CD59 Expression in Skeletal Muscles and Its Role in Myasthenia Gravis

Kazuo Iwasa, MD, PhD, Yutaka Furukawa, MD, PhD, Hiroaki Yoshikawa, MD, PhD, Masahito Yamada, MD, PhD, and Kenjiro Ono, MD, PhD

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Abstract

Background and Objectives
Complement regulatory proteins at the neuromuscular junction (NMJ) could offer protection against complement-mediated damage in myasthenia gravis (MG). However, there is limited information on their expression at the human NMJ. Thus, this study aimed at investigating the expression of the cluster of differentiation 59 (CD59) at the NMJ of human muscle specimens and demonstrating the overexpression of CD59 mRNA and protein in the muscles of patients with MG.

Methods
In this observational study, muscle specimens from 16 patients with MG (9 and 7 patients with and without thymoma, respectively) and 6 nonmyopathy control patients were examined. Immunohistochemical stains, Western blot analysis, and quantitative real-time reverse transcription PCR were used to evaluate the CD59 expression.

Results
A strong localized expression of CD59 was observed at the NMJ in both patients with and without MG. Moreover, the CD59/glyceraldehyde-3-phosphate dehydrogenase protein ratio in patients with MG was significantly higher than that in the nonmyopathy controls (MG; n = 16, median 0.16, interquartile range (IQR) 0.08–0.26 and nonmyopathy controls; n = 6, median 0.03, IQR 0.02–0.11, p = 0.01). The proportion of CD59 mRNA expression relative to AChR mRNA expression ($\Delta$CtCD59/AChR) was associated with the quantitative MG score, MG activities of daily living score, and MG of Foundation of America Clinical Classification ($r$ = 0.663, $p$ = 0.01; $r$ = 0.638, $p$ = 0.014; and $r$ = 0.715, $p$ = 0.003, respectively).

Discussion
CD59, which acts as a complement regulator, may protect the NMJ from complement attack. Our findings could provide a basis for further research that investigates the underlying pathogenesis in MG and the immunomodulating interactions of the muscle cells.
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Glossary

Ab = antibodies; ACh = acetylcholine; AChE = acetylcholinesterase; AChR = acetylcholine receptor; C = complement component; CD59 = cluster of differentiation 59; Ct = cycles to threshold; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GPI = glycosylphosphatidylinositol; IgG = immunoglobulin G; IQR = interquartile range; MAC = membrane attack complex; MG = myasthenia gravis; MG-ADL = myasthenia gravis activities of daily living; MGFA = Myasthenia Gravis Foundation of America; NMJ = neuromuscular junction; QMG = quantitative myasthenia gravis; qRT-PCR = quantitative real-time polymerase chain reaction; SPSS = Statistical Package for the Social Sciences; WB = western blotting; α-BTx = α-bungarotoxin.

Myasthenia gravis (MG) is an autoimmune disorder in which a disturbance at the neuromuscular junction (NMJ) induces fatigable muscle weakness.¹ Most patients (80%–85%) with MG have autoantibodies against the muscle acetylcholine receptor (AChR) at the NMJ.² The pathogenic actions of AChR-antibodies (AChR-Ab) are attributed to 3 mechanisms as follows: (1) antibody blocking, which refers to a direct block of ACh attached to the AChR; (2) modulating antibody, which includes AChR degradation through cross-linking and endocytosis; and (3) complement activation of immunoglobulin G3 (IgG3) and IgG1 AChR-Ab, which leads to postsynaptic membrane damage of the NMJ.³⁴

AChR-Ab and the complement factors of complement component (C) 3 or C9 are deposited at the NMJ in MG.⁵ Moreover, patients with MG demonstrated impaired NMJ membrane folds through the activation of complements, which results in the formation of membrane attack complexes (MAC).⁵ Complement inhibitors that target molecules in the complement activation cascade have been useful therapeutic agents in MG and other complement-related diseases.⁶ Eculizumab targets C5 in the plasma and prevents the cleavage of C5 to C5a and C5b, which inhibit MAC assembly, leading to an improvement in 86% of the patients with MG. The effects of eculizumab in MG suggested that a reduction in MAC formation at the NMJ inhibits endplate destruction.⁷

The cluster of differentiation 59 (CD59), also known as the MAC inhibitory protein, is a membrane-anchored complement regulatory protein that can inhibit the cytolytic activity of binding to the C9 and C5b-8 complex.⁸⁹ CD59 is widely distributed in normal human tissues and cells, such as blood cells, heart, liver, spleen, kidney, endothelia, various epithelial cells, and skeletal muscles.¹⁰ CD59 deficiency is mainly associated with paroxysmal nocturnal hemoglobinuria. Primary neurologic diseases that have been associated with sequence variants in CD59 were reported as recurrent Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, and recurrent aseptic meningitis. Sustained hyperglycemia in diabetes mellitus can lead to increased glycation of CD59 and functional impairment of CD59. In tissues with impaired CD59 function, a repair mechanism can aggravate inflammation and tissue destruction.⁶

The NMJ presumably might express the complement regulatory proteins, and it could be protected against complement attack by regulatory mechanisms. In this study, we aimed at assessing CD59 expression at the NMJ of muscles and demonstrating the overexpression of CD59 mRNA and proteins in muscles of patients with MG.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The study protocol was approved by the medical ethics committee of the Kanazawa University School of Medicine (693-5). Consecutive patients with MG who underwent thymectomy between 2004 and 2013 provided written informed consent before undergoing muscle biopsy during thymectomy. Nonmyopathy controls provided written informed consent to use the specimens for experiments before undergoing muscle biopsy.

Patient Samples

A total of 16 pectoralis muscle specimens were assessed at the Kanazawa University to identify patients with MG. MG was diagnosed based on clinical features with fatigability, positive AChR-Ab, decremented response on repetitive nerve stimulation, and/or positive edrophonium test. In total, 9 of the 16 patients had thymoma (Table 1). The various MG scores (quantitative MG [QMG] scores, MG activities of daily living [MG-ADL] scores, and MG of Foundation of America [MGFA] Clinical Classification) were assessed at the time of admission which was a couple of weeks or within a month before undergoing thymectomy. In addition, control biopsy specimens of the peroneus brevis and the biceps brachii muscles were obtained from 6 nonmyopathy controls (peripheral neuropathy, n = 3, and nonorganic disease, n = 3, respectively). Accordingly, these findings reflect normal or nonspecific neurogenic changes without affecting the muscles. Six patients with MG were given immunotherapy at the time of obtaining the muscle biopsy (Table 1). The nonmyopathy controls had not received any immunosuppressive medications before undergoing biopsy.

Immunohistochemistry of CD59 and α-Bungarotoxin

Immunohistochemistry was performed according to previously described standard methods.¹¹ We had not selected tissues according to nonspecific esterase staining that
identifies the endplates; however, we could detect the NMJ of muscle specimens by $\alpha$-bungarotoxin ($\alpha$-BTx). Tissue sections were incubated with rabbit monoclonal antibody (EPR6452[2]) against CD59 (1:100; Abcam, UK) and detected with Alexa Fluor®488-conjugated goat anti-rabbit IgG (1:100; Abcam). To detect the AChR of NMJ, Alexa Fluor®594-conjugated $\alpha$-BTx (1:100; Invitrogen) was simultaneously incubated with the second antibody. Cross-sectional images were obtained by using an all-in-one fluorescence microscope (BZ-X710; KEYENCE, Osaka, Japan) equipped with an optical sectioning module (BZ-H4XF; KEYENCE, Osaka, Japan). The Z-stack images were captured over 6 μm at 10-step sizes and projected onto a complete focus image using the BZ-analyzer.

**Western Blot of CD59**

Western blotting (WB) was performed as previously described. Briefly, muscle proteins were resolved on Bis-Tris 4%–12% gels (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene difluoride membrane using an iBlot system (NuPAGE Western Transfer Protocol; Invitrogen). The membrane was incubated with a rabbit monoclonal anti-CD59 antibody (1:1,000; Abcam) and an antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2,000; Abcam), which was used as a loading control. Protein bands were visualized by chemiluminescence, and digital images were acquired using a LAS-4000 mini system and Multi Gauge v.3.X software (Fujifilm, Tokyo, Japan). The CD59 expression level relative to that of GAPDH was evaluated using ImageJ software v.1.53 (NIH, Bethesda, MD).

**Quantitative Real-Time PCR Analysis of CD59 and AChR mRNA Expression**

Quantitative real-time PCR (qRT-PCR) was performed using TaqMan probes (Thermo Fisher Scientific) for the target genes CD59 (Hs00174141_m1) and CHRNA1 (Hs00175578_m1), and an internal control gene GAPDH (Hs00265705_g1) using QuantStudio3 instrument (Thermo Fisher Scientific). The relative values of mRNA expression were determined according to the cycle threshold (Ct) of qRT-PCR. Ct represents the cycle number in which the amount of amplified DNA reaches a threshold level. A smaller starting copy number generates a higher Ct value.

Gene expression was normalized to that of GAPDH; the relative amounts of CD59 mRNA and AChR mRNA were expressed as follows: $\Delta$Ct = (target Ct – GAPDH Ct). Moreover, we calculated the fold change with the $2^{-\Delta\Delta$Ct} method. For the second analysis of the relative expression of

### Table 1 Clinicodemographic Characteristics of Patients With MG

<table>
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<tr>
<th>Patient no.</th>
<th>Age at thymectomy (y)</th>
<th>Sex</th>
<th>Thymoma (WHO)</th>
<th>AChR Ab (nM)*</th>
<th>MGFA</th>
<th>QMG score</th>
<th>ADL score</th>
<th>Medication at the time of thymectomy</th>
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<td>nd</td>
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<td>3</td>
<td>PSL 30 mg/AD, FK506 2 mg</td>
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<td>M</td>
<td>nd</td>
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<td>9</td>
<td>4</td>
<td>PB, PSL 30 mg/AD</td>
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<tr>
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Abbreviations: AChR Ab = anti-acetylcholine receptor autoantibody titer; AD = alternate-day; ADL score = myasthenia gravis activities of daily living score; FK506 = tacrolimus hydrate; IAPP = immunoabsorption plasmapheresis; IVlg = IV immunoglobulin; IVMP = IV methylprednisolone; MGFA = Myasthenia Gravis of Foundation of America Clinical Classification; mPSL = methylprednisolone; nd = no data; PB = pyridostigmine bromide; PSL = prednisolone; QMG score = quantitative MG score; WHO = WHO classification of thymic epithelial tumors, including thymomas, thymic carcinomas, and neuroendocrine tumors.

* Controls <0.3 pmol/mL.
CD59 mRNA to AChR mRNA, $\Delta C_t_{\text{CD59/AChR}}$ was calculated as follows: $\Delta C_t_{\text{CD59/AChR}} = \text{AChR Ct} - \text{CD59 Ct}$.

**Data Availability**
Anonymized data used and analyzed during this study will be shared on reasonable request from any qualified investigator.

**Statistical Analyses**
Owing to the small sample size, exact nonparametric tests were performed to determine the significance. Pearson correlation or Spearman correlation coefficient was used to evaluate the association between variables. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) statistical software (v21.0. SPSS Inc;
Chicago, IL). All reported p-values were based on 2-tailed statistical tests, with a significance threshold of 0.05.

Results

CD59 Cluster at the NMJ With AChR on Muscles
The NMJ of muscles was detected by α-BTx using the Z-stack technique. CD59 was weakly localized on the cytoplasmic membrane of the muscle fibers. In addition, we detected CD59 staining at the NMJ because of its coexpression with α-BTx. CD59 was strongly localized at the NMJ not only in nonmyopathy controls but also in patients with MG (Figure 1A). In MG, CD59 immunostaining at the NMJs exhibited a weaker staining and smaller regions (Figure 1A; patients 11 and 10). It is of interest that the relative mRNA expression of CD59 in the muscle cells (Figure 1A; patients 9 and 13).

Overexpressed CD59 Protein in MG Muscles
All muscle specimens demonstrated CD59 protein expression in WB analysis (Figure 1B, eTable 1 and eTable 2, links.lww.com/NXI/A762, links.lww.com/NXI/A763). The expression ratio of CD59/GAPDH in patients with MG was significantly higher compared with that in the nonmyopathy controls (MG; n = 16, median 0.39, IQR 0.28–1.43 and nonmyopathy controls; n = 6, median 0.28, IQR 0.10–0.60, p = 0.231). The ratio of CD59/GAPDH in nonmyopathy controls and patients with MG. The AChR mRNA expression showed no significant difference between the nonmyopathy controls and the patients with MG (MG; n = 16, median 1.08, IQR 0.40–1.87 and nonmyopathy controls; n = 6, median 0.55, IQR 0.18–0.83, p = 0.095). AChR = acetylcholine receptor; CD59 = cluster of differentiation 59; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IQR = interquartile range; MG = myasthenia gravis.

CD59 mRNA and AChR mRNA in MG Muscles
The qRT-PCR analysis revealed that CD59 mRNA and AChR mRNA expression showed no significant differences between the nonmyopathy controls and the patients with MG (Figure 2, B and C). The values of the abundance of ΔCt and fold changes of each mRNA among the patients with MG and nonmyopathy controls are summarized in eTable 1 and eTable 2, respectively, links.lww.com/NXI/A761). The ratio of CD59/GAPDH in WB was associated with the fold change of CD59 mRNA (r = 0.602, p = 0.003) (Figure 3A).

ΔCtCD59/AChR Was Significantly Correlated With the Clinical Parameter Scores
It is of interest that the relative mRNA expression of CD59 to AChR (ΔCtCD59/AChR) was associated with the QMG scores and MG-ADL scores (r = 0.54, p = 0.043 and r = 0.56, p = 0.037, respectively), but only under a certain condition of excluding a patient who was in a stage of crisis (eFigure 1, links.lww.com/NXI/A761). Meanwhile, there was a correlation between CD59 mRNA expression and the QMG scores and MG-ADL scores (r = 0.715, p = 0.003) (Figure 3B).
was no association between CD59 expression (including ΔCtCD59/AChR) and AChR-Ab titers.

**Discussion**

In MG, a major role of the AChR-Ab is conferring damage to the postsynaptic membrane by inducing complement activation leading to MAC formation.\(^5\) The NMJ of patients with MG contained deposits of IgG, C3, and C9.\(^5\) Complement inhibitors that target molecules in the complement activation cascade have been useful therapeutic agents in MG.\(^6\) Eculizumab targets C5 in the plasma and prevents cleavage of C5 to C5a and C5b that inhibits MAC assembly, leading to an improvement in 86% of the patients with MG.\(^7\) The effects of eculizumab in MG suggested that the reduction of MAC formation at the NMJ inhibits endplate destruction.\(^7\) Control of MAC assembly to ensure protection from autologous complement attack and MAC formation is mainly mediated by CD59, one of the complement regulatory proteins. CD59 inhibited MAC formation by binding to C9 and C5b-8 complexes.\(^8,9\) CD59 is widely distributed in normal human tissues and cells,\(^9\) including skeletal muscles.\(^10\)

This study revealed that CD59 is strongly localized at the NMJ in addition to the cytoplasmic membrane of the muscle in both patients with MG and nonmyopathy controls. CD59 expression at the NMJ has been reported in amyotrophic lateral sclerosis and experimental autoimmune MG.\(^13,14\) However, the strongly localized CD59 expression at the NMJ in patients with MG and nonmyopathy controls has not been reported previously.

Compared with nonmyopathy controls, patients with MG demonstrated weaker expressions of CD59 at the NMJ with an irregular expression of AChR. Correlation between CD59 expression and clinical scores of MG was analyzed. (A) Correlation between the fold changes in CD59 mRNA and the protein ratio of CD59/GAPDH by western blotting in nonmyopathy controls and patients with MG. Pearson correlation was used to calculate the association. (B) Correlation between the fold changes in CD59 mRNA and AChR mRNA in nonmyopathy controls and patients with MG. Pearson correlation was used to calculate the association. (C) Association between ΔCtCD59/AChR (=AChR Ct−CD59 Ct) and QMG scores in patients with MG. Pearson correlation was used to calculate the association. (D) Association between ΔCtCD59/AChR (=AChR Ct−CD59 Ct) and MG-ADL scores in patients with MG. Pearson correlation was used to calculate the association. (E) Association between ΔCtCD59/AChR (=AChR Ct−CD59 Ct) and MGFA Clinical Classification in patients with MG. Spearman correlation was used to calculate the association. AChR = acetylcholine receptor; CD59 = cluster of differentiation 59; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; MG = myasthenia gravis; MG-ADL = MG-specific activities of daily living; MGFA = Myasthenia Gravis of Foundation of America; QMG = quantitative MG.
NMJ against the attack by the formed MAC which was induced by complement activation. The destructive condition of NMJ by complement activation resulted in the decreased expression of both CD59 and AChR.

Moreover, this study revealed that CD59 expression was concentrated in the NMJ without α-BTx staining, and the α-BTx-stained region partially overlapped with the CD59 localized area. CD59 expression might be concentrated at the NMJ by different mechanisms of AChR clustering in patients with MG. CD59 is a glycosylphosphatidylinositol (GPI)-anchored protein; GPIs are on the lipid raft of the membrane. Caveolin-3 is a muscle-specific caveolin member and functions as a lipid raft. In other words, CD59 might be expressed on caveolin-3. Caveolin-3 expression was strongly localized to the NMJ and cytoplasmic membrane. In addition, caveolin-3 interacts with muscle-specific tyrosine kinase and dystrophin-dystroglycan complex. The collagen-tailed form of acetylcholinesterase (AChE) was expressed on the muscle surface and clustered at the NMJ with dystroglycan-perlecan complex during NMJ formation. Strong localization of CD59 expression at the NMJ might be associated with dystroglycan-perlecan and caveolin-3 complex, which are associated with AChE clustering.

It is of interest that up-regulation of CD59 protein was induced in the muscles of patients with MG and CD59 mRNA expression was correlated with the AChR mRNA levels. In addition, a higher ratio of CD59 to AChR mRNA (ΔCtCD59/AChR) expression was associated with better clinical parameters of the QMG scores, MG-ADL scores, and MGFA Clinical Classification. These findings suggest that CD59 overexpression compared with AChR in MG might have a sturdy effect on the inhibition of MAC formation at the NMJ. In addition, we hypothesized that NMJ reconstruction in MG might require CD59 overexpression compared with AChR. Moreover, over-expression of CD59 over the entire muscle cell surface might have consequently made up for the expression at NMJ and be an essential factor for clinical improvement in MG.

The ratio of CD59/AChR mRNA overexpression can be a biomarker of disease severity; however, this index was calculated from the mRNA of the muscles. Accordingly, it is challenging to use this index in daily clinical practice due to the difficulty in obtaining muscle specimens from patients with MG. To use this index in clinical practice, it would be necessary to identify serum biomarkers that reflect the ratio of CD59/AChR mRNA in the muscles.

Furthermore, CD59 has other immunosuppressive functions such as the suppression of T-cell activity and anti-inflammatory function. Its overexpression in the muscles may induce the immunosuppressive condition of MG.

However, no significant differences in CD59 mRNA expression were found between the nonmyopathy controls and the patients with MG possibly owing to the small number of patients. The mRNA and protein expression of CD59 might show an increase in patients with MG in future large-scale studies. Regarding polymyositis, dermatomyositis, and the other inflammatory myopathies, complement is always associated with muscle fiber necrosis. Moreover, in dermatomyositis, there is complement-mediated microangiopathy in the patients’ biopsies. In addition, conditions characterized by muscle damage, such as Duchenne muscular dystrophy, polymyositis, dermatomyositis, amyotrophic lateral sclerosis, and sepsis, are associated with increased muscle expression of CD59. The findings demonstrate that muscle cell damage may be susceptible to complement attack and may require protection by complement regulatory proteins in the pathologic conditions of the muscles.

Our study had some limitations. First, the muscle tissues were obtained from different anatomical locations; thus, they may exhibit different histologic and molecular findings. This difference may affect the CD59 and AChR expression levels, which might pose significant sampling bias. Second, a small number of patients were used, and there was a lack in specific molecular testing. Further studies should investigate other complement regulatory factors (e.g., CD46 and CD55 expression) in MG muscles.

In conclusion, CD59 expression at the NMJ may be an important factor for the protection of the NMJ against complement attack in humans. In addition, CD59 overexpression might be required for the NMJ reconstruction as one of the essential steps for improving the pathophysiology of MG. The induction of CD59 expression at the NMJ would have the potential to be a novel therapeutic strategy in MG.

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**Disclosure**
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Appendix (continued)

Name                      | Location                                                                 | Contribution                                                                                                                                 |
---------------------------|---------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|
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Hiroaki Yoshikawa, MD, PhD | Health Service Center, Kanazawa University, Kanazawa, Japan                | Drafting/revision of the manuscript for its content, including medical writing for its content, and the analysis or interpretation of data |
Masahito Yamada, MD, PhD   | Department of Neurology, Kudanzaka Hospital, Tokyo, Japan Department of Neurology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan | Drafting/revision of the manuscript for its content, including medical writing for its content, and the analysis or interpretation of data |
Kenjiro Ono, MD, PhD       | Department of Neurology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan | Drafting/revision of the manuscript for its content, including medical writing for its content, and the analysis or interpretation of data |

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