivity was counted after two washes of the pellets with saline. All positive sera were titrated, and results expressed as nanomoles of ^{125}I-MuSK precipitated per liter of serum.

Epitope mapping

Muscle-specific tyrosine kinase Ab-positive sera were tested by an RIA for the presence of IgG Abs to MuSK 1–2 or MuSK 3–4. In brief, 5 μl of each sample was incubated overnight at 4°C with ^{125}I-his-tag MuSK (40 000 cpm), ^{125}I-his-tag MuSK 1–2 (30 000 cpm), or ^{125}I-his-tag MuSK 3–4 (30 000 cpm), after which 50 μl of anti-human IgG was added. The samples then were incubated for another 2 h at room temperature. Radioactivity was counted after two washes of the pellets with saline.

IgG subclasses of MuSK Ab

Microtiter plates (Breakapart plate, Nunk-Immuno Module, Roskilde, Denmark) were coated with 100 μl of 10 μg/ml of each Ab to IgG subclasses (sheep polyclonal anti-human IgG1, 2, 3 and 4; Binding Site, Birmingham, UK) diluted with 10 mM sodium carbonate-bicarbonate buffer, pH 9.3 and kept for 1 h at room temperature. Nonspecific binding sites were saturated with 200 μl PBS containing 5% skimmed milk and 10% Blockace (Dainippon Seiyaku, Osaka, Japan) for 2 h at room temperature. A serum sample (20 μl), first incubated for 2 h at room temperature with ^{125}I-MuSK (30 000 cpm), was added to a plate, and the whole incubated for 2 h at room temperature. After four washes, ^{125}I was counted in each well.

Statistical analysis

Statistical analysis was performed by regression analysis, Kruskal–Wallis, one-way analysis of variance, and Student *t* test. A *P*-value of <0.05 was considered significant.

Results

MuSK Abs

The cut-off value (0.01 nM) was calculated from the mean + 3SD of the healthy subjects' values obtained by an RIA constructed with ^{125}I-MuSK extracellular domains. MuSK Ab was present in 23 (27%) of the 85 SNMG patients but not in any of the 272 SPMG patients, healthy subjects and patients with other neurological or immunological diseases (Fig. 1). Ab-positive samples were confirmed by serial dilution tests, and titers shown as nanomoles of ^{125}I-MuSK precipitated per liter of serum. MuSK Ab titers ranged from 8.4 to 240 nM (median, 57 nM). All the positive serum samples had extremely high titers on ^{125}I-human MuSK immunoprecipitation.

Clinical features of patients with MuSK Abs

Table 1 shows the clinical features of 23 MuSK Ab-positive patients. MuSK Ab in generalized SNMG showed female predominance (five men, 18 women) but not in ocular MG. Age at onset ranged from 18 to 72 years old (median 45 years). Clinical features of MuSK Ab-positive patients were confined to ocular [ptosis, 13/23 (57%) and double vision, 18/23 (78%)]; bulbar [dysphagia: 23/23 (100%), dysarthria: 19/23 (83%)]; neck extensor, 13/23 (57%); respiratory 8/23 (35%) muscle weaknesses. Prevailing weaknesses affected the oculobulbar and respiratory muscles of MuSK Ab-positive patients. About 48% (11/23) had no limb weakness. No thymomas were detected by CT. Six (26%) of the 23 MuSK Ab-positive patients who were thymectomized, had histological abnormalities including small hyperplastic features.

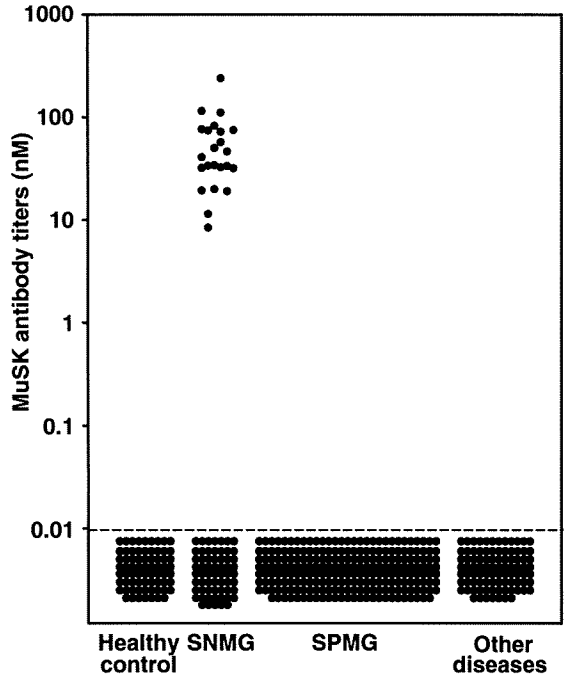

Figure 1 RIA-detected MuSK Ab titers of 85 patients with SNMG, 272 patients with SPMG, 91 patients with other neurological or immunological diseases, and 70 healthy participants. Broken line, the cutoff (0.01 nM) for MuSK Abs.

Table 1 Clinical features of MuSK Ab-positive patients

MuSK Ab positivity in SNMG	23/85 (27%)
MuSK Ab titers	8.4–239 (median 57 nM)
F:M	18:5
Age at onset	18–72 years (median 45 years)
Distribution of weakness	
Ptosis	13/23 (57%)
Ocular motor dysfunction	18/23 (78%)
Bulbar	23/23 (100%)
Neck	13/23 (57%)
Respiratory (crises)	8/23 (35%)
Limb	12/23 (52%)
Thymus	
Thymoma	0/23 (0%)
Hyperplasia	6/23 (26%)

Serial studies of clinical status and MuSK Abs

We measured MuSK Ab titer serially during the disease's course. Table 2 shows anti-MuSK Ab titers in relation to disease severity and duration, and immunosuppressive treatment (A), plasma exchange (B), or thymectomy (C). Disease severity was graded according to the Myasthenia Gravis Foundation of America (MGFA) classification [16] at the onset of myasthenic symptoms, in the maximally deteriorated state, and at

Table 2 Changes in MuSK Ab titers and in clinical status in MuSK Ab-positive patients

Case	Gender	Age at onset (years)	Duration (days)	MuSK Ab (nM)	MGFA classification	treatment
(A) Early steroid therapy						
P-1	F	18	0	39.3	IIIb	
			56	39.0		Pred
			82	40.2		Pred
			138	38.0		Pred
			175	35.0	IIb	Pred
			313	33.0	PR	Pred
			577	21.0		Pred
P-2	F	32	0	113.0	IVb	Pred
			141	17.0		Pred
			261	15.0	IIb	Pred
			409	16.0		Pred
P-3	F	48	0	80.0	IVb	Pred
			46	28.0		Pred
			101	5.0		Pred
			1,641	4.2	PR	Pred
P-4	F	53	0	36.8	IIIb	
			41	31.0		Pred
			97	15.2		Pred
			111	10.0	IIb	Pred
P-5	F	52	0	240.0	V	Pred
			49	57.0		Pred
			77	22.9		Pred
			101	8.4		Pred
			129	3.0	IIb	Pred
P-6	F	76	0	33.0	IIb	Pred
			83	0.5		Pred
			118	0.2	PR	Pred
(B) Simple plasma exchange (PE)						
P-7	M	53	0	74.4	V	
			42	59.0		
			52	47.0		
			PE ⟶			
			62	28.5	IIb	
			PE ⟶			
			67	17.2		
			125	16.0		Pred
			132	11.5		Pred
			138	9.0	IIb	Pred
P-8	M	71	0	113.9	IVb	
			PE ⟶			
			11	32.1		
			45	32.0		Pred
			219	31.0		Pred
			616	28.0	IIb	Pred
P-9	F	66	0	30.0	IIIb	
			45	40.5		
			361	32.0		
			PE ⟶			
			374	14.0	IIb	
			379	21.2		
			389	29.9		
			403	35.5	IIIb	Pred, Cyclo
			441	25.0		Pred, Cyclo
			476	20.5	IIb	Pred, Cyclo

Table 2 (Continued)

(C) Thymectomy (Tx)						
P-10	F	47	0	20.0		
			47	26.0	IIb	
			Tx ⟶			
			95	47.2	IIIb	Pred
			270	19.8	IIb	Pred
P-11	F	52	0	22.6	IIb	
			58	23.2		
			Tx ⟶			
			170	21.1		Pred
			255	25.0	IIb	
P-12	F	48	0	16.5	IIb	
			95	17.6		
			Tx ⟶			
			210	15.7		Pred
			274	17.0	IIb	

PR, Pharmacological Remission; Pred, Predonisolone; Cyclo, Cyclosporine.

the last clinic visit after or during treatment. As shown in Table 2a, six patients (P1–P6) who underwent early steroid therapy showed impressive clinical improvement associated with a sharp decrease in anti-MuSK Ab titer; from 39.3 to 21.0 nM (P-1), 113.0 to 16.0 nM (P-2), 80.0 to 4.2 nM (P-3), 36.8 to 10.0 nM (P-4), 240.0 to 3.0 nM (P-5), and 33.0 to 0.2 nM (P-6). MG severities showed clinical improvement from class IIIb to pharmacological remission (PR) (P-1), class IVb to IIb (P-2), class IVb to PR (P-3), class IIIb to IIb (P-4), class V to IIb (P-5), and class IIb to PR (P-6).

Muscle-specific tyrosine kinase Ab titers of three patients were measured in serial samples taken before and after simple plasma exchange (Table 2b). The patients responded dramatically to that therapy, Ab titers decreasing from 74.4 to 9.0 nM (P-7), 113.9 to 28.0 nM (P-8), and from 30.0 to 20.5 nM (P-9), indicative of clinical improvement from class V to IIb (P-7), class IVb to IIb (P-8), and class IIIb to IIb (P-9). Moreover, conventional immunosuppression maintained the clinical improvement initially achieved by plasma exchange. In one patient (P-9), the effect had tapered off 45 days after plasma exchange, and Ab titer and disease severity returned to the level before treatment. Prednisolone and cyclosporin administered after MG relapse resulted in slower improvement.

Three patients who had histological abnormalities, including a small hyperplastic thymus, underwent thymectomies (Table 2c). After surgery one patient (P-10) immediately had worsening of dysphagia from class IIb to IIIb associated with a rapid increase in MuSK Ab titer from 26.0 to 47.2 nM. Thymectomy was not effective for the other two patients (P-11, P-12) who showed no change in disease severity and MuSK Ab titer.

We analyzed MuSK Ab titers in relation to quantitative clinical scores on the MGFA scale in serial studies of 12 individuals. MuSK Ab titers and disease severity were correlated ($P = 0.01$ by Kruskal–Wallis).

Epitopes in the extracellular domains of human MuSK

Eighteen sera with MuSK Abs were examined for ^{125}I-MuSK 1–2 and ^{125}I-MuSK 3–4 binding. All predominantly bound to ^{125}I-MuSK 1–2, range 68–97%. Only five of the 18 sera also showed slight binding (20–30%) to ^{125}I-MuSK 3–4 (Table 3).

IgG subclasses of MuSK Abs

In a solid phase RIA with sheep polyclonal antibodies to human IgG subclasses, in all the 18 sera tested MuSK Abs were exclusively IgG4 (Table 4).

Discussion

The MuSK Ab-positive rate found for generalized SNMG patients in Japan was 27% with female predominance (M:F = 5:18). This rate is lower than the 70% positivity originally reported [1] and the 40–50% recently reported [2–7]. It is consistent with the 27–33% reported for Japanese and Korean population [8–10] but significantly higher than the 3.8% Chinese positivity rate [11]. Age at onset ranged from 18 to 72 years old (median, 45 years); 61% of the patients presenting at >40 years of age, later than for Caucasians, and 57–71% of patients presenting at <40 years of age, but the differences was not significant [3,7,17,18].

Table 3 Ratio of MuSK Ig 1–2 and 3–4 Ab in MuSK 1–4 Ab titers

Case	MuSK Ab (nM)	Ig 1–2 domain (%)	Ig 3–4 domain(%)
1	8.4	97.1	2.9
2	19.3	68.1	31.9
3	32.5	81.2	18.8
4	50.0	82.7	17.3
5	33.4	97.3	2.7
6	31.8	95.7	4.3
7	19.9	91.0	9.0
8	11.4	95.2	4.8
9	40.7	87.9	12.1
10	32.0	91.0	9.0
11	33.6	95.0	5.0
12	74.7	95.4	4.6
13	72.0	76.2	23.8
14	46.2	80.8	19.2
15	74.7	96.9	3.1
16	82.2	74.2	25.8
17	110.7	89.5	10.5
18	114.6	71.2	28.8

Table 4 Ratio of IgG subclasses of MuSK Abs

Case	MuSK Ab (nM)	IgG 1 (%)	IgG 2 (%)	IgG 3 (%)	IgG 4 (%)
1	114.6	0.0	0.0	0.0	100.0
2	110.7	0.0	0.0	4.9	95.1
3	82.2	0.0	0.0	0.0	100.0
4	74.7	11.0	21.0	19.0	49.0
5	74.0	4.1	5.0	5.6	85.3
6	72.0	0.0	1.0	0.0	99.0
7	46.2	0.0	0.0	0.0	100.0
8	40.7	5.3	0.0	7.1	87.6
9	33.6	15.1	15.7	0.0	69.2
10	33.4	0.0	0.0	0.0	100.0
11	32.5	5.3	0.0	0.0	94.7
12	32.0	4.2	2.6	1.2	92.0
13	31.8	6.7	6.7	8.3	78.3
14	19.9	0.0	0.0	1.7	98.3
15	19.5	0.0	0.0	0.0	100.0
16	19.3	0.0	0.0	0.0	100.0
17	11.4	0.0	28.9	26.1	45.0
18	8.4	0.0	0.0	0.0	100.0

All the Ab-positive patients had similar patterns of muscle weakness, with prevalent involvement of the bulbar muscles in 100%, ocular symptoms (blepharoptosis and/or double vision) in 80%, and of the respiratory muscles in 35% with frequent myasthenic crises. Limb muscle involvement was comparatively less severe and inconsistent. Japanese MuSK Ab-positive patients therefore have clinical features similar in terms of the predominance of bulbar involvement to those reported for Caucasians.

We evaluated the correlation between MuSK Ab titers and disease severity. Table 2 shows patients who had a good response to early immunosuppressive therapy or simple plasma exchange. Their MuSK Ab titers sharply decreased in parallel with clinical improvement, whereas their Ab titers remained positive. We evaluated the effect of thymectomy in three individuals by measuring MuSK Ab titers in serum samples taken pre- and post-thymectomy. One patient's condition deteriorated after thymectomy and her Ab titer greatly increased. The two others showed neither progression nor Ab titer change during the observation period. Thymectomy therefore did not produce good results. Histological changes in the thymus of MuSK Ab-positive subjects are reported to be minimal and to include rare small germinal centers [19,20] in contrast to SPMG patients who had lymph node-type infiltrates. These findings, together with the lack of benefit of thymectomy, are evidence against a role for the thymus in antigen presentation and antibody production.

Serial studies showed a statistically close correlation between MuSK Ab titers and disease severity. MuSK Ab titers also recently were found to correlate with MG severity [21]. MuSK Ab titers were extremely high in all the positive cases (Fig. 1). The close relationship between clinical status and MuSK Ab, found by monitoring Ab titers, suggests that MuSK Ab has a significant pathogenic role in MG patients. Circulating MuSK Abs, however, are reported not to cause a MuSK or AChR deficiency at the endplates [22]. Recent experimental models (rabbits [13] and mice [23]), developed by immunization with recombinant MuSK ectodomain protein, produced MG-like muscle weakness with reduced AChR clustering at neuromuscular junctions. These findings clarified the pathogenic MG mechanisms produced by MuSK Ab.

The paramount MuSK Ab IgG subclass in our eighteen patients was IgG4. Limb and intercostal muscle biopsies found neither reduction in AChR numbers nor complement deposition [9,24]. The absence of complement deposits at a patient's end plates is explained by the fact that MuSK Ab is mainly IgG class 4 which does not fix complement [5,25]. The MuSK extracellular domain consists of four MuSK immunoglobulin-like (Ig) domains. Binding analysis of MuSK Abs to ^{125}I-MuSK Ig 1–2 or ^{125}I-MuSK Ig 3–4 showed that the eighteen sera tested predominantly bound to the ^{125}I-MuSK Ig 1–2 domain. The epitope was the N-terminal of the extracellular domain of human MuSK as described previously [5]. Furthermore, MuSK Abs have been shown to inhibit agrin-induced clustering of AChRs [26]. In fact, MuSK Ig 1–2 domains are more responsible for agrin responsiveness of MuSK, in contrast to Ig 3-4 domains which are more responsible for rapsyn association. We postulate that this is relevant to our findings of predominant binding analysis to MuSK Ig 1–2. The characteristics of the MuSK IgG subclass and Ab binding epitope in Japanese patients therefore are similar to those of Caucasians.

Muscle-specific tyrosine kinase Ab-positive patients often suffer facial and tongue muscle atrophy [3,27]. Benveniste *et al.*[28] reported that MuSK Ab plasma may affect the expression of atrophy-related protein and that a facial muscle, the masseter, is the most susceptible. Amongst our MuSK Ab-positive patients, four patients had detectable tongue atrophy from a relatively early phase of illness; weakness was moderate in 2 patients and mild in two patients. More *in vitro* and *in vivo* studies are needed to clarify the pathologic mechanisms that cause the muscle weakness produced by MuSK Ab. MuSK Ab detection provides a valuable biological means of support for the clinical diagnosis of MG and a way to monitor its clinical course.

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Anti-alkaline phosphatase antibody positive myasthenia gravis

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Abstract

Anti-alkaline phosphatase antibody (AP Ab) was specific in 9% of 249 anti-acetylcholine receptor (AChR) Ab-positive myasthenia gravis (MG) (SPMG) patients but not in patients with AChR Ab-negative MG (SNMG), other neurological and immunological diseases, or healthy volunteers. No cross-reactivity and no significant titer correlation were found between AP Ab and AChR Ab. We confirmed immunologically by radioimmunoassay and western blot analysis the presence of antibodies directed against AP. AP Ab-positive SPMG patients were characterized clinically as having female predominance and a more severe form of generalized MG than AP Ab-negative SPMG patients, and about half required artificial ventilation at maximum severity. AP Ab's pathogenic role in MG is yet unclarified, but our findings show AP to be a novel antigen among the various autoantigens present in MG patients and in whom AP Ab may modify clinical symptoms.

Keywords: Alkaline phosphatase antibody; AChR antibody; Myasthenia gravis

1. Introduction

Myasthenia gravis (MG), a T-cell-dependent chronic autoimmune disorder, is induced by sustained production of an antibody (Ab) to nicotinic acetylcholine receptor (AChR) at neuromuscular junctions [1]. Unexpectedly, we recently found alkaline phosphatase (AP) Ab in AChR Ab-positive MG (seropositive MG: SPMG) sera in a radioimmunoassay (RIA) for the muscle-specific receptor tyrosine kinase (MuSK) Ab, in which a recombinant MuSK protein was fused with placental AP. AP Ab was detected in 9% (20/229) of the SPMG patients, but was absent in the AChR Ab-negative MG (seronegative MG:SNMG) patients studied [5].

Here, we show immunologically the presence of AP Ab in SPMG and investigate the disease specificity and clinical features of AP Ab-positive patients.

2. Materials and methods

2.1. Subjects

Serum samples were obtained from 319 MG (249 SPMG, 70 SNMG) patients and from 136 patients with other neurological or immunological diseases (10 Lambert–Eaton myasthenic syndrome, 10 polymyositis, 15 multiple sclerosis, 5 spinal progressive muscular atrophy, 5 chronic inflammatory demyelinating polyneuropathy, 28 ALS, 10 epilepsy, 15 progressive muscular dystrophies, 15 thyroiditis, 10 type 1 diabetes mellitus, and 13 rheumatoid arthritis). Positive diagnostic features of MG was confirmed by electromyography (>10% decrement on repetitive nerve stimulation at 3 Hz in a hand or shoulder muscle), and an unequivocally positive response to edrophonium or other anticholinesterase agents.

Abbreviations: AChR, acetylcholine receptor; AP, alkaline phosphatase; MuSK, muscle-specific receptor tyrosine kinase; MG, myasthenia gravis.

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MG patients were classified according to the MG Foundation of America (MGFA) [3]. The control population comprised 70 healthy volunteers.

Ninety-nine SPMG patients, whose medical records were detailed, were divided into two groups for comparison; ten with AP Ab-positive MG and eighty-nine AP Ab-negative MG. Clinical features investigated were gender, age at disease onset, disease duration, MGFA clinical classification at maximum severity, thymic pathology, and the use of mechanical ventilation, steroids or other immunosuppressants. The study was approved by the ethics committee of Utano National Hospital. All persons gave their informed consent prior to their inclusion in the study.

2.2. AChR Ab assay

AChR Abs were measured with a standard AChR Ab kit (RSR Limited, Cardiff, UK).

2.3. Human MuSK and AP Ab assay

Recombinant MuSK proteins were constructed from the extracellular domains of human MuSK. AP-tag-MuSK was the fusion protein of MuSK and human placental alkaline phosphatase (AP). AP-MuSK and His-MuSK were secreted from COS 7 cells. These recombinant MuSK (rMuSK) proteins were purified in a nickel affinity column then labeled with ^{125}I. Anti-MuSK Ab were measured by the previously described radioimmunoprecipitation method [5]. AP Ab was detected by an RIA. In brief, 5 μL of each serum sample was incubated with ^{125}I-AP-MuSK (40,000 cpm) overnight at 4 °C, after which 50 μL of anti-human IgG was added, and the sample were incubated for 2 h at room temperature. After two washes of the pellets with saline, radioactivity was counted. AP antibodies were also detected by an RIA using ^{125}I-AP. Human placental AP was purchased (Calzyme Laboratories, CA) and further purified (95% purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) by affinity chromatography on anti-AP monoclonal antibody coupled to agarose (Sigma, St. Louis, MO). AP was assayed in 5 μL of serum samples with ^{125}I-AP (40,000 cpm). Results expressed as nanomoles of ^{125}I-AP-MuSK precipitated per liter of serum. Specific radioactivity of ^{125}I-AP-MuSK was 3100 cpm per pM. Furthermore, the assay was modified to demonstrate inhibition of AP specific counts employing unlabeled AP.

2.4. Western blot analysis

Alkaline phosphatase (AP) (Calzyme Laboratories Inc. California) was separated in 4–20% sodium dodecyl sulfate polyacrylamide gels at a constant current of 40 mA then transferred to a nitrocellulose membrane at 80 V for 1.5 h. After non-occupied protein binding sites had been blocked by 5% skim milk, the membrane was cut into small strips (3 × 70 mm), which were incubated at room temperature for 2 h with the test serum diluted 1:500. The strips were washed four times and again incubated at room temperature for 2 h with horseradish peroxidase-conjugated anti-human IgG (Amersham Biosciences, Tokyo, Japan) diluted 1:5000. After a final wash, the membrane was soaked in chemiluminescence solution containing ECL Western blotting detection reagents (Amersham Biosciences, Tokyo, Japan).

2.5. Statistical analysis

All p-values given were 2-tailed by Fisher's exact test. The frequencies of the MGFA clinical classifications at maximum severity were compared for AP status for trend by the Mann–Whitney U test. $p<0.05$ was considered statistically significant.

3. Results

The AP Ab titer cut-off value (0.01 nM) was calculated from the mean+3SD (0.0025+0.0075) of the values of the

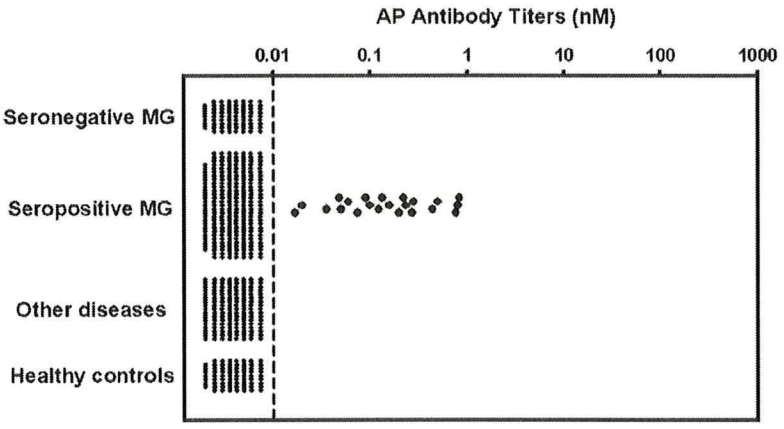

Fig. 1. Alkaline phosphatase antibody titers detected by ^{125}I-human placental AP assays. Titers of 70 patients with seronegative MG, 249 patients with seropositive MG, 136 patients with other neurological and immunological diseases, and 70 healthy volunteers. The dashed line denotes the cut off value (0.01 nM) calculated from the mean+3SD of values for the 70 healthy control participants.

70 healthy participants in RIA using a AP-MuSK protein. The mean+3SD of AP-MuSK titers for SNMG and other diseases was 0.0031+0.0061 nM, and 0.0026+0.0070 nM, respectively. AP Ab was detected in 9% (two men, twenty women) of the 249 SPMG patients (70 men and 179 women) but not in the 70 patients with SNMG (19 with, 51 without MuSK Ab). No Ab was detected in the non-MG patients or healthy participants (Fig. 1). In this population (n=22), AP Ab-positive MG patients showed significant female predominance by Fisher's exact test ($p<0.05$) as compared with those with AP Ab-negative SPMG. The mean AP Ab value for SPMG was 0.265 nM (n=22; 0.017 to 0.8 nM).

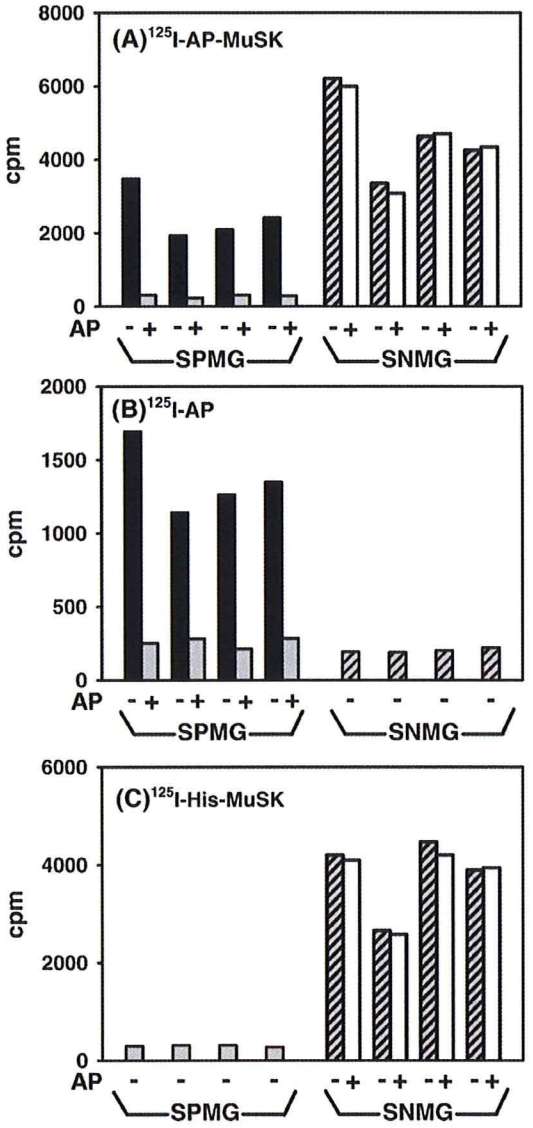

Fig. 2. Antibody specificity detected in 4 SPMG and 4 SNMG sera by the ^{125}I-AP-MuSK assay. Inhibition studies of sera co-incubated with an excess of unlabeled AP. (A) ^{125}I-AP-MuSK precipitation assay. (B) ^{125}I-AP precipitation assay (C) ^{125}I-His-MuSK precipitation assay. 5 μL of SPMG sera were used for assay (A) and 5 μL of a 1:300 dilution of SNMG sera were used for assay (C). AP Abs were present in SPMG but not in SNMG.

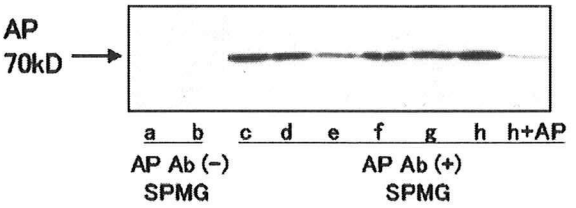

Fig. 3. Immunostaining patterns of AP Ab-negative SPMG (a and b) and AP Ab-positive SPMG (c–h) sera. Six AP Ab-positive sera recognized a single AP band which disappeared when the samples were preabsorbed with AP protein (Fig. 3h+AP). No band was detected in sera from the AP Ab-negative SPMG patients or the healthy controls.

All AP-MuSK Ab-positive patients were tested in the inhibition assay, in the ^{125}I-AP RIA, and ^{125}I-His-MuSK RIA. As shown in Fig. 2A, AP-MuSK Ab-positive sera was found in both SPMG and SNMG by the ^{125}I-AP-MuSK assays. AP-MuSK Ab was detected in 22 of the 249 SPMG patients as shown in Fig. 1. SNMG sera had extremely high Ab titers, whereas SPMG sera had relatively low ones. The specificity of the assay system was determined by AP inhibition experiments with all AP-MuSK Ab-positive sera. Co-incubation of SPMG sera with an excess of unlabeled AP (27 μg) decreased ^{125}I precipitation to the control levels in all sera tested, whereas co-incubation of SNMG sera with AP did not alter precipitation (Fig. 2A). ^{125}I-AP precipitation assays also detected AP Abs in all SPMG patients with AP-MuSK Ab, but not in SNMG sera. Co-incubation of positive sera with unlabeled AP decreased ^{125}I precipitation to the control levels in all sera tested (Fig. 2B). His-MuSK precipitation assays detected MuSK Abs in the SNMG but not the SPMG sera (Fig. 2C). Thus AP Abs were present in SPMG but not in SNMG.

We also performed the immunoblot analysis on sera from patients with high AP Ab titers, and tested in the inhibition assay. Western blotting was used on sera from 2 AP Ab-negative (Fig. 3a, b) and 6 AP Ab-positive (Fig. 3c–h) SPMG patients, and from 4 healthy participants. Immunostaining patterns of these sera are shown in Fig. 3. Molecular weights of the proteins reactive with those in the sera were calibrated with standard proteins. Coomassie brilliant blue staining showed transblotted purified AP protein of approximately 70 kD. Six AP Ab-positive sera recognized a single 70 kD band which disappeared when the sera were preabsorbed with the AP protein (Fig. 3h+AP). No band was present in sera from the healthy controls or AP Ab-negative SPMG patients.

We evaluated the relationship between AP Ab and AChR Ab antibodies in 18 AP Ab-positive patients. There was no correlation between AChR and AP Ab titers (Fig. 4), and no cross-reactivity was found between AChR and AP on the addition of AP to the AChR assay system (data not shown). The relationships between variations in AP Ab and AChR

Ab in individuals, were determined by measuring both antibody titers in serial samples of serum taken from seven participants who had antibodies against both AChR and AP. In some cases, there were temporary discrepancies between the changes in both titers, but there also was a broad correlation between AChR and AP Ab titer fluctuations in certain patients (data not shown). Those patients showed less improvement after undergoing various therapies and had consistently high antibody titers for 5 to 24 years after disease onset.

The clinical features of the AP Ab-positive among the 99 SPMG patients (27 men, 72 women) (Table 1) were characterized. Ten (10%) had AP Abs (mean±SD: 0.241± 0.235 nM). All were women, and there was significant female predominance in the AP Ab-positive group by Fisher's exact test ($p<0.05$). Although the mean age of AP Ab-positive patients was older compared to AP Ab-negative patients, the statistical analysis did not show significant difference among Ab positive and negative patients even excluding ocular type MG patients. There was no difference in the disease duration between the AP Ab-positive and -negative patients. Fifteen participants with AChR Ab-positive ocular MG (MGFA class I) had no AP Ab. The MGFA clinical classification at maximum severity was significantly more severe in the AP Ab-positive than AP Ab-negative group, including patients with and without ocular MG ($p<0.05$ by the Mann–Whitney U test). The frequency of intubation requiring mechanical ventilation, defined as MGFA class V, was increased in the AP Ab-positive group (5/10, 50%) but did not differ statistically from that in the AP Ab-negative generalized MG group (18/74, 24%). Nor did the frequency of thymoma differ between the groups. The mean AChR Ab titer was higher but did not differ significantly in patients with AP Ab. There was no significant correlation between the AP and AChR Abs titers for either Ab-positive patient group.

Table 1
Baseline clinical characteristics of SPMG patients with and without AP Ab

Characteristic	AP Ab-positive patients ($n=10$)	AP Ab-negative patients ($n=89$)
Age: mean±SD (range)	49.3±13.9 (24–67)	38.0±20.8 (1–85)
Men/women⋆	0/10	27/62
Mean disease duration (year)	21.5 (3–35)	18.2 (1–51)
MGFA at max severity†		
I	0	15
II	0	20
III	4	29
IV	1	7
V	5	18
Thymoma (%)	2 (20%)	27 (30%)
Thymectomy	10	79
Prednisolone or immunosuppressants	8	74
Anti-AChR Ab: mean±SD (nM) (range)	209±285 (0.4–1750)	25.3±30.1 (0.2–400)
Anti-AP Ab: mean±SD (nM) (range)	0.24±0.21 (0.011–0.80)	

⋆: $p<0.05$ by Fisher's exact test. †: $p<0.05$ by the Mann–Whitney U test.

Most patients in both groups had lessened MG symptoms after prednisolone treatment with or without other immunosuppressants. Only a few in each group showed improvement after receiving anti-cholinesterase medication without immunosuppressants.

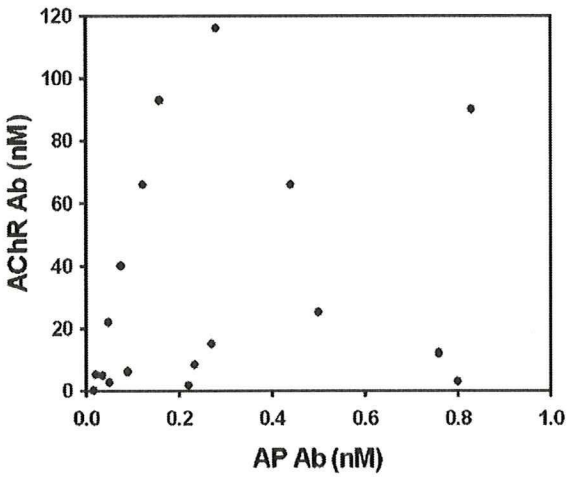

Fig. 4. There is no significant correlation between the AP Ab and AChR Ab titers.

4. Discussion

AP Ab was detected exclusively in 9% of SPMG patients tested, similar to the value given in our earlier report [5]. In contrast, no AP Ab was detected in the SNMG and non-MG patients, or in the healthy participants [5]. We confirmed immunologically by western blot analysis the presence of antibodies against AP in SPMG patients and showed there was no cross-reactivity or correlation between AChR and AP. The other aim of this study was clarification of the clinical characteristics of AP Ab-positive SPMG patients as compared to those who had AP Ab-negative SPMG. Clinical characteristics found for the AP Ab-positive patients are female predominance, absence of ocular MG, as well as a severe clinical course trend that included myasthenic crisis requiring artificial ventilation. Female predominance also has been reported in MuSK Ab-positive SNMG [2,4,6,7], but the reason for it is not clear. Whether AP Ab has a modifying effect on the clinical symptoms of SPMG and whether it represents a useful clinical indicator of SPMG requires further study.

Our findings indicate that AP Ab is a novel autoantibody found only in SPMG patients and that its presence may result in a more severe form of generalized MG as compared to patients with AP Ab-negative SPMG. The location of AP in the

cell and cell membrane with relevance to its antigenicity must be clarified to notice how AP antibody concerns pathophysiology of SPMG.

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Myasthenia gravis induced by autoantibodies against MuSK

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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 have remained 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, experimental autoimmune MG, muscle-specific kinase

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 worsens, 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 MG in 1973 (3). While a number of studies showed the pathogenic roles of AChR antibodies in causing structural and functional damage of the neuromuscular junction (NMJ) (4-8), autoantigens of the remaining MG patients (~20%) were undefined (5). Although these patients do not have AChR antibodies, they respond to immunotherapy (1, 2), and their serum antibodies can transfer a defect in neuromuscular transmission to mice (2), indicating that the muscle weakness is also induced by autoantibodies against neuromuscular junctions (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 common (1, 2). The response to AChE inhibitors is generally unsatisfactory with risk of worsening symptoms, especially when starting treatment in patients with bulbar symptoms or an impending respiratory crisis. Thymectomy does not alleviate the symptoms of MuSK-positive MG. In short-term therapy, patients with MuSK MG respond as well to plasma exchange and intravenous immunoglobulin as those with AChR seropositive MG. Even so, those patients whose

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neck and shoulder muscles are affected often experience respiratory weakness. MG in which weakness is limited to the ocular muscle is not frequent but does occur.
Some workers in this field are now coming to believe that MuSK MG must constitute a distinct subclass of the disease (4, 6, 7). The reason is that many patients with MuSK antibodies develop severe muscle weakness and eventual atrophy, which is rare in AChR seropositive MG, and the former respond differently to therapy than persons in the latter group. After the identification of MuSK antibodies in an MG patient, laboratory testing is now required to confirm the diagnosis of MG, to seek AChR antibodies and to formulate the clinical treatment.

MuSK functions in NMJ

MuSK plays multiple roles in clustering AChR during development of the postsynaptic membranes of NMJ (12, 13). Contact of the motor-nerve growth cone with the muscle induces a narrow, distinct endplate zone in the mid-muscle that is marked by a high density of AChR clustering. In this step, agrin released from motoneurons activates MuSK and redistributes AChR clusters to synaptic sites (12). However, the direct physical interaction between MuSK and agrin has so far not been demonstrated despite many attempts to do so (13). Thus, the mechanism(s) of MuSK activation and the following events remain obscure, although a co-receptor of MuSK, a co-ligand of agrin or alternative post-translational modification of either agrin or MuSK have been postulated (13). Intriguingly, MuSK is also required for organizing a primary synaptic scaffold to establish the post-synaptic membrane (12). Prior to muscle innervation, 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. However, establishing a scenario for MuSK's participation in the process is somewhat complicated. For example, an element other than agrin may activate MuSK and trigger the postsynaptic specialization at NMJ. Simultaneously or alternatively, MuSK could act as a primary scaffold molecule without activation. The listed pleiotropic roles of MuSK in AChR clustering at developmental NMJ could also be required for the maintenance of mature NMJ (14-16). Studies performed *in vivo* have shown that synaptic AChRs intermingle among themselves completely over a period of four days and that many extra-synaptic AChRs are incorporated into the synapse at the mature NMJ, although the synaptic membrane in adult muscle appears macroscopically to be stable (17). Therefore, the mechanisms at play during AChR clustering in developing NMJ are also required in mature NMJ where postsynaptic complexes including those with AChR and MuSK are dynamically turning over for the maintenance of muscle function.

Do MuSK antibodies cause myasthenia?

Research on the mechanisms of synaptic transmission at the NMJ has uncovered some pathogenic effects of antibodies to AChR that could underlie MG (18). Effective neuromuscular transmission depends on numerous interactions between acetylcholine and its receptor, AChR, and the failure of neuromuscular transmission results in myasthenic weakness and fatigue. To evoke action potential for the contraction of muscle fibers, a large enough number of AChR must be present at postsynaptic membranes. In 1973, Fambrough and colleagues found an abnormal decrease in the number of AChR at postsynaptic membranes of the NMJ of patients with MG (1, 2). Others showed that AChR antibodies affect neuromuscular transmission by three main mechanisms: (a) Complement-mediated lysis of post-synaptic membranes follows the binding and activation of complement at the NMJ; (b) the degradation of AChR molecules accelerates upon cross-linking of those molecules by antibodies (antigenic modulation); (c) AChR antibodies block AChR function. The predominant pathogenicity is caused by the complement-mediated mechanisms, but all three mechanisms tend to reduce the number of available AChR and, thereby, decrease neuromuscular transmission between motor nerve endings and postsynaptic membranes. Therefore, an individual nerve impulse cannot generate enough postsynaptic depolarization to achieve the crucial firing threshold required for opening of sufficient voltage-gated sodium channels to initiate an action potential in the muscle fiber (18).
In contrast to the well-accepted mechanisms by which AChR antibodies function in MG, the pathogenic role of MuSK antibodies has been unclear (19). First, no significant loss of AChR at NMJ was observed in biopsies from biceps brachii muscles of MuSK-positive patients with MG (20). Second, MuSK antibodies are mainly in the IgG4 subclass, which does not activate complement (9), and complement-mediated damage to postsynaptic membranes is considered a major source of pathogenicity in MG patients with AChR antibodies. Third, passive transfer of MuSK serum in MG patients cannot generate the equivalent disease in mice. Fourth, no experimental animal model induced by MuSK had been developed. Although none of these studies seems to support a pathogenic role for MuSK antibodies in human MG, MuSK antibodies from MG patients effectively inhibit MuSK functions *in vitro* (5).

An experimental animal model of myasthenia (EAMG) induced by MuSK antibodies

The pathogenicity of AChR antibodies was shown experimentally by the induction of muscle weakness

and development of paralysis in rabbits immunized with AChR protein purified from the electric eel (3). This AChR protein induced the production of antibodies that cross-reacted with rabbit AChR at the NMJ. The flaccid paralysis that followed and electrophysiological studies of these animals provided a model that resembled the MG of humans (21). Furthermore, this EAMG could be transferred by injecting sera from the paralyzed rabbits into naïve animals, indicating that the antibodies rather than cellular immunity caused the disease. Subsequently, EAMG was also induced in other species by repeated inoculations with purified AChR protein. The pathogenic nature of these antibodies from MG patients was demonstrated by passive transfer of the IgG fraction into mice. In addition to these experimental studies indicating the pathogenicity of AChR antibodies, clinical laboratory analyses determined that the patients had serum antibodies that were specific for AChR. Therefore, the next step was using MuSK antibodies to induce an EAMG model, which was essential for proving their pathogenicity and investigating their mechanisms of eliciting MG.

Recently we demonstrated that immunization of rabbits with MuSK ectodomain caused myasthenic weakness and produced electromyographic findings that were compatible with a diagnosis of MG (16), as shown by Patrick and Lindstrom. The extracellular segment of MuSK comprised five distinct domains, i.e., four immunoglobulin-like domains and one cysteine-rich region. The fusion protein expression constructs, which consisted of mouse MuSK ectodomain with the Fc region of human IgG1 or His-tag, were generated and transfected in COS-7 cells. The secreted recombinant MuSK-Fc and MuSK-His proteins were purified by using protein-A Sepharose and histidine affinity columns, respectively. New Zealand White rabbits were then immunized with 100 to 400 mg of purified MuSK recombinant protein. After three to four injections of MuSK protein, all of six rabbits manifested flaccid paralysis (Fig. 1A). Sera from the paretic rabbits contained a high titer of MuSK antibodies that reacted specifically with MuSK molecules on the surfaces of C2C12 myotubes as observed in sera from MG patients who were positive for MuSK antibodies. Histological studies of the muscle tissues from the paretic rabbits, which had manifested severe exhaustion, revealed alterations in muscle fibers ranging from subtle to angular atrophy intermingled with normal muscle tissue (Fig. 1B). The histological changes typical of atrophied muscle fibers can result from MG, reduced mechanical ability or cachexia. In repetitive electromyograms from one of these paretic rabbits, the retroauricular branch of facial nerve was stimulated at 20 Hz, and recordings were taken from adjacent retroauricular muscle (Fig. 1C). The compound muscle action potential (CMAP) showed a decremental pattern, consistent with MG. However, injections of acetylcholine esterase inhibitor did not significantly reverse either the CMAP defect or the paralytic symptoms. Importantly, the induction of EAMG by MuSK antibodies is not confined to rabbit, as we and others can also elicit EAMG in mice by injection of MuSK protein (22).

How do antibodies to MuSK cause myasthenia?

Next, we focused on demonstrating how MuSK antibodies cause MG. The pathogenicity of MuSK antibodies in MG has been questioned, since MuSK-positive patients with MG do not have a decrease in the number of AChRs nor is complement deposited at the NMJ of their biceps brachii muscles (20). Although the mechanisms of MG caused by AChR antibodies are well delineated, the same pattern does not necessarily apply to MG caused by MuSK antibodies. MuSK antibodies have been identified as predominantly of the IgG4 subclass, which does not activate complement. However, the binding of MuSK antibodies to MuSK molecules could accelerate the latter's degradation (antigenic modulation) and/or inhibit MuSK functions directly. MuSK is essential for AChR clustering at the developing NMJ, and its deficiency may lead to the complete loss of junctional ultrastructure (12, 13). To reveal the pathogenic role of MuSK antibodies in MG, we still need to know how MuSK acts at mature NMJ and whether MuSK is also required for the maintenance of AChR clustering and the structural stability of mature NMJ.

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 autophosphorylation by MuSK. *In vitro*, this event represents a major cascade of AChR clustering at the NMJ after innervation by motoneurons. Laminin-1 and the *N*-acetylgalactosamine (GalNAc)-specific lectin *Vicia villosa* agglutinin (VVA-B4) also induce AChR clustering on C2C12 myotubes, without activation of MuSK. 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 formation and maintenance of NMJ, the latter via agrin-independent pathways as shown by genetic studies (16).

In a previous study, Hoch et al. observed that the MuSK antibodies of MG patients inhibited agrin-induced AChR clustering in C2C12 myotubes (5). 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 (16). These results showed that the MuSK antibodies effectively inhibited the formation of agrin-induced AChR clustering. Intriguingly, the monovalent Fab fragments of MuSK antibodies from rabbits with EAMG also inhibited AChR clustering