

Improved methodology to obtain large quantities of correctly folded recombinant N-terminal extracellular domain of the human muscle acetylcholine receptor for inducing experimental autoimmune myasthenia gravis in rats

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Abstract

Introduction: Human myasthenia gravis (MG) is an autoimmune disorder of the neuromuscular system. Experimental autoimmune myasthenia gravis (EAMG) is a well-established animal model for MG that can be induced by active immunization with the *Torpedo californica*-derived acetylcholine receptor (AChR). Due to the expensive cost of purifying AChR from *Torpedo californica*, the development of an easier and more economical way of inducing EAMG remains critically needed.

Material and methods: Full-length cDNA of the human skeletal muscle AChR $\alpha 1$ subunit was obtained from TE671 cells. The DNA fragment encoding the extracellular domain (ECD) was then amplified by polymerase chain reaction (PCR) and inserted into pET-16b. The reconstructed plasmid was transformed into the host strain BL21(DE3)pLysS, which was derived from *Escherichia coli*. Isopropyl- β -D-thiogalactopyranoside (IPTG) was used to induce the expression of the N-terminal ECD. The produced protein was purified with immobilized Ni²⁺ affinity chromatography and refolded by dialysis.

Results: The recombinant protein was efficiently refolded to soluble active protein, which was verified by ELISA. After immunization with the recombinant ECD, all rats acquired clinical signs of EAMG. The titer of AChR antibodies in the serum was significantly higher in the EAMG group than in the control group, indicating successful induction of EAMG.

Conclusions: We describe an improved procedure for refolding recombinant ECD of human muscle AChR. This improvement allows for the generation of large quantities of correctly folded recombinant ECD of human muscle AChR, which provides for an easier and more economical way of inducing the animal model of MG.

Key words: experimental autoimmune myasthenia gravis, extracellular domain, acetylcholine receptor, prokaryotic expression, refolding.

Introduction

Myasthenia gravis (MG) is an antibody-mediated autoimmune disorder that is caused by autoantibodies directed against neuromuscular junction proteins [1–4]. Experimental autoimmune myasthenia gravis (EAMG) is

a well-established animal model for exploring the mechanism underlying the pathophysiology of MG and developing novel therapeutic strategies [5]. Experimental autoimmune myasthenia gravis can be induced by the passive transfer of serum from patients with MG to mice or by active immunization against the *Torpedo californica*-derived acetylcholine receptor (AChR) [6]. Due to the limited yield of serum from patients with MG, and the high cost of AChR purification from *Torpedo californica*, development of an easier and more economical way to induce EAMG remains an unmet need in this field.

The nicotinic AChR (nAChR) molecule is an oligomeric transmembrane glycoprotein that consists of five homologous subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ (embryonic) or $\alpha_2\beta\epsilon\delta$ (adult), which form a cation channel [7]. The N-terminal extracellular domain (ECD) of the α chain of the human muscle AChR contains both the binding sites for cholinergic ligands and the main immunogenic region (MIR). It has been shown that anti-nAChR antibodies produced in patients with MG are high-affinity immunoglobulin G (IgG) antibodies that predominantly bind to the MIR [4].

Previous studies have demonstrated that both B and CD4+ T cells are involved in the pathogenesis of MG [8–11]. Since the ECD of AChR includes both the binding sites for MIR and the T and B cell epitopes, we postulated that the ECD can be used as an immunogen to stimulate the growth of antigen-specific T and B cells, which may thereby induce EAMG. In this context, we designed a modified protocol based on a previous method for obtaining large quantities of correctly folded recombinant human muscle N-terminal ECD AChR protein. The immunogenicity of the purified protein was then verified by inducing EAMG in an animal model using the protein.

We aimed to establish a repeatable, reliable, and economical methodology for inducing an animal model of MG.

Material and methods

Cloning and expression of human ECD AChR

The DNA fragment encoding the ECD (amino acids 1–207) of the human muscle AChR was amplified by PCR (MJ Research company, US) using cDNA from the human neuromedulloblastoma TE671 cell line as the template [12], and the primers 5'-GCG CAT ATG TCC GAA CAT GAG ACC CGT CT-3' and 5'-AT CGG ATC CTC CAG GCG CTA CAT GAC GAA GT-3', which contained a G→A substitution (shown in bold) in order to create a TAG stop codon as well as NdeI and BamHI endonuclease sequences (underlined, respectively). The amplified fragment and prokaryotic expression vector pET16b were digested with the endonucleases and ligated together using a T4 DNA

ligase reaction (TaKaRa Biotechnology, China). The reconstructed plasmid was sequenced to confirm the correct insert and then transformed into the expression host *Escherichia coli* BL21(DE3)pLysS (Invitrogen, US). Induction of protein expression was carried out according to the pET system manual. Briefly, the *E. coli* BL21(DE3)pLysS were grown at 37°C until reaching an optical density (OD) of 0.6 and then induced with 1 mM isopropyl- β -D thiogalactopyranoside (IPTG) for 4 h at 37°C.

Purification of inclusion bodies

Frozen *E. coli* cell pellets were thawed in phosphate-buffered saline (PBS, pH 8.0). The released *E. coli* genomic DNA was sheared in an ice bath using an ultrasonic disruption apparatus (Ningbo Xin-zhi Biotechnology Co. Ltd., China). The lysed suspension was then centrifuged at 13,000 rpm with a Beckman JA-14 rotor (Beckman, USA). The sediments containing the inclusion bodies were washed twice with distilled water. The inclusion bodies were then resolved in 100 ml of denaturing buffer containing 5 mM imidazole (Sigma, USA), 6 M guanidine-HCl (Beijing Dingguo Biological Technology, China), and 50 mM phosphate-buffered saline (PBS) (pH 7.5). The solubilized inclusion bodies were purified using immobilized Ni²⁺ affinity fast protein liquid chromatography (FPLC) columns (GE Healthcare company, Germany). The target protein was eluted in a buffer containing 0.8 M imidazole, 20 mM 2- β -mercaptoethanol, 8 M urea, 0.5 M NaCl, and 20 mM PBS (pH 7.5). The protein concentration was determined using the Bradford method [13].

Refolding of the inclusion bodies using various methods

For the first method of refolding the inclusion bodies, a refolding buffer consisting of 0.02% Triton X-100, 60 mM Tris, 500 mg/l cysteine, and various concentrations of L-arginine-HCl was used in a rapid dilution experiment [14, 15]. For the second method, the fractions were resolved in denaturing buffer containing 8 M urea and 20 mM Tris (pH > 10) to a terminal concentration of 500 μ g/ml. The fractions were then buffer exchanged using dialysis against 4 M urea and 20 mM Tris, followed by 2 M urea and 20 mM Tris, and finally 20 mM Tris with an over-saturated concentration of cysteine.

Determination of the biological activity of the refolded ECD protein

The activity of the refolded AChR ECD protein was determined by measuring the absorbance value of the supernatant at a wavelength of 495 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan, Switzerland) [16]. The

anti-AChR (mAb35) and horseradish peroxidase (HRP)-labeled rabbit anti-rat IgG (Cell Signaling, US) antibodies used in these experiments were prepared as previously described [17]. The refolded proteins were then concentrated by dialyzing the samples against glycerin in dialysis bags. The protein concentration of the final protein yield was again determined using the Bradford method.

Induction of the EAMG model

Eight-week-old female Lewis rats weighing 140–160 g were purchased from Beijing Hufukang Biotech AG. The study was approved by our Institutional Animal Care and Use Committee (IACUC). All rats were microorganism-free animals. Three experimental groups of rats were subcutaneously (s.c.) injected with 50, 200, or 400 µg of ECD protein ($n = 3$ in each group) every seven days over a total of 21 days. Using a blinded protocol, two different examiners assessed clinical signs and body weight of the rats twice a week. The clinical severity of EAMG was graded using criteria previously described by Wu *et al.* [6]. Briefly, the grading criteria were as follows: no definite weakness – 0; weak grip or cry with fatigability – 1; hunched posture at rest, head down, forelimb digits flexed, or tremulous ambulation – 2; and severe generalized weakness, no cry or grip, or moribund – 3. Rats with intermediate signs were assigned grades of 1.5 or 2.5, as appropriate.

Determination of AChR IgG antibody titers in serum

On the 30th day after immunization, 1 ml of serum was isolated from rats treated with ECD AChR protein and control rats, and antibody titers were determined using an ELISA assay [16].

Statistical analysis

Data were presented as mean values \pm standard deviations (SD). The Statistical Program for Social Sciences 14.0 (SPSS, IBM, US) was used to analyze all data. One-way analysis of variance (ANOVA) and Kruskal-Wallis tests were used to compare values among groups followed by Student's *t*-test or Mann-Whitney *U*-test to compare values between groups. All tests were two-tailed and the level of significance was set to $p < 0.05$.

Results

Cloning and expression of human ECD

Sequencing results showed that all of the reconstructed plasmids contained wild-type sequences as expected without any mutations, including the codon-optimized sequences. After screening several clones, a restriction digest and subsequent gel

electrophoresis analysis revealed a 650 bp fragment, indicating proper insertion of the ECD fragment in the vector (Figure 1). Human ECD was efficiently overexpressed as a fusion protein with an amino-terminal histidine tag containing 10 residues in the pET16b vector.

Purification of inclusion bodies

The results of SDS-PAGE analysis before and after IPTG induction and inclusion body purification are summarized in Figure 2. The *E. coli* exhibited no leaky protein expression in the absence of IPTG, but efficiency produced a protein of approximately 26 kDa after the addition of IPTG (Figure 2, lane 1 vs. 2). According to the Bioedit software, the predicted molecular weight (MW) of the ECD protein is 26.65 kDa, and therefore we presumed that the protein obtained from the induced *E. coli* corresponded to recombinant ECD. In addition, the purified inclusion body preparation demonstrated a protein band of similar size (Figure 2, lane 7).

Determination of the biological activity of refolded human ECD

In the rapid dilution experiment, none of the L-arginine concentrations tested could prevent the formation of an insoluble dimer and multimer. As

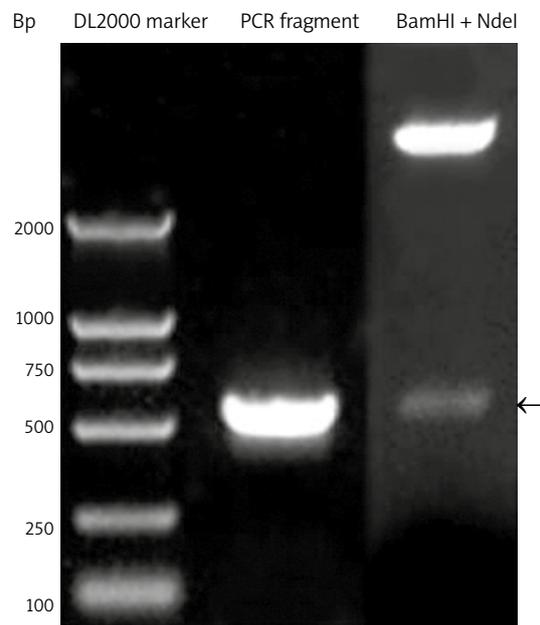


Figure 1 Gel electrophoresis showing the AChR ECD fragment generated by PCR from AChR $\alpha 1$ subunit cDNA of TE671 cells and the recombinant pET 16b-ECD plasmid after ligation and restriction endonuclease digest. 1: DL 2000 marker; 2: AChR ECD PCR fragment; 3: The fragment after insertion into pET16b and subsequent restriction endonuclease digestion with BamHI and NdeI. The arrow indicates the AChR ECD fragment, which was the smaller band of approximately 650 bp

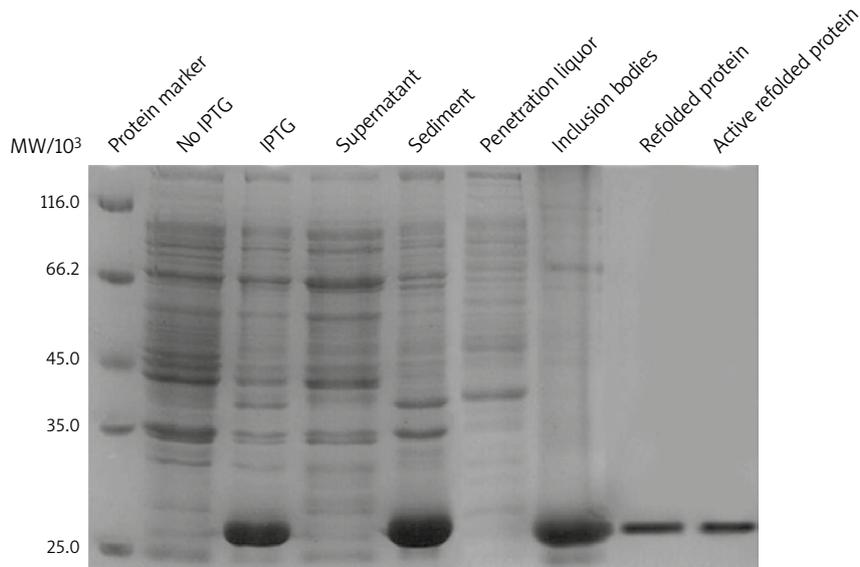


Figure 2. The expression of AChR α 1 subunit ECD in *E. coli*. The samples were resolved by SDS-PAGE and visualized by Coomassie blue stain. Lane 1: Protein marker. Lane 2: BL21(DE3) pLysS bacteria not induced by IPTG. Lane 3: BL21(DE3) pLysS bacteria induced by 1 mM IPTG for 4 h. Lane 4: Supernatant of induced culture after centrifugation. Lane 5: Sediment of the induced culture after centrifugation. Lane 6: Supernatant from washed inclusion bodies after purification on an Ni^{2+} column. Lane 7: Purified inclusion bodies. Lane 8: Non-hydrogenated re-folded AChR ECD protein. Lane 9: Hydrogenated re-folded AChR ECD protein

shown in Figure 3, we determined that the optimal method for the re-folding of recombinant ECD was the dialysis method. In addition, we found that a concentration of 5 $\mu\text{g}/\text{ml}$ recombinant protein was optimal for dialysis. Importantly, prior to concentrating the recombinant protein, we found that the protein was prone to precipitation after

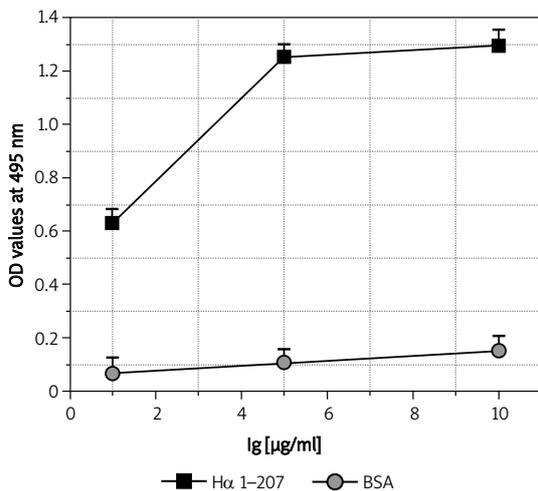


Figure 3. The anti-AChR mAb35 antibody binds to re-folded recombinant human AChR ECD protein re-folded using the dialysis method. The absorbance values as shown by ELISA were significantly increased by more than 10-fold in the experimental group compared to the control group ($p < 0.05$), indicating that the recombinant protein had biological activity after dialysis refolding. Representative data from one out of three independent experiments are presented as mean value \pm standard deviation (SD)

a freeze/thaw cycle, suggesting that the protein was inherently unstable. However, after determination of protein concentration, the protein did not exhibit sedimentation and was able to bind the anti-AChR antibody (mAb35) in an ELISA after repeated freeze/thaw cycles, indicating that the protein was stable and maintained the native conformation (Figure 4). Previous studies failed to indicate how protein stability was maintained, and therefore our optimized procedure provides a sufficient method for maintaining long-term stability of the recombinant protein. The refolding of pro-

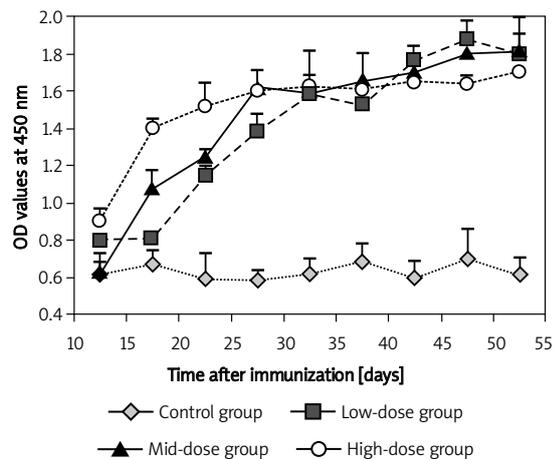


Figure 4. The AChR antibody titers in serum from each experimental group were significantly higher than the control group ($p < 0.05$). Representative data from one out of three independent experiments are presented as mean value \pm standard deviation (SD); $n = 3$ in each group

teins isolated from inclusion bodies has been shown to generally have a low efficiency rate of less than 20% [18]. However, in our study, we obtained 4.55 g of refolded recombinant protein from 14 g of inclusion bodies generated from 1000 g of *E. coli*. Based on these amounts, the refolding efficiency of recombinant human ECD using the urea dialysis method was determined to be 32.5%.

Induction of EAMG

All rats immunized with recombinant ECD acquired EAMG. For the evaluation of the EAMG animal model, we referred to the diagnostic criteria for human MG as previously described [6]. Acetylcholine receptor antibody testing is an important basis for the diagnosis of MG. Acetylcholine receptor antibody titers in each of the experimental groups were significantly higher than those in the control group ($p < 0.05$, Figure 4). Approximately 28 days (d) post-injection (p.i.), the EAMG reached the nadir in all of the groups. The rats gradually recovered from EAMG after approximately 35 d p.i., and by 49 d p.i., almost all of the rats had recovered from the disease. The rats in the 400 µg group showed the most severe clinical signs and at 14 d p.i., one rat in the 400 µg group died, followed by those immunized with 200 µg ECD protein. Rats in the 50 µg group and the control group did not exhibit any clinical signs ($p < 0.05$; Table I). No complication was observed in any of the groups.

Discussion

Experimental autoimmune myasthenia gravis can be induced by the passive transfer of serum from patients with MG to mice and rats or by active immunization against the *Torpedo californica*-derived AChR [6]. Previous studies exploring EAMG have used AChR extracted from *Torpedo californica*; however, the cost to purchase or capture this species is substantially more expensive than TE671 cell cultures. TE671 cells express functional AChRs that have marked resemblance to muscle AChRs [19]. Since the α subunit of AChRs purified from TE671 cells is very similar to that of muscle AChR, we hypothesized that the ECD protein generated from the TE671 AChR α subunit cDNA sequence could be

used to induce EAMG in rats efficiently. Moreover, it is challenging to humanely remove *Torpedo* electric organs. It has been shown that previous purification methods of recombinant AChR yield proteins in inclusion bodies that can bind to α-¹²⁵I-bungarotoxin (α-¹²⁵I-BuTx) prior to re-folding, but cannot bind to an anti-AChR antibody (mAb35); however, refolded protein can bind both the toxin and antibody, indicative of full bioactivity [17].

Moreover, the cost of reagents using the α-¹²⁵I-BuTx method is markedly higher than the antibody approach used in our study. Therefore, our modified methodology costs much less than the previous methods for inducing the EAMG model.

Based on the study by Talib *et al.*, we used molecular cloning and recombinant protein expression methods to obtain large quantities of properly folded human muscle recombinant AChR ECD protein [20]. Our revised methodology exhibited improvements in terms of protein expression, refolding, and yield of ECD protein compared to the previous method, all of which contribute to a much simpler, faster, and cheaper production of protein for use in the EAMG model. Importantly, these findings will greatly facilitate future studies of MG. The coding sequence of the ECD from the AChR α subunit (a.a. 1–210) was originally identified in the early 1990s and successfully expressed in *E. coli* [20]. Using the expressed product, Lennon *et al.* successfully induced EAMG in Lewis rats [21]; however, the RNA sequence determined in that study was obtained from human muscle by RT-PCR, and isolated human muscle AChR has a short half-time and degrades quickly *in vitro*. Because of these characteristics, the product is easily contaminated and the yield can be limited. Therefore, in this study we cloned the ECD fragment by directly purifying the cDNA sequence of the AChR α subunit from TE671 cells. TE671 cells express functional AChRs that have marked resemblance to muscle AChRs [22]. Since the α subunit of AChRs purified from TE671 cells is very similar to that of muscle AChR, we hypothesized that the ECD protein generated from the TE671 AChR α subunit cDNA sequence could be used to induce EAMG in rats efficiently.

One technical difficulty in obtaining a bioactive AChR ECD protein is how to ensure proper folding

Table I. Clinical signs from each experimental group

Dose [µg]	No. positive (fatalities)	Clinical features		
		Day of onset*	Maximum grade of severity*	Duration [days]*
0	0	–	0	–
50	1 (0)	30	1.0	29
200	2 (0)	28; 35	1.5; 1.5	37; 39
400	3 (1)	28.0 ± 2.4	2.0 ± 0.2	50.0 ± 10.3

*Mean ± standard error of the mean; n = 3 in each group

of the protein. In this study, we explored and determined the optimal conditions for protein refolding. Initially, we assessed the rapid dilution method as described by White *et al.* to refold recombinant human ECD [15]. However, precipitate gradually developed when we were using this method, which may have resulted from an insufficient dilution ratio. Therefore, we next assessed various dilution ratios up to 1 : 200, and the protein concentration was below 20 µg/ml. However, even under these conditions, precipitation was still unavoidable and the refolding efficiency was only about 1%. Another possibility was that the N-terminal 10-histidine tag, which was fused to the recombinant protein, may have a negative effect on protein refolding. In the rapid dilution experiment, none of the L-arginine concentrations tested could prevent the formation of soluble dimers and multimers. Based on these findings, we hypothesized that the rapid formation of aggregates might be due to drastic and sudden changes in the concentrations of the denaturant during the dilution procedure. Therefore, we employed a dialysis strategy in order to gradually decrease the concentration of the denaturant for proper re-folding of the recombinant human ECD. In the refolding process during dialysis, the protein underwent a structural transition and began to fold when a critical concentration of urea was reached. If the concentration of the reductant decreased fast enough to enable the formation of disulfide bonds before the urea concentration reached the critical point, then we postulated that the protein may predominantly form inter-chain disulfide bonds. To explore these hypotheses, we tested different refolding solutions with or without cysteine, or with cysteine at different stages of the dialysis experiment. Since the exact transition point of this protein in urea was unknown, the formation of disulfide bonds had to be delayed in order to avoid the formation of inter-chain disulfide bonds as the concentration of urea started to decrease. In this context, we devised a simpler protocol that would simulate this process. We estimated that after the dialysis against 4 M urea (1 : 50), the concentration of 2-ME would drop from 20 mM to approximately 0.4 mM. When an equal volume of the buffer was decanted and an equal volume of 20 mM Tris was added, the concentration of urea and 2-ME would further drop to 2 M and 0.2 mM, respectively. The oversaturated concentration of cysteine that was added was sufficient for reaction with the 2-ME in the buffer. As a result, the addition of cysteine in the diluted dialysis buffer would transform the strong reductive environment to a relatively mild reductive environment, which enables the formation of disulphide bonds in the hydrophobic core of the protein [19]. The solubility of cysteine in water is approximately 0.45 mM. As a consequence, the

cysteine (500 mg/l) added to the buffer was not readily resolved and decreased the rate of reaction with 2-ME. Therefore, a weak reductive environment was also formed in a relatively slow manner. This postulated dynamic change from a reductive environment to an oxidative environment in the dialysis buffer is also in agreement with the model reported by Lu *et al.* [19]. This concentration method using glycerin prevented the formation of insoluble multimers, and specifically the refolded recombinant protein was more stable. Therefore, the two key steps determined in our optimized dialysis protocol were: a) a shallow urea gradient that materializes from a stepwise dialysis; and b) the optimal time for adding a suitable concentration of cysteine.

The objective of our study is to explore a new therapy for MG. Autoantibody-induced complement activation, which causes disruption of the postsynaptic membrane, is recognized as a key pathogenic factor in MG. Therefore, the specific targeting of complement inhibitors to the site of complement activation is a potential therapy for MG. We had previously expressed single-chain antibody fragment-decay accelerating factor (scFv-DAF) [23], which was composed of a single-chain fragment scFv1956 based on the rat complement inhibitor DAF in prokaryotic systems, and studied its inhibitory effect on complement deposition *in vitro*. We will further test whether scFv-DAF may serve as a candidate for *in vivo* protection of MG.

In conclusion, we have described an improved procedure of refolding recombinant AChR ECD from human muscle. Our new method excludes the use of L-arginine, which is an expensive material, in the refolding solution, and significantly increases the refolding efficiency. We also found that glycerin dialysis enhances the stability of the recombinant human ECD. These improvements in the purification method allow for the production of large quantities of correctly folded recombinant AChR ECD from human muscle, which provides material for an easier and more economical way of inducing the EAMG animal model. Wu *et al.* [24] and Yang *et al.* [25], respectively, used the AChR γ subunit and the recombinant human AChR α subunit to immunize HLA-DQ8 transgenic mice to develop ocular myasthenia gravis (oMG). The oMG model is a tool for studying the mechanism of oMG and generalized myasthenia gravis (gMG) pathogenesis in humans as well as for preclinical therapeutic analysis. Therefore, in future studies, after we clarify that scFv-DAF is a candidate for *in vivo* protection of AChR in the rat MG model, we will attempt to study the effect of DAF in the oMG model.

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