Dehghan et al. (2008) report that rs2231142 in *ABCG2* is associated with gout by a genome-wide association study (OR = 1.74 and 1.71 in white and black participants, respectively). Woodward et al. (2009) showed a significant association between rs2231142 and hyperuricemia (OR = 1.68) in a population-based study of 14,783 individuals. Kolz et al. (2009) demonstrated that rs2231142 elevated the serum urate concentration more strongly in men than in women by meta-analysis of 28,141 individuals. Stark et al. (2009) analyzed 683 patients with gout and indicated a significant association between rs2231142 and gout (OR = 1.37). Although rs2231142 is an attractive causative SNP, our normouricemic subjects were also heterozygous for rs2231142.

Graessler et al. (2006) analyzed 389 German individuals with primary hyperuricemia and found that rs3825016 and rs11231825 in *SLC22A12* were significantly associated with reduced fractional excretion of urate in the kidney. Tabara et al. (2010) analyzed 1,526 normal Japanese individuals retrospectively and longitudinally, and clarified that rs11231825 was associated with reduced urate excretion and with future development of hyperuricemia. Again, although the two SNPs are attractive causes of hyperuricemia, we observe variable dosages of these SNPs even in our normouricemic subjects.

We next looked into neighboring genes of *COL1A1* without considering the functions of the gene products, and identified that two missense variants in *ZPBP2* and *GPATCH8* cosegregated with the *COL1A1* mutation in F1. Neither variant was detected in 300 normal alleles or in dbSNP132. *ZPBP2* p.T69I, however, is unlikely to be pathogenic for three reasons: lack of conservation in mammals; two missense/frameshifting SNPs at or close to the variant site (Fig. 3a); and the benign predicted outcome by PolyPhen-2 and SIFT. On the other hand, p.A979P in *GPATCH8* substitutes an amino acid in the highly conserved serine-rich region (Fig. 3b), and the substitution is predicted to damage the structure and function of the protein by in silico analysis. *GPATCH8* encodes the G patch domain-containing protein 8 that harbors both an RNA-processing domain and a zinc finger domain. *GPATCH8* is expressed in a wide variety of human tissues including skeletal muscles, brain, heart, pancreas, liver and kidney (McKinney et al. 2004). Functions of the *GPATCH8* gene product, however, have not been studied to date. The p.A979P variant in *GPATCH8* is highly likely to be associated with hyperuricemia in F1, but it may also cause another yet unidentified phenotype that cosegregates with OI.

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Anti-MuSK autoantibodies block binding of collagen Q to MuSK

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ABSTRACT

Objective: Muscle-specific receptor tyrosine kinase (MuSK) antibody-positive myasthenia gravis (MG) accounts for 5%–15% of autoimmune MG. MuSK mediates the agrin-signaling pathway and also anchors the collagenic tail subunit (ColQ) of acetylcholinesterase (AChE). The exact molecular target of MuSK-immunoglobulin G (IgG), however, remains elusive. As acetylcholine receptor (AChR) deficiency is typically mild and as cholinesterase inhibitors are generally ineffective, we asked if MuSK-IgG interferes with binding of ColQ to MuSK.

Methods: We used 3 assays: in vitro overlay of the human ColQ-tailed AChE to muscle sections of *Colq*−/− mice; in vitro plate-binding assay to quantitate binding of MuSK to ColQ and to LRP4; and passive transfer of MuSK-IgG to mice.

Results: The in vitro overlay assay revealed that MuSK-IgG blocks binding of ColQ to the neuromuscular junction. The in vitro plate-binding assay showed that MuSK-IgG exerts a dose-dependent block of MuSK binding to ColQ by but not to LRP4. Passive transfer of MuSK-IgG to mice reduced the size and density of ColQ to ~10% of controls and had a lesser effect on the size and density of AChR and MuSK.

Conclusions: As lack of ColQ compromises agrin-mediated AChR clustering in *Colq*−/− mice, a similar mechanism may lead to AChR deficiency in MuSK-MG patients. Our experiments also predict partial AChE deficiency in MuSK-MG patients, but AChE is not reduced in biopsied NMJs. In humans, binding of ColQ to MuSK may be dispensable for clustering ColQ, but is required for facilitating AChR clustering. Further studies will be required to elucidate the basis of this paradox. *Neurology*® 2011;77:1819–1826

GLOSSARY

AChE = acetylcholinesterase; **AChR** = acetylcholine receptor; **ColQ** = collagen Q; **IgG** = immunoglobulin G; **LRP4** = low-density lipoprotein receptor-related protein 4; **MG** = myasthenia gravis; **MuSK** = muscle-specific receptor tyrosine kinase; **NMJ** = neuromuscular junction; **SDS-PAGE** = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

During development of the neuromuscular junction (NMJ), neural agrin released from the nerve terminal binds to the postsynaptic transmembrane protein LRP4.[1,2] Dimerized LRP4 forms a heterotetramer with the dimerized muscle-specific receptor tyrosine kinase (MuSK).[3] MuSK together with Dok-7 promotes clustering of acetylcholine receptor (AChR) on the junctional folds by rapsyn.[4] The clustering effect of MuSK is mediated by distinct pathways involving Rho GTPase.[5]

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At the NMJ, 3 tetramers of catalytic subunits of acetylcholinesterase (AChE) are linked to ColQ, the triple helical collagenic subunit.[6] ColQ-tailed AChE is anchored to the synaptic basal lamina by 2 mechanisms: 2 sets of heparan sulfate proteoglycan residues in the collagen

Supplemental data at www.neurology.org

Supplemental Data

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domain of ColQ[7] bind to heparin sulfate proteoglycans, such as perlecan[8]; and the C-terminal domain of ColQ binds to MuSK.[9]

Five percent to 15% of patients with myasthenia gravis (MG) carry antibodies against MuSK (MuSK–immunoglobulin G [IgG]).[10,11] MuSK-MG patients respond favorably to immunotherapy, but usually do not respond to, or are even worsened by, cholinesterase inhibitors.[12–15] Anti-AChR antibodies comprise IgG1 and IgG3 moieties that bind complement whereas anti-MuSK antibodies are largely IgG4 that do not activate complement, and complement deposits at the NMJ are sparse.[16–18] However, the exact target of MuSK-IgG remains elusive. We therefore examined an effect of MuSK-IgG on an interaction between ColQ and MuSK by in vitro and in vivo assays, and found that MuSK-IgG blocks this interaction.

METHODS **Patients.** We obtained serum from 4 MuSK-MG patients (patients 1–4) and a patient with limb-girdle muscular dystrophy as a control (control 1). We obtained 10 mL peripheral blood from patients 1, 3, 4, and control and residual plasmapheresis fluid from patient 2. We also obtained expired fresh-frozen plasma (control 2) from Dr. Isao Takahashi at the Aichi Red Cross Blood Center with institutional approval. We used sera of patient 2 and control 2 for all the experiments, and sera of patients 1, 3, 4, and control 1 only for the in vitro overlay and in vitro plate binding assays because only small amounts of sera were available from these patients.

Ages and genders of patients 1–4 were a 48-year-old woman, a 30-year-old woman, a 59-year-old man, and a 45-year-old woman, respectively. The titers of anti-MuSK antibodies of patients 1–3 were 22.0 nM, 11.2 nM, and 0.12 nM, respectively (normal <0.01 nM). Patient 4 was positive for anti-MuSK antibody, but the titer was not determined.

Standard protocol approvals, registrations, and patient consents. We performed all human studies under the institutional review board approvals of the Nagoya University Graduate School of Medicine and the Mayo Clinic, and obtained written informed consents from each patient and a control. We also obtained approvals of the *Colq*−/− mice studies and the passive IgG transfer studies by the Animal Care and Use Committee of the Nagoya University.

Plasmids. We previously made CMV-based mammalian expression vectors, pTargeT-*COLQ* and pTargeT-*ACHE*.[19] To generate hMuSKect-myc, we cloned the extracellular domain (aa 1–393) of human *MUSK* cDNA (Open Biosystems) into a mammalian expression vector pAPtag-5 (GenHunter) at the *Nhe*I and *Xho*I sites upstream of a myc epitope. For hLRP4N-FLAG, we cloned the extracellular domain (aa 1–1722) of human *LRP4* cDNA (Open Biosystems) into the *Hin*dIII and *Xba*I sites upstream of a 3xFLAG epitope of a mammalian expression vector p3XFLAG-CMV-14 (Sigma Aldrich).

Preparation of recombinant human ColQ-tailed AChE. We prepared human ColQ-tailed AChE for in vitro overlay assay and for in vitro plate-binding assay. Both pTargeT-*COLQ* and pTargeT-*ACHE* were transfected into HEK293 cells in a 10-cm dish using the calcium phosphate method as described elsewhere.[20] We extracted proteins from the cells in Tris-HCl buffer (50 mM Tris-HCl [pH 7.0], 0.5% Triton X-100, 0.2 mM EDTA, leupeptin [2 μg/mL], and pepstatin [1 μg/mL]) containing 1 M NaCl, and diluted the extracts containing ColQ-tailed AChE in Tris-HCl buffer containing 0.2 M NaCl and loaded onto the HiTrap Heparin HP columns (GE Healthcare). We washed the columns with 5 volumes of Tris-HCl buffer containing 0.2 M NaCl, and eluted ColQ-tailed AChE with Tris-HCl buffer containing 1 M NaCl. We concentrated the eluate with an Amicon Ultra-4 Centrifugal Filter (50K) (Millipore) to 12-Ellman units per mL. The units were normalized with the Torpedo-derived AChE (C2888, Sigma-Aldrich).

Preparation of hMuSKect-myc and hLRP4N-FLAG proteins. We prepared hMuSKect-myc and hLRP4N-FLAG for in vitro plate-binding assays. We introduced a construct carrying either hMuSKect-myc or hLRP4N-FLAG into HEK293 cells in a 10-cm dish using the calcium phosphate method as above. We purified the hMuSKect-myc with the c-myc-Tagged Protein Mild Purification Kit version 2 (MBL), and purified the hLRP4N-FLAG with the Anti-DYKDDDDK-tag Antibody Beads (Wako). We detected purified hMuSKect-myc and hLRP4N-FLAG by anti-myc antibody (9E10, Abcam) and anti-FLAG antibody (M2, Sigma-Aldrich), respectively (data not shown), and also detected hMuSKect-myc by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein staining with the Oriole Fluorescent Gel Stain (Bio-Rad).

Purification of plasma IgG. We purified IgG as described elsewhere[21] with minor modifications. We adjusted plasma to pH 8.0 with 1 M NaOH. While stirring 1 volume of plasma, we slowly added 3.5 volumes of 0.4% rivanol (Tokyo Chemical Industries) in water for 30 minutes. We left the solution overnight at RT, and removed a tenacious yellow precipitate. After filtering the supernatant through Whatman no. 1 paper to remove residual precipitates, we added 8 g of activated charcoal (Wako Chemicals) for 100 mL of the IgG solution and incubated overnight at 4°C to remove rivanol. We then slowly added an equal amount of saturated ammonium sulfate, and again incubated overnight at RT to precipitate crude IgG. We centrifuged the solution at 3,000 × *g* for 30 minutes, and added saline to the precipitate to form a slurry, which was then transferred to a dialysis tube (Spectra/Por MWCO 50,000, Spectrum Laboratories). We dialyzed the solution in saline at 4°C for 3 hours, followed by dialysis in PBS at 4°C for 2 hours and then overnight. We removed residual charcoals by filtering through a 0.22-μm Millex-GP filter (Millipore), and concentrated IgG using Amicon Ultra 50K (Millipore). We confirmed purity of isolated IgG by 6% SDS-PAGE under a nonreducing condition. We also reduced IgG in 4% 2-mercaptoethanol and fractionated the heavy and light chains by 10% SDS-PAGE.

Incubation of purified IgG to a muscle section of *Colq*−/− mice. We prepared 10-μm-thick sections of quadriceps muscles of *Colq*−/− mice[22] with a Leica CW3050–4 cryostat at −20°C. We blocked nonspecific binding of a muscle section with the blocking buffer that contained 5% sheep serum in PBS at RT for 2 hours. We suspended the purified IgG in the blocking buffer at 50 μg/mL, and overlaid it on a muscle section at 4°C overnight. We detected human IgG by FITC-labeled

anti-human IgG antibody (02–10-06, KPL), and AChR by Alexa594-labeled α-bungarotoxin (Molecular Probes).

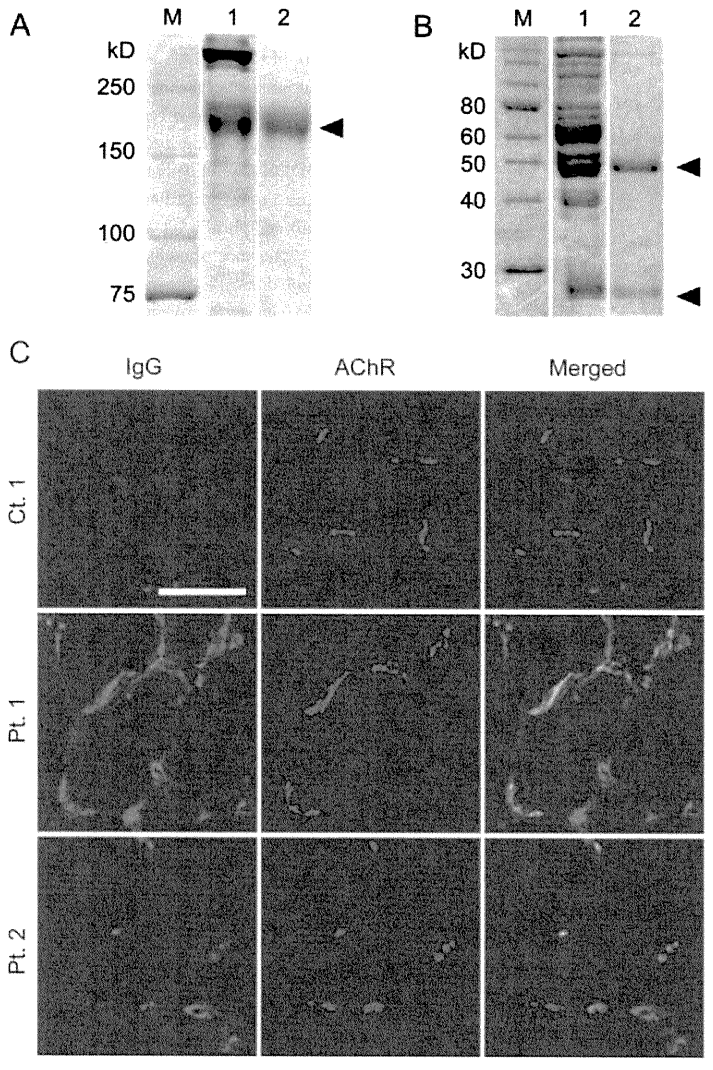
In vitro overlay assay. The overlay binding method was essentially as previously described.[23] We overlaid 600 μg IgG of patients at 4°C overnight before adding 120-milli-Ellman units of ColQ-tailed AChE.

In vitro plate-binding assay for quantifying ColQ-MuSK interaction. We coated the Maxi-Sorp Immuno Plate (Nunc) with 0.15 μg of purified hMuSKect-myc at 4°C overnight and then incubated it with a blocking buffer that contained 50 mM Tris-HCl (pH 7.4), 0.5% BSA, 0.5% ovalbumin, and 0.5 M NaCl at RT for 1 hour. We incubated the wells with 1 pg to 100 μg of IgG of controls 1 and 2 and patients 1–4 at 4°C for 6 hours. We added 0.12-Ellman units of ColQ-tailed AChE as described above. We then quantified the bound ColQ-tailed AChE by the Ellman method in the presence of 5×10^{-5} M ethopropazine.[19] Each time before we moved to the next step, we washed the plate 3 times with PBS.

In vitro plate-binding assay for quantifying LRP4-MuSK interaction. We coated the Maxi-Sorp Immuno Plate with 0.15 μg of purified hMuSKect-myc as described above, and then blocked with 1% BSA in PBS at RT for 1 hour. We incubated the wells with 1 pg to 100 μg of IgG of control 2 and patient 2 at 4°C for 6 hours. We added 0.12 μg of purified hLRP4N-FLAG on each well at RT for 2 hours. We then quantified the bound hLRP4N-FALG by anti-FLAG-HRP using the TMB substrate kit (Pierce). Again, between each step, we washed the plates 3 times with PBS.

Passive transfer of human IgG to mice. We made passive transfer model mice as described elsewhere.[24] We intraperitoneally injected 40 mg IgG of control 2 and patient 2 into 6-week-old female C57BL/6J mice every day for 15 days. We sterilized IgG with a 0.22-μm filter (Millipore) and dissolved it in 400 μL PBS. The mice were killed on day 16 under deep anesthesia. To suppress any active immune response to the human protein,[25] we injected 300 mg/kg of cyclophosphamide monohydrate (10 mg/mL in 0.9% NaCl) intraperitoneally 24 hours after the first IgG injection. We also injected IgG of patient 2 into 2 additional mice to confirm consistency, and analyzed a representative mouse in detail. We detected AChR by Alexa594-labeled α-bungarotoxin (Molecular Probes), ColQ by 1:100 of a newly raised anti-ColQ antibody (figure e-1 on the *Neurology*® Web site at www.neurology.org), and MuSK by 1:100 of anti-MuSK antibody (C-19, Santa Cruz). We quantified signals by the BZ-9000 microscope (Keyence) equipped with the Dynamic Cell Count software BZ-H1C (Keyence).

Figure 1 **Muscle-specific receptor tyrosine kinase (MuSK)-immunoglobulin G (IgG) recognizes the neuromuscular junction (NMJ) of *Colq*−/− mice**

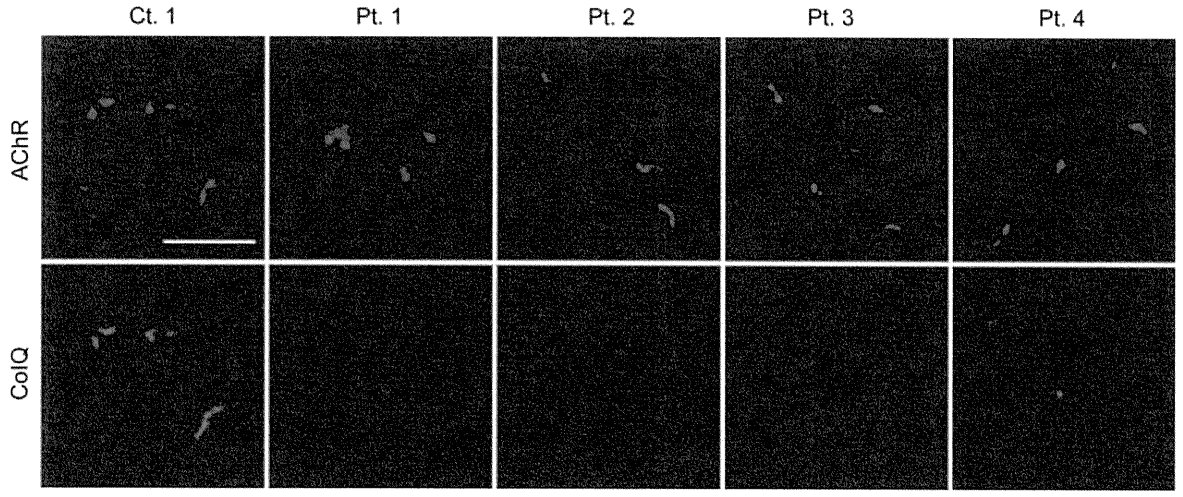


Nonreducing (A) and reducing (B) sodium dodecyl sulfate-polyacrylamide gel electrophoresis of serum proteins and purified IgG of patient 1. Gels are stained with Coomassie brilliant blue. M = molecular weight markers; 1 = serum before purification; 2 = purified IgG. Arrowheads point to IgG of 160 kD (A), as well as the heavy (50 kD) and light (25 kD) chains of IgG (B). (C) In vitro overlay of purified IgG on a 10-μm skeletal muscle section of *Colq*−/− mice. IgG is visualized with FITC-labeled antihuman IgG and acetylcholine receptor with Alexa594-labeled α-bungarotoxin. Scale bar = 50 μm.

RESULTS **MuSK-IgG recognizes NMJ of a muscle section of *Colq*−/− mouse.** We first confirmed that human MuSK-IgG recognizes the mouse NMJ. We isolated IgG from serum of MuSK-MG patients and confirmed the purity of IgG by Coomassie staining of nonreducing (figure 1A) and reducing (figure 1B) SDS-PAGEs. We then overlaid MuSK-IgG on quadriceps muscle sections of *Colq*−/− mice.[22] IgG of control 1 was not bound to the NMJ, whereas IgGs of patients 1 and 2 colocalized to the NMJs (figure 1C). Human MuSK-IgG thus has the potential to bind to the mouse NMJ.

In vitro overlay assay discloses that MuSK-IgG blocks binding of ColQ-tailed AChE to the NMJ of a muscle section of *Colq*−/− mouse. We previously demonstrated that the purified recombinant human ColQ-tailed AChE protein complex could bind to sections of the frog NMJs[23] and the mouse NMJs (in preparation) in vitro. Using the in vitro overlay assay, we next examined whether MuSK-IgG blocks anchoring of ColQ-tailed AChE to the mouse NMJs. We incubated a muscle section of *Colq*−/− mice with MuSK-IgG overnight at 4°C and overlaid human ColQ-tailed AChE followed by histologic visualization of ColQ and AChR (figure 2). In the presence of

Figure 2 **In vitro overlay assays**



Purified recombinant collagen Q (ColQ)-tailed acetylcholinesterase (AChE) was overlaid on a 10-μm quadriceps muscle section of *Colq*−/− mice in the presence of the indicated purified muscle-specific receptor tyrosine kinase–immunoglobulin G. ColQ is stained with anti-ColQ antibody and acetylcholine receptor (AChR) with Alexa594-labeled α-bungarotoxin. Scale bar = 50 μm.

IgG of control 1, ColQ was colocalized with AChRs, whereas, in the presence of 4 MuSK-IgGs, no ColQ signal was observed at the NMJs.

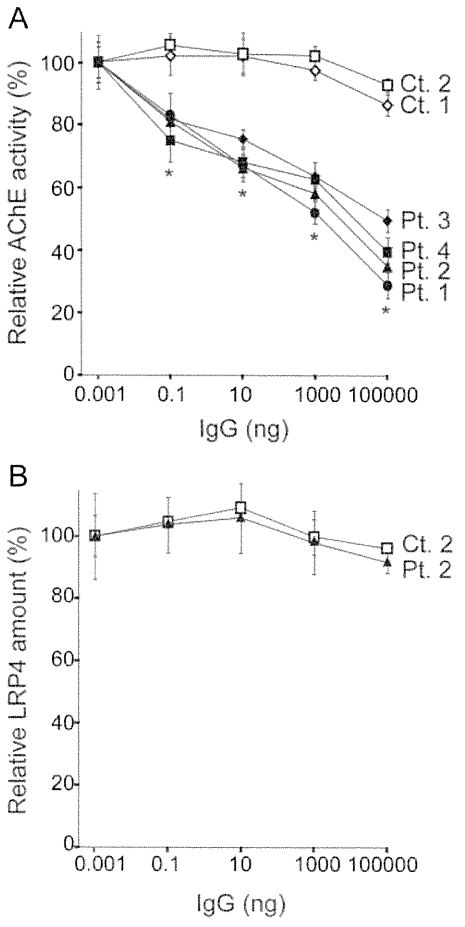
In vitro plate-binding assay shows that MuSK-IgG blocks binding of ColQ-tailed AChE but not of LRP4 to MuSK. We next quantified an effect of MuSK-IgG on an interaction of human ColQ and human MuSK by an in vitro plate-binding assay. We synthesized and purified the myc-tagged extracellular domain of human MuSK (hMuSKect-myc). We then incubated an hMuSKect-coated plate with variable concentrations of control IgG or MuSK-IgG, and added a fixed amount of the purified recombinant human ColQ-tailed AChE. In 2 controls, AChE remained bound even in the presence of 100 μg of IgG, whereas in 4 MuSK-MG patients the numbers of bound AChE were proportionally decreased with increasing amounts of the patient's IgG (figure 3A).

We also examined the effect of MuSK-IgG on the interaction between the extracellular domain of MuSK and LRP4. We found that even at 100 μg IgG of control 2 or patient 2 did not block binding of LRP4 to MuSK (figure 3B).

Passive transfer model exhibits reduced ColQ signals at the NMJs. As described in the introduction, active and passive immunization of model animals reveals reduction of AChRs at the NMJs,[24,26–29] but an effect of MuSK-IgG on ColQ-tailed AChE has not been examined to date. We thus injected IgG of control 2 and patient 2 for 14 days to C57BL/6J female mice and visualized the expression of AChR, ColQ, MuSK, and AChE in quadriceps muscle sections. Signal intensities of ColQ and AChE were markedly attenuated, but the AChR and MuSK signal intensities were only moderately reduced (figure 4, A and B). Quantitative analysis of the fluorescence signals revealed that signal areas (figure 4C), intensities (figure 4D), and densities (figure 4E) of ColQ in mice injected with patient 2 IgG were significantly reduced. Conversely, signal areas (figure 4C), intensities (figure 4D), and densities (figure 4E) of AChR were only moderately reduced. Similarly, the same parameters of the MuSK signal were moderately reduced (figure 4, C, D, and E). Moderate reductions of the areas and intensities of AChR and MuSK signals are likely due to reduced sizes of the NMJs, because the densities of AChR and MuSK were only marginally affected. In addition, whereas the number of MuSK per AChR remained essentially the same, the number of ColQ per AChR was prominently reduced (figure 4F). To summarize, MuSK-IgG compromised anchoring of ColQ-tailed AChE and had a less prominent effect on the expression of MuSK and AChR.

DISCUSSION Molecular basis of MuSK-MG has been examined in cultured cells[30,31] as well as in active[28,29] and passive[24,26,27] immunization models. Application of MuSK-MG antibodies to TE671 muscle cells induces inhibition of cell proliferation and secondarily leads to downregulation of AChR and rapsyn.[30] Similarly, MuSK-MG antibodies have no or minimal effect on the cell surface expression of AChR in TE671 and C2C12 muscle cells.[31] Conversely, mice[29] and rabbits[28] immunized with recombinant MuSK develop myasthenic symptoms and

Figure 3 **In vitro plate-binding assays**

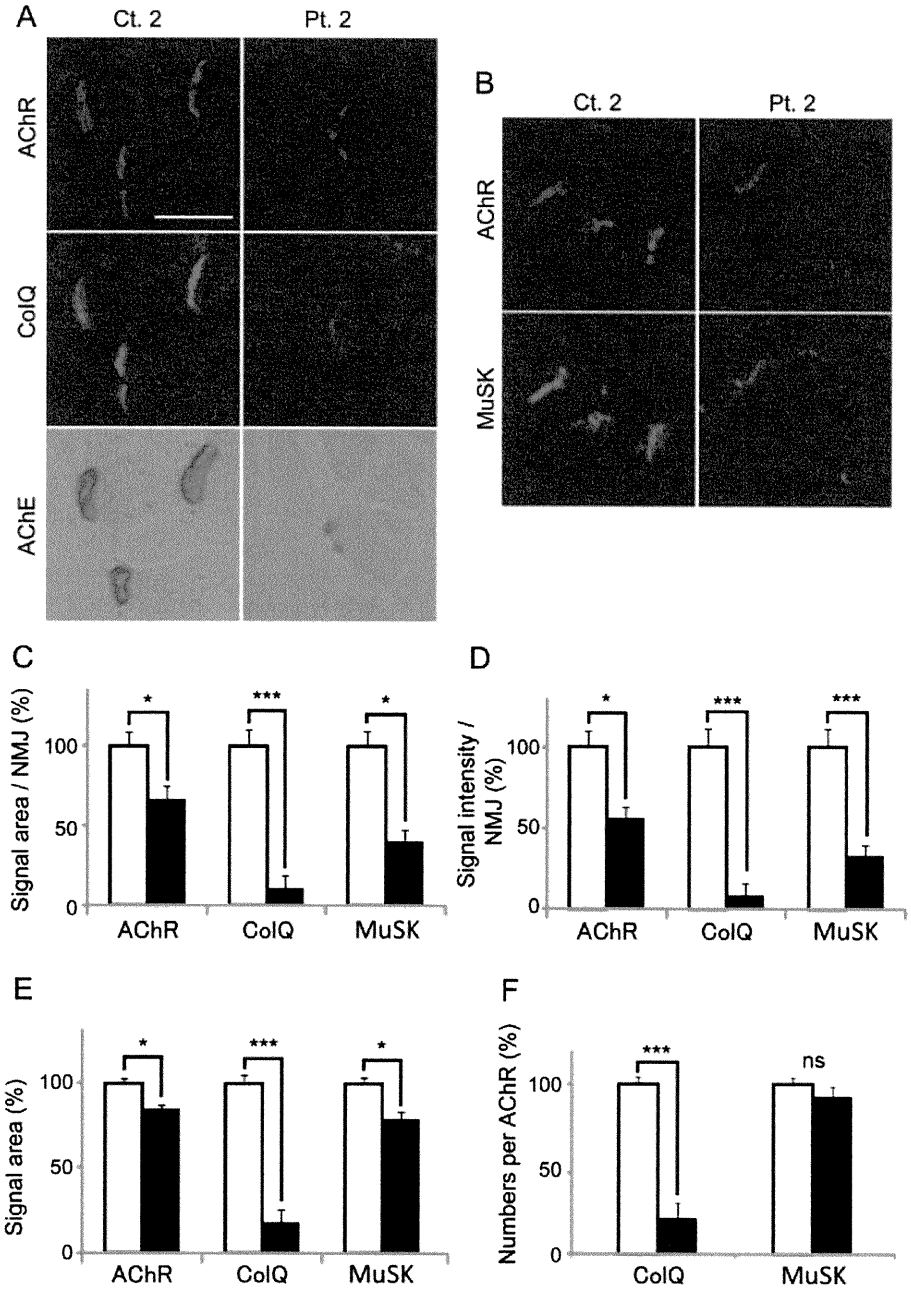


(A) Increasing amounts of muscle-specific receptor tyrosine kinase (MuSK)-immunoglobulin G (IgG) block binding of the purified recombinant collagen Q (ColQ)-tailed acetylcholinesterase (AChE) to the extracellular domain of human MuSK that is coated on a 96-well plate. Bound ColQ-tailed AChE is quantified by AChE activity. AChE activities are normalized for that at 1 pg IgG of each sample. Mean and SEM of 3 experiments are plotted. *$p < 0.01$ between controls and patients. (B) MuSK-IgG does not block binding of the purified FLAG-tagged extracellular domain of human LRP4 (LRP4N-FLAG) to MuSK that is coated on a 96-well plate. Bound LRP4N-FLAG is quantified with anti-FLAG-HRP. HRP activities are normalized for that at 1 pg IgG of each sample. Mean and SEM of 3 experiments are plotted.

NMJ AChR deficiency. Similarly, injection of MuSK-IgG into mice reduces the number of AChRs at the NMJ to 22% of controls, compromises the apposition of the presynaptic and postsynaptic components of the NMJ,[24] and reduces muscle contractility.[27] A recent report demonstrates that MuSK-IgG enhances internalization of MuSK from plasma membrane, which leads to progressive dispersal of postsynaptic AChRs by disruption of the MuSK scaffold and not by disruption of the agrin/LRP4/MuSK signaling pathway.[26] To summarize, MuSK-IgG does not reduce AChR expression in cultured cells, but active and passive immunization of model animals results in AChR deficiency, which is not likely due to blocking of the agrin/LRP4/MuSK pathway. Our findings that MuSK-IgG blocks binding of ColQ but not of LRP4 to MuSK are consistent with these findings. In myotubes of *Colq*−/− mice, the number of membrane-bound MuSK is prominently reduced, and agrin-mediated phosphorylation of the AChR β subunit and the subsequent clustering of AChR are reduced to 30%–50% of the wild type.[32] Thus, compromised clustering of AChRs at the NMJs in some MuSK-MG patients could result from blocking of ColQ binding to MuSK but not from blocking of LRP4 binding to MuSK.

Although our results predict endplate AChE deficiency in MuSK-MG patients, we found no AChE deficiency in intercostal muscles of one reported[33] and two unreported cases of MuSK-MG. In vitro microelectrode studies showed a normal EPP decay time constant.[34] In the 3 MuSK-MG patients observed by us, the MEPC decay times were shorter than normal, normal, and 2-fold prolonged[33] compared to controls. Thus, our biopsy findings do not indicate that MuSK-MG patients have endplate AChE deficiency. There are 2 plausible explanations for the apparently contradicting observation on the human biopsies and the in vitro and in vivo studies. First, MuSK-IgG does not block binding of ColQ-tailed AChE to the NMJ to a detectable extent in the patients. ColQ is localized to the synaptic basal lamina via 2 mechanisms: one is by binding to heparin sulfate proteoglycans including perlecan,[7,8] and the other is by binding to MuSK.[9] We previously reported that both mechanisms are required for in vitro anchoring of human ColQ to the frog NMJ.[23] Reduced clustering of ColQ in our passive transfer model suggests that ColQ needs to bind to at least MuSK in mice. However, binding of ColQ to MuSK is dispensable for clustering ColQ in humans, but is required for facilitating AChR clustering.[32] Second, AChE could be deficient in severely affected muscles but not in the biopsied intercostal muscles. However, the respiratory functions of the patients who had intercostal muscle biopsies were severely compromised. Expression levels of MuSK[35] and ColQ[36] were reported to be different between slow- and fast-twitch muscles in model animals. In active[37] and passive[26] immunization models, slow-twitch diaphragm was more severely affected than fast-twitch tibialis anterior and intercostal muscles. Similar uneven distributions of affected muscles are reported in MuSK-MG patients.[12] Further studies will be required to elucidate the basis of the discrepant observations between mice and humans.

Figure 4 **Passive transfer of muscle-specific receptor tyrosine kinase (MuSK)–immunoglobulin G (IgG) of control 2 and patient 2 to C57BL/6J mice**



(A, B) Quadriceps muscle sections of mice injected with IgG of control 2 or patient 2 are stained for acetylcholine receptor (AChR) by Alexa594-labeled α-bungarotoxin, collagen Q (ColQ) and MuSK by immunostaining, acetylcholinesterase (AChE) by cytochemical staining. Scale bar = 40 μm. Signal areas (C), intensities (D), and densities (intensity/area) (E) of the indicted molecules per neuromuscular junction (NMJ) are shown in mean and SEM. (F) Densities of ColQ and MuSK are normalized for the density of AChR to estimate the number of ColQ and MuSK per AChR. For AChR, ColQ, and MuSK, we analyzed 44 NMJs of control 2 and 23 NMJs of patient 2. For MuSK, we analyzed 82 NMJs of control 2 and 42 NMJs of patient 2. Areas and intensities are quantified by the BZ-9000 microscope (Keyence). Open and closed bars represent control 2 and patient 2, respectively. $^{*}p < 0.05$, $^{***}p < 0.001$. NS = not significant.

AUTHOR CONTRIBUTIONS

Dr. Kawakami designed and conducted experiments and wrote the paper. Dr. Ito designed and conducted experiments. Dr. Hirayama conducted experiments. Dr. Sahashi diagnosed a patient and conceived the study. Dr. Ohkawara designed experiments. Dr. Masuda designed experiments. Dr. Nishida diagnosed a patient and conceived the study. Dr. Mabuchi diagnosed a patient and conceived the study. Dr. Engel diagnosed a patient, conceived studies, designed experiments, and wrote the paper. Dr. Ohno conceived study, designed experiments, and wrote the paper.

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DISCLOSURE

Dr. Kawakami, Dr. Ito, Dr. Hirayama, Dr. Sahashi, Dr. Ohkawara, Dr. Masuda, Dr. Nishida, and Dr. Mabuchi report no disclosures. Dr. Engel serves as an Associate Editor of *Neurology*®; receives publishing royalties for *Myology 3rd ed.* (McGraw-Hill, 2004); and has received research support from the NIH and the Muscular Dystrophy Association. Dr. Ohno has received Grants-in-Aids from the Japan Society for the Promotion of Science, the Ministry of Health, Labour and Welfare, and the Japan Science and Technology Agency.

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MEDICAL GAS RESEARCH

COMMENTARY Open Access

The 2011 Medical Molecular Hydrogen Symposium: An inaugural symposium of the journal *Medical Gas Research*

Shigeo Ohta[1*], Atsunori Nakao[2] and Kinji Ohno[3]

Abstract

This report summarizes a brief description/history of the Hydrogen Research Meetings as well as key presentations/oral abstracts delivered in the most recent symposium. Additionally, we introduced 38 diseases and physiological states for which hydrogen exhibits beneficial effects.

Introduction

Novel medical gases are expected to provide us with more effective therapeutic interventions and preventive medicine. In the past decades, there has been extraordinary, rapid growth in our knowledge of gaseous molecules, including nitric oxide, carbon monoxide, and hydrogen sulfide, which have been known to play important roles in biological systems. Additionally, since Dr. Shigeo Ohta's group's pioneering paper was published in the June 2007 *Nature Medicine* showing the potency of hydrogen as a therapeutic gas for oxidative stress-mediated diseases including cerebral infarction [1], basic and clinical hydrogen research has resurfaced. In Japan, the birthplace of hydrogen gas research, Dr. Ohta (Nippon Medical School), who is currently serving as an Associate Editor of *Medical Gas Research*, organized annual "Medical Molecular Hydrogen Research Meetings" in 2009 and 2010 to provide investigators with focused opportunities to share their rapid scientific progress. Most recently, we organized the Medical Molecular Hydrogen Symposium on February 18-19, 2011 at the Nagoya University Hall (Figure 1). The latest meeting is a "kick-off" inaugural meeting for the newly launched journal *Medical Gas Research* (MGR), which aims to promote the exchange and dissemination of the latest scientific findings.

This report summarizes a brief description/history of the Hydrogen Research Meetings as well as key presentations/oral abstracts delivered in the most recent symposium.

First Medical Molecular Hydrogen Research Meeting, 2009

The first scientific meeting organized by Dr. Ohta was held on February 7, 2009 in Tokyo. 42 scientists and clinicians from 30 individual institutes were invited. The aim of the meeting was to unite innovative investigators to discuss and propagate medical hydrogen research. Dr. Ohta delivered the keynote presentation, in which he gave a brief history of hydrogen medicine and emphasized the huge impact of his report published in *Nature Medicine*. He pointed out the great interest in the field, expressed in more than 30 personal communications with investigators, and the resulting need to widen the scope of basic/clinical research to the whole world. He mentioned the successful application of hydrogen gas in a rat neonatal hypoxic brain injury model in collaboration with Dr. Xuejun Sun (Second Military Medical School, Shanghai, China) [2], who is currently serving as an Associate Editor. Dr. Atsunori Nakao (Department of Surgery, University of Pittsburgh), who is also an Associate Editor, presented the promising preliminary results of a collaborative study with Dr. Ohta's group in which hydrogen water was applied in a rat kidney transplant model. Dr. Nakao's report clearly showed survival benefits for transplant recipients. He received a research award at this meeting and his report was later published in *Kidney International* [3,4]. Dr. Takahisa Kawai (Forsythe Research Institute, Boston, MA, USA), who is an editorial

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Figure 1 A snap shot of the Medical Molecular Hydrogen Symposium in 2011.

board member, focused on hydrogen generated by intestinal bacteria. His initial studies elegantly demonstrated the critical physiological roles of gut microflora-derived hydrogen [5]. There was a general consensus that both clinicians and researchers in the field of molecular hydrogen research should gather and exchange accumulating knowledge in future annual meetings.

Second Medical Molecular Hydrogen Research Meeting, 2010

The second meeting was also organized by Dr. Ohta on February 3, 2010 in Tokyo. 47 basic scientists and clinical physicians, as well as 23 corporate participants were invited and shared the latest developments in medical issues related to hydrogen. This meeting hosted a keynote lecture, an invited lecture, two special lectures, and twelve platform presentations. After Dr. Ohta began his keynote lecture by remarking on the impressive progress over the last year, Dr. Sun gave an invited lecture and introduced the great effects of intraperitoneal administration of saline dissolved with H_2 in several model animals. Dr. Takashi Asada (Department of Psychiatry, Tsukuba University), an authority on Alzheimer disease, presented the results of clinical studies involving patients with mild cognitive impairment (MCI). He started clinical intervention studies on MCI patients by orally administering hydrogen water; the project is still in progress. Dr. Toru Yoshikawa (Kaohsiung Medical University, Taiwan) also gave a special lecture on the physical aspects of hydrogen effects. He presented the physical characteristics of molecular hydrogen's interaction with water in biological systems.

Third Medical Molecular Hydrogen Research Meeting, 2011

The third meeting was organized by Dr. Kenji Ohno (Nagoya University Graduate School of Medicine), an editorial board member, and held in Nagoya on Feb 18-19, 2011. This symposium mainly focused on molecular hydrogen and covered a wide-range of therapeutic gases, including hydrogen sulfide (H_2S), nitric oxide (NO), and carbon monoxide (CO). A total of 98 academic and 53 corporate registrants attended the meeting from Japan (144), USA (5), Korea (1), and Taiwan (1). The meeting's timetable, titles, and speakers are summarized in Table 1. We also introduced 38 diseases and physiological states for which hydrogen exhibits beneficial effects (Table 2).

Hydrogen

Dr. Ohta gave a keynote lecture and introduced a number of hydrogen's potent efficacies on a broad spectrum of diseases in animal and human models, as well as the emerging molecular bases of hydrogen's effects. He emphasized the following points: (i) In the three and a half years since the first hydrogen paper was published in *Nature Medicine*, more than 70 original papers have been published in leading biological/medical journals. Based on cumulative knowledge, beneficial biological effects of hydrogen have been established with no doubt. (ii) There are several ways to intake or consume hydrogen, including inhaling hydrogen gas, drinking water dissolved with hydrogen (hydrogen water), taking a hydrogen bath, injecting hydrogen saline, dropping hydrogen saline into the eye, and increasing production of intestinal hydrogen by bacteria. (iii) Hydrogen shows not only anti-oxidative stress effects, but also has various anti-inflammatory and anti-allergic effects. (iv) The primary molecular target of hydrogen remains unknown. In their first report published in 2007 [1], Dr. Ohta's group indicated that cells cultured in H_2-rich medium were protected against oxidative stress by the hydroxyl radical-scavenging activity of H_2; however, recent evidence clearly shows that the scavenging property is not the only explanation for the potent beneficial effects of hydrogen. For example, the amount of orally administered H_2 may not be enough to scavenge hydroxyl radicals. In addition, it is likely that the dwell time of H_2 in the body is too short to scavenge a large amount of hydroxyl radicals that are continuously generated. (v) Several reports demonstrate an effect on the regulation of gene expressions and protein-phosphorylations; however, the transcriptional factors and kinases involved in the functions afforded by H_2 have not been identified. It also remains unknown whether the regulations are directly performed by H_2. (vi) The amount of administered H_2 is independent of the extent of effects. Intestinal bacteria seem to produce more than 1 liter of H_2 gas per day, whereas the amount of H_2 originating from drinking hydrogen water is less than 50 ml. Nevertheless, additional H_2 in drinking hydrogen water is

Table 1 Scientific Program of the Medical Molecular Hydrogen Symposium in 2011

Feb. 18, 2011	Title	Speaker
13:00	**Opening Remarks**	Kinji Ohno, Nagoya Univ.
13:05	**Keynote Lecture** "Recent progress towards hydrogen medicine"	Shigeo Ohta, Nippon Medical Sch.
13:35	**Scientific Session I**	
	1. Effects of hydrogen-rich water on kidney functions in SHR.Cg-*Lepr*^{cp}/NDmcr rat - a metabolic syndrome model rat	Michio Hashimoto, Shimane Univ.
	2. Hydrogen-rich UW solution attenuates renal cold ischemia reperfusion injury	Toyofumi Abe, Osaka Univ.
	3. Therapeutic effect of maternal hydrogen water administration in a rat model of fetal brain damage	Yukio Mano, Nagoya Univ.
	4. Effects of hydrogen water on human skin and hair	Yoshiaki Kurita, Hosei Univ.
14:23	Coffee Break	
14:50	**Scientific Session II**	
	5. Consumption of hydrogen water prevents memory impairment accompanying neurodegeneration in transgenic mice	Ikuroh Ohsawa, Metropolitan Insttitue of Gerontology
	6. Appearance of hydrogen gas in the human skin after the ingestion of hydrogen-rich water and inhalation of hydrogen gas	Kazutoshi Nose, National Cerebral and Cardiovascular Center
	7. Effectiveness of long-term intake of hydrogen rich water in a patient with MELAS	Mikio Hirayama, Kasugai Municipal Hospital
	8. Effectiveness of hydrogen rich water on myopathy	Tohru Ibi, Aichi Medical Univ.
	9. Inhaled hydrogen gas therapy for prevention of lung transplant-induced ischemia/reperfusion injury in rats	Tomohiro Kawamura, Univ. of Pittsburgh
15:50	**Invited Lecture I** "Deadly gas can save a life! -Preclinical studies using carbon monoxide/hydrogen-"	Atsunori Nakao, Univ. of Pittsburgh
16:30	Break	
16:40	**Invited Lecture II** "Medical Gas Research"	John Zhang, Loma Linda Univ.
17:20	**Invited Lecture III** "A hypothesis on biological protection from space radiation through the use of therapeutic gases"	Michael P. Schoenfeld, NASA Marshall Space Flight Center
18:10	Banquet	
Feb. 19, 2011		
9:30	**Symposium I** "CO as a therapeutic modality"	Organizer: Atsunori Nakao, Univ. of Pittsburgh
	S1-1. Is exhaled carbon monoxide a marker of oxidative stress?	Tohru Takahashi, Okayama Prefecture Univ.
	S1-2. A tracer analysis study demonstrates redistribution of endogenous carbon monoxide from blood to tissue in human body	Makoto Sawano, Saitama Medical Univ.
	S1-3. Translational research of carbon monoxide therapy using miniature swine	Hisashi Sahara, Kagoshima Univ.
10:30	**Special Lecture** "Function of hydrogen sulfide and its therapeutic applications"	Hideo Kimura, National Center of Neurology and Psychiatry
11:10	Break	
11:20	**Scientific Session III**	
	10. Hydrogen from intestinal bacteria is protective for Con A-induced Hepatitis	Mikihito Kajiya, The Forsyth Institute
	11. Abnormal breath hydrogen production by ingestion of lactulose in Parkinson's disease	Masaaki Hirayama, Nagoya Univ.
	12. A new portable breath hydrogen analyzer and its clinical application	Akito Shimouchi, National Cerebral and Cardiovascular Center
	13. Application of hydrogen water in the dental field	Noriyuki Tanaka, Uchida Dental Clinic
12:08	Lunch	
12:45	**Symposium II** "Biological Effects of NO"	Hirosuke Kobayashi, Kitasato Univ.
	S2-1. NO-ROS cellular signaling mediated via nitrated cyclic nucleotide	Hideshi Ihara, Osaka Prefecture Univ.
	S2-2. Relationship between protein S-nitrosylation and neuronal death	Takashi Uehara, Wakayama Mediccal Univ.
	S2-3. Role of nitrosative stress in chronic obstructive pulmonary disease	Ryujiro Sugimoto, Iwakuni Clinical Center
	S2-4. Effects of simultaneous inhalation of nitric oxide and hydrogen on mouse myocardial ischemia-reperfusion injury	Hirosuke Kobayashi, Kitasato Univ.
14:15	**Scientific Session 4**	
	14. Effect of hydrogen rich water against a progression of disease and a formation of liver tumor in NASH model mouse	Daisuke Kawai, Okayama Univ.
	15. The dynamic movement of H2 in a liver and its effects	Naomi Kamimura, Nippon Medical Univ.
	16. Molecular hydrogen effectively protects cereulide-induced liver injury by suppressing apoptosis	Sayaka Sobue, Chubu Univ.
14:51	**Closing Remarks**	Kinji Ohno, Nagoya Univ.

Table 2 Thirty-eight diseases and physiological states for which hydrogen effects are reported

Disease/Physiology	Species	Source of H_2	Reference
Brain			
Cerebral infarction	rodent	gas	[1]
Superoxide in brain	rodent	water	[6]
Neonatal brain hypoxia	rodent	gas	[2,7]
	rodent	saline	[8]
	pig	gas	[9]
Restraint-induced dementia	rodent	water	[10]
Alzheimer's disease	rodent	saline	[11]
Senile dementia	rodent	water	[12]
Parkinson's disease	rodent	water	[13,14]
Hemorrhagic cerebral infarction	rodent	gas	[15]
Traumatic brain injury	rodent	gas	[16]
Spinal cord			
Spinal cord injury	rodent	saline	[17]
Eye			
Glaucoma	rodent	eye drop	[18]
Corneal alkali-burn	rodent	eye drop	[19]
Ear			
Hearing disturbance	rodent	medium	[20]
	rodent	gas	[21]
	rodent	water	[22]
Lung			
Lung cancer	Cells	medium	[23]
Oxygen-induced lung injury	rodent	saline	[24,25]
Lung transplantation	rodent	gas	[26]
Heart			
Myocardial infarction	rodent	gas	[27]
	rodent	saline	[28]
Heart transplantation	rodent	gas	[29]
Irradiation-induced heart injury	rodent	water	[30]
Liver			
Hepatic ischemia	rodent	gas	[31]
Hepatitis	rodent	bacteria	[5]
Obstructive jaundice	rodent	saline	[32]
Kidney			
Cisplatin nephropathy	rodent	gas, water	[33]
	rodent	water	[34]
Hemodialysis	human	dialysis	[35,36]
Kidney transplantation	rodent	water	[4]
Pancreas			
Acute pancreatitis	rodent	saline	[37]
Intestine			
Intestinal graft	rodent	gas	[3]
	rodent	saline	[38,39]
Ulcerative colitis	rodent	gas	[40]
Blood vessel			
Atherosclerosis	rodent	water	[41]
Metabolism			
Diabetes mellitus type 2	human	water	[42]
Metabolic syndrome	human	water	[43]
Obesity/Diabetes	rodent	water	[44]
Cancer			
Tongue carcinoma	cells	medium	[45]
Inflammation and allergy			
Allergy type I	rodent	water	[46]
Sepsis	rodent	gas	[47]
Zymosan-induced inflammation	rodent	gas	[47]
Others			
Multipotent stromal cells	cells	gas	[48]
Radiation injury	cells	medium	[49,50]

Although the observations are not directly relevant to diseases, Turmeric [51] and acarbose [52] increase hydrogen production by intestinal bacteria in humans.

unambiguously effective. Many mysteries of hydrogen therapy remain unsolved. He closed his talk by emphasizing that the molecular mechanisms underlying the amazing effects of a very small amount of H_2 remain elusive.

Sixteen platform speakers presented clinical and basic aspects of the medical application of molecular hydrogen. Among them were three treatments for patients with neurological and dental diseases. Dr. Mikio Hirayama (Department of Neurology, Kasugai City Hospital) and his colleagues presented their clinical report of treatment for a patient with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode (MELAS) syndrome. He reported that a 33-year-old female patient was successfully treated by drinking hydrogen-containing water for one and half years, which reduced the frequency of episodic cerebral ischemia. Dr. Tohru Ibi (Department of Neurology, Aichi Medical School) conducted an open label trial on mitochondrial disorders and inflammatory myopathies, and demonstrated a remarkable reduction of several serum markers specific for myelopathy. He also conducted a double-blind crossover trial; however, the trial showed no significant effects, which was likely due to a small amount of hydrogen water and to a short observation period. Both reports suggest that oral administration of hydrogen water is likely to be effective for mitochondrial diseases. Dr. Noriyuki Tanaka (Uchida Dental Clinic) reported that direct dental application of hydrogen water on injured regions reduced inflammation and promoted healing in dental operations, including tooth extraction.

Medical Gas Research's editor-in-chief Dr. John Zhang (Department of Neurosurgery, Loma Linda University, CA) gave a greeting talk, introducing the aims and scope of the journal. Dr. Zhang pointed out the importance of stimulating medical gas research and collaborating with a wide-range of people in various fields.

Dr. Michael P. Schoenfeld from NASA gave a special lecture on the potential application of H_2 to protect astronauts from radiation-mediated injury during long space travel. Cosmic radiation induces serious oxidative stress, which is one of the major issues to be resolved by hydrogen research.

Other Medical Gases (NO, CO, and H_2S)

Mammals produce NO, CO, and H_2S by their native enzymes; however, mammals lack an enzyme to produce H_2. All four gases modulate signaling pathways and have some therapeutic effects. Thus, we invited leading NO, CO, and H_2S researchers to the symposium.

Dr. Hideo Kimura (National Center of Neurology and Psychiatry), an editorial board member, gave a special lecture on hydrogen sulfide (H_2S). Dr. Kimura discovered biological roles for H_2S. He reviewed studies on H_2S from past to present and emphasized the potential for actual medical applications.

Dr. Nakao organized a mini-symposium on carbon monoxide (CO). In the mini-symposium, the discrepancy between the animal and human models was discussed. CO binds to human hemoglobin more strongly than to murine hemoglobin; this seems to be the reason why CO is more toxic in clinical studies.

Dr. Hirosuke Kobayashi (Department of Respiratory Medicine, Kitasato University), an editorial board member, organized a mini-symposium on nitric oxide (NO). Dr. Kobayashi explained the contradictory properties of NO; while it is medically beneficial, it is also toxic and enhances oxidative stress. NO has been approved as a therapeutic agent in clinical practice. He presented the amazing effects of inhaling an NO and H_2 mixture. NO enhances oxidative stress and induces production of peroxynitrite, and H_2 reduces peroxynitrite derived from NO. Indeed, he presented that the mixture of NO and H_2 improves the number of surviving myocytes in a rodent model of myocardial infarction. The mixture of NO and H_2 may be a promising modality for clinical applications.

In addition to NO, CO, and H_2S, Dr. Zhang addressed potential effects of inhaled xeon and helium in animal models in his invited lecture.

Commercial products

Six companies financially supported the meeting by demonstrating their new commercial products. Melodian Co Ltd. presented a sports drink containing H_2, and Miz Co Ltd. introduced an interesting device to prepare a clinically applicable H_2-rich infusion solution by simply soaking an infusion bag in hydrogen water. With this method, no other treatment is necessary to prepare hydrogen infusion solution without opening the infusion bag. Taiyo Co Ltd. presented reasonably priced devices to measure H_2 gas in breath. Takaoka Co Ltd. demonstrated a tiny home apparatus to produce hydrogen water. Doctor's Choice Co Ltd. demonstrated powders to prepare a hydrogen bath at home using a new material that releases H_2 gas. These new products will help expand our knowledge and applicability of hydrogen research.

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Misfolded SOD1 forms high-density molecular complexes with synaptic molecules in mutant SOD1-linked familial amyotrophic lateral sclerosis cases

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ABSTRACT

Mutations in the *superoxide dismutase 1* (*sod1*) gene cause familial amyotrophic lateral sclerosis (FALS), likely due to the toxic properties of misfolded mutant SOD1 protein. Here we report identification of various synaptic molecules forming molecular complexes with misfolded SOD1 in mutant SOD1-associated FALS patient tissues as well as in cellular FALS models. In the FALS cellular model system, we found that membrane depolarization that mimics synaptic hyperactivation/excitotoxicity could cause misfolding of mutant SOD, as well as acceleration of misfolded SOD1–synaptic protein complex formation. These results suggest that inhibition of synaptic release mechanism by association of misfolded SOD1 with synaptic molecules plays a role in the dysfunction of FALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal, progressive neurodegenerative disease characterized by motor neuron cell death in the brain and spinal cord, accompanied by rapid loss of muscle control and eventually complete paralysis [1,2]. Most cases of ALS are of unknown etiology, while 5–10% are familial (FALS). Although the cause of sporadic ALS remains unclear, 15%–20% of FALS patients have point mutations in cytosolic Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1) [3]. SOD1 is an antioxidant enzyme ubiquitously expressed in the cytosol, which converts the superoxide anion radical to hydrogen peroxide. More than 115 disease-causing mutations, affecting all regions of the SOD1 gene product, have been identified.

Previous studies using transgenic animal models expressing mutated human SOD1 have shown that the disease is not caused by a loss of its dismutase activity, but by the gain of toxic properties [1]. Many mutant SOD1 proteins tend to become easily misfolded and form aggregates especially under oxidative stress [4]. Intracellular aggregates containing SOD1 were specifically detected in affected regions of both patients and animal models possessing *sod1* mutations. In spinal cords of mutant SOD1-Tg mice, misfolded intermediates of mutant SOD1 proteins, which showed decrease in solubility and increase in size, were found prior to disease onset. These results suggest that misfolded mutant SOD1 may contribute to motor neuron-specific damage in ALS.

We have previously reported that misfolded SOD1 specifically associates with KAP3, a component of the kinesin-2 motor complex [5]. Kinesin-2 constitutes a part of kinesin superfamily of molecules that mediate anterograde axonal transport [6]. We showed that misfolded SOD1 inhibits transport of choline acetyl-transferase (ChAT), a kinesin-2 cargo, and that resultant decrease of Ach release at presynaptic terminals may play a role in motor dysfunction observed in an early stage of FALS [5]. In an effort to show association of misfolded SOD1 species with KAP3 in FALS patient tissue homogenates, we examined migration profiles of SOD1- and KAP3-immunoreactivity in density-gradient centrifugation. In addition to identify that KAP3 co-migrates with misfolded SOD1 in this experiment, we realized that this method would be suitable in screening molecules that associate with misfolded SOD1 by using FALS patient tissues.

Here we report identification of various synaptic molecules that co-migrate with misfolded SOD1 in SOD1-associated FALS patient tissues as well as in cellular FALS models. In the FALS cellular model system, we found that membrane depolarization that mimics synaptic hyperactivation/excitotoxicity could cause misfolding of mutant SOD1 molecules. These results suggest that inhibition of synaptic release mechanism by association of misfolded SOD1 with synaptic molecules plays a role in the dysfunction of FALS.

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2. Materials and methods

2.1. Reagents

FLAG-tagged human SOD1 cDNA (wild-type and a mutant bearing L126S mutation) at the carboxyl terminus were cloned into pcDNA3 and pTRE2hyg (Clontech) as previously described [5]. The integrity of each clone was verified by nucleotide-sequence analysis. The antibodies used in this study were obtained and used as previously described [5,7].

2.2. Autopsy specimens

The studies were performed on frozen tissues (indicated regions of the brain, spinal cord, femoral nerve, dorsal root ganglia) obtained at autopsy from 2 FALS patients (A: 71 years old, female; B: 46 years old male) who were members of a family, 2 sporadic ALS patients (61 years old, female and 67 years old, male) , and 1 control patient (73 years old, male) suffered from schizophrenia. Leucine to serine substitution at codon 126 in the SOD1 gene in the two FALS cases was identified by sequence analysis. Consent for autopsy was obtained from legal representatives in accordance with the requirements of local institutional review boards.

2.3. Isolation of misfolded SOD1-containing protein complexes by density gradient centrifugation

The autopsy tissue samples were homogenized and the lysates were separated by Nycodenz linear density gradient centrifugation as previously described [5]. Briefly, the tissue samples were homogenized in a buffer containing 20 mM HEPES, pH 7.4, 120 mM NaCl, 2 mM EDTA, pH 8.0, 1%Triton X-100, and complete protease inhibitor cocktail (Roche)). The lysates were added to 3.2 ml of Nycodenz linear density gradient in 10 mM HEPES, pH 7.4 and 1 mM EDTA, pH 8.0 (initial concentration of Nycodenz was 30%). After a centrifugation at 87,480×g for 2 hr at 4 °C, in Optima MAX-E using MLS 50 rotor (Beckman Coulter), a 160 μl of aliquot was collected from the top to the bottom of the tube, totaling 22 fractions. Twenty microliters of each fraction was collected and analyzed by immunoblot.

2.4. Cell culture

NG108-15 cells (a kind gift from Dr. Akazawa in Tokyo Medical and Dental University) were stably transfected with pTRE2hyg-WT SOD1-Flag or pTRE2hyg-L126S SOD1-Flag as previously described [5]. Transgene expression in the transfected NG108-15 cells was induced by treating the cells with doxycycline per manufacturer's protocol. Differentiation of the transfected NG108-15 cells was performed as previously described for 7 days. Hyperstimulation model was generated by treating the differentiated NG108-15 cells stably expressing WT or mutant SOD1 with 100 μM glutamate as previously described [8].

3. Results

Misfolded SOD1 species observed in SOD1-associated FALS patients likely show toxic properties in cells by associating with other molecules and thereby inhibit their function [1,2]. Therefore, to gain mechanistic insights on misfolded SOD1 toxicity, it is important to identify molecules that specifically bind to misfolded SOD1. In our previous report, we found by analyzing tissue lysate from *SOD1*^{G93A}-Tg mice in linear density gradient centrifugation that some of the molecular motor components, such as KAP3, co-migrate with misfolded SOD1, which lead us to identify direct association of misfolded SOD1 with KAP3 [5]. To extend this observation in mutant SOD1-linked human FALS cases and identify molecules that bind to misfolded SOD1 in human FALS cases directly, we compared sedimentation of misfolded SOD1 in a linear density gradient centrifugation with that of various molecules in lysates of motor areas of FALS postmortem brain. We first examined representative molecules that compose kinesin motor complex to confirm the findings that we reported previously. Lysates of postmortem brain tissues from FALS cases bearing L129S mutation in SOD1 were subject to linear Nycodenz density gradient centrifugation analysis. Similar to what we observed in the rodent FALS model, we found that misfolded SOD1 species migrate to a heavy fraction (Fig. 1A). Among kinesin motor complex components, a fraction of KAP3 co-migrate with misfolded SOD1, which confirmed our previous observations, while immunoreactivity for p62, a nuclear protein, was not observed in the fraction in which misfolded SOD1 is observed. These results confirmed specificity of this assay and assured our previous observation that KAP3 specifically associates with misfolded SOD1 in disease affected neuronal tissues in FALS.

Next, to identify other candidate molecules that associate with misfolded SOD1, we examined migration profiles of the candidates in this analysis. We chose to examine synaptic molecules as important candidates, since inhibition of synaptic vesicle recycling has been noted in other neurodegenerative disorders such as Parkinson's disease. By applying the tissue processing and density gradient analysis strategy that we used above, we analyzed brain tissue lysate from control patients and found that presynaptic molecules including SNAP-25 (a SNARE complex component), and AMPA-type glutamate receptor GluR2/3, as well as a post-synaptic molecule, PSD95 mainly migrate to low density fractions (No1–8) as well as high density fractions (No. 16–19), while synaptophysin (a synaptic vesicle protein) migrates only to the low density fractions (Fig. 1A). We presumed that the molecules that migrate to lower and higher density fractions represent "free" molecules and molecules forming structural complexes, respectively. These results suggest that the current experimental condition allows most of the synaptic molecular structures containing pre- and postsynaptic structural and signaling molecule complexes to retain their association in the lysate. Analysis of the FALS patient brain tissue lysates revealed that the pre- and postsynaptic proteins show different distribution profile in the density gradient. We found that major amount of synaptic molecules, GluR2/3, SNAP-25, and PSD-95, as well as a synaptic vesicle protein, synaptophysin, migrate to a very high density fraction (No. 19), in which misfolded SOD1 species are observed (Fig. 1A). Decreased amount of synaptic molecules located in the lower density fractions relative to the amount in No. 19 was also noted. These results suggest that, in mutated SOD1-linked FALS patients, misfolded SOD1 promotes formation of very high density molecular complex in the synaptic terminal region involving a large variety of synaptic molecules.

To examine how specific this observation is to motor regions of mutated SOD1-linked FALS cases, we performed the same analysis using tissue lysates from non-motor neuronal tissues of FALS cases and precentral gyrus of sporadic ALS (SALS) cases. We found that the distribution profiles of the pre- and post-synaptic proteins in these tissues show patterns very similar to those in control tissues (Fig. 1B, C). These results suggest that the shifted migration profile of synaptic proteins in the density gradient analysis is observed specific to FALS motor brain regions. Taken together, our data suggest that formation of high-density molecular complex containing synaptic molecules may contribute specifically to mutated SOD1-linked FALS pathogenesis.

A number of studies have supported the importance of excitotoxic mechanisms in ALS pathogenesis [9–12]. We have previously shown that decreased Ca^{2+} permeability of AMPA-type glutamate receptors expressed in motor neurons resulted in delayed onset and extended lifespan in a mouse model of mutated SOD1-linked FALS, which suggested that glutamate excitotoxicity promotes misfolding of mutated SOD1 and motor neuron death [7]. To correlate the formation of high-density molecular complex involving synaptic molecules in mutated

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