Prevention of passively transferred experimental autoimmune myasthenia gravis by an in vitro selected RNA aptamer

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Abstract Myasthenia gravis (MG) and its animal model, experimental autoimmune MG (EAMG), are mainly caused by autoantibodies directed against acetylcholine receptors (AChR) located in the postsynaptic muscle membrane. Previously, we isolated an RNA aptamer with 2'-fluoropyrimidines using in vitro selection techniques that acted as an effective decoy against both a rat monoclonal antibody called mAb198, which recognizes the main immunogenic region on the AChR, and a significant fraction of patient autoantibodies with MG. To investigate the therapeutic potential of the RNA, we tested the ability of the RNA aptamer to protect the receptors in vivo from mAb198. Clinical symptoms of EAMG in rats engendered by passive transfer of mAb198 were efficiently inhibited by a truncated RNA aptamer that was modified with polyethylene glycol, but not by control scrambled RNA. Moreover, the loss of AChR in the animals induced by the antibody was also significantly blocked with the modified RNA aptamer. These results suggested that RNA aptamers could be applied for antigen-specific treatment for autoimmune diseases including MG.

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Key words: Experimental autoimmune myasthenia gravis; In vitro selection; RNA aptamer; Acetylcholine receptor; Autoantibody

1. Introduction

Myasthenia gravis (MG) is a neuromuscular disorder associated with muscular weakness and fatigability. The pathogenesis of MG mainly results from antibody-mediated autoimmune response to nicotinic acetylcholine receptors (AChR) located in the postsynaptic muscle cell membrane [1]. Experimental autoimmune myasthenia gravis (EAMG) can be generated in animals by active immunization with purified AChR or passive transfer of anti-AChR antibodies [2]. The majority of anti-AChR antibodies found in both human patients with MG and rats with EAMG bind to the main immunogenic region (MIR) on the extracellular domain of the AChR \( \alpha \)-subunit [3,4]. Such autoantibodies cause internalization and degradation of the AChR available on the skeletal muscle at neuromuscular junctions, resulting in the failure of neurotransmission and paralysis [1,2]. Because the current MG therapies including general immunosuppression often cause toxic side effects [1], development of agents that can more specifically inhibit MG is warranted.

Since the pathogenesis of MG is mainly due to autoantibodies to AChR, much effort has been directed toward the development of small molecules that block interaction of the antibodies with the receptors [5–7]. Short RNA aptamers that have been isolated to a wide range of proteins with iterative in vitro selection techniques from a combinatorial RNA library [8,9] have been reported to act as specific decoys and inhibit the physiological functions known to be associated with the target proteins in cells and in animal models [10]. The rationale of these experiments is that a short RNA molecule with a certain sequence can form a fairly stable and unique tertiary structure via intramolecular base-pairing [11]. Noticeably, attachment of a polyethylene glycol (PEG) to the 5’ end of an aptamer has been shown to enhance in vivo bioavailability of the aptamer [12,13]. Thus, RNA molecules could be alternative candidates for specific and effective inhibitors of MG autoantibodies.

Previously, we employed in vitro selection methods and successfully isolated a short and nucleasresistant RNA aptamer with 2’-amino- [14] or 2’-fluoro-modified pyrimidines [15] that can bind both a rat monoclonal antibody, called mAb198, that is specific to the MIR on human AChR, and patient autoantibodies with MG with high specificity and high affinity. Surprisingly, the sequence of the RNA aptamer with 2’-fluoropyrimidines was the same as that of RNA with 2’-aminopyrimidines [15]. These RNA aptamers have been shown to act as decoys to protect AChR from the effects of the antibodies in human cells. Moreover, we could improve binding activity and bioactivity of the RNA aptamer with 2’-amino by extended sequence selection [16]. In this study, we present the ability of the RNA aptamer to inhibit EAMG in animal models. We demonstrated that the selected RNA aptamer with 2’-fluoropyrimidines that was truncated and modified by attachment of PEG at its 5’ end showed no significant differences in binding affinity to mAb198 and bioactivities protecting AChR on human cells from both mAb198 and patient autoantibodies, compared to the unmodified 2’-fluoro RNA aptamer. Moreover, the modified aptamer protected clinical symptoms of EAMG in animals passively transferred by mAb198 and blocked the antibody-mediated loss of muscle AChR in the animals.

2. Materials and methods

2.1. Antibodies, AChR, and cells

Rat anti-MIR mAb198 and rat hybridoma cell lines producing...
mAb198, kindly provided by Jon Lindstrom (University of Pennsylvania, Philadelphia, PA, USA), were prepared, and characterized as described [6]. Serum samples from a patient with MG (batch #2051) were purchased from The Binding Site (San Diego, CA, USA). According to the manufacturer, the radioimmunoassay titer of anti-human AChR in the patient’s serum was very high. The major extracellular domain of the human AChR α-subunit (amino acids 1–210) was a gift from Sohel Talib (Gencell, Santa Clara, CA, USA). The TE671 human medulloloblastoma cell line (ATCC 8805–CRL) of rhabdomyosarcoma origin [17] expresses receptors for acetylcholine apparently identical to human AChR [18] and was used in AChR down-modulation experiments.

2.2. Preparation and analysis of selected RNA aptamer

Full-length RNA aptamer with 2′-deoxy-2′-fluoropyrimidines (Ambion) previously selected against mAb198 or its truncated form (tMG RNA or tMG RNA, respectively) was synthesized by in vitro transcription of synthetic DNA templates, radiolabeled, and isolated as described [19]. A 5′-GGGAGAGCCGAAGCGUCUGCU GGGCC-15,19 of fMG RNA was 5′-transcribed in vitro of mAb198 and RNA aptamer after preincubation for 1 h at 37°C. In order to increase its residence time in animal plasma as described [20], the aptamer was coupled to 40-kDa PEG to its 5′ end (Peg-tMG RNA). Where the underlined sequence represents selected nucleotides (nt) and the 46-nt-long truncated sequence in italics is a minimal binding domain. Truncated RNA aptamer was coupled to 40-kDa PEG to its 5′ end (Peg-tMG RNA) to increase its residence time in animal plasma as described [20]. For binding affinity assays, competitive binding was performed by incubating 100 pM of32P-labeled fSE RNA, 50 nM mAb198, and 10 nM of fMG RNA, and excess amount of unlabeled competitor RNA in 100 μl binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 2 mM dithiothreitol, and 1% bovine serum albumin) for 30 min at room temperature with shaking. Antibody–RNA complexes were immunoprecipitated with 20 l of protein G-Sepharose beads (Pharmacia Biotech), and bound RNAs eluted from the pellets and analyzed on a 6% polyacrylamide gel with urea. The percentage of RNA bound to mAb198 was calculated as the fraction of radioactivity present in the antibody–RNA complexes in the absence of any competitors. The KD of the competitor RNA was estimated to be equivalent to its 50% inhibitory concentration as described [21].

2.3. Assay of AChR down-modulation

AChR down-modulation was monitored as described [14–16]. MAb198 (5 nM) or autoantibodies from a patient with MG were preincubated in 90 μl of Dulbecco’s modified Eagle’s medium containing 10 mM MgCl2, 1% bovine serum albumin, and 10% fetal bovine serum with tested RNA for 30 min at room temperature. These mixtures were then added to TE671 cells in the presence of 40 μg/ml of cycloheximide and incubated at 37°C for 4 h. Next, 10 μl of medium with 50 nM of125I-labeled α-bungarotoxin (α-Bgt) was added and incubated for 3 h more. The cells were then washed, and the bound α-Bgt was quantitated using a γ 5000 counter (Packard Instruments). Incubation of TE671 cells with 5 nM of mAb198 by itself reproducibly reduced AChR expression of the cell surface by approximately 50%. This level of antigenic down-modulation was considered 100%, and other values were normalized to this amount. For antigenic down-modulation experiments with patient autoantibodies, patient sera were added to TE671 cells at concentrations determined to engender similar levels of antigenic down-modulation as 5 nM of mAb198. 1 μl of 1:2 dilution of patient sera (data not shown).

2.4. Prevention of EAMG in rats

Six-week-old female Lewis rats weighing 100–150 g were used for i.p. passive transfer of 100 μg of mAb198 in phosphate-buffered saline (PBS). The effect of RNA aptamer was analyzed by injecting a mixture of tMG RNA and RNA aptamer after preincubation for 1 h at room temperature. To ensure that intact RNA aptamer was continuously present in the circulation, the aptamer was injected two more times. There was no significant inhibition of EAMG with only initial amount present in the circulation, the aptamer was injected two more times. There was no significant inhibition of EAMG with only initial amount present in the circulation. The rats were assessed for EAMG symptoms and scored according to the following grade: 0, normal; 1, weak grasp after a few trials; 2, incomplete paralysis of hind limbs; 3, severe paralysis and moribund; and 4, death. Rats with intermediate symptoms were scored with intermediate scales. At the end of the experiment, the rats were killed and the AChR content in the extract of hind limb muscles of each rat was monitored by radioimmunoassay as described [22]. The values were expressed as a percentage of the AChR amount in rats injected only with PBS.

3. Results and discussion

3.1. Binding affinity of RNA aptamer to mAb198

Previously, we isolated an 89-nt-long nucleoside-resistant RNA aptamer, termed tMG RNA, which bound to mAb198 very avidly (KD ≈ 25 nM) and specifically, from a large (~1012 molecules) combinatorial RNA library with every pyrimidine modified at its 2′ position by a fluoro group [15]. For the scale of efficient synthesis necessary for in vivo applications, however, tMG RNA must be shortened to at most 50 nt. A 46-nt-long truncated version of tMG RNA, termed tMG RNA, was observed to contain the most critical sequences for mAb198 binding from deletion mapping analysis (Fig. 1A, data not shown). Scrambling the five nucleotides that form the internal loop of tMG RNA disrupted the aptamer binding to mAb198 (Fig. 1B), hence this RNA (Scramble RNA) was used as a negative control for tMG RNA. For the assessment of in vivo activity of the aptamer, Peg-tMG RNA was generated because its conjugation to an RNA ap-
tamer with small size has been shown to reduce rapid tissue diffusion and renal clearance of the aptamer [12,13].

To determine the binding affinity of the truncated aptamers, a competition binding assay was carried out by incubation of trace amounts of radiolabeled iMG RNA and increasing amounts of unlabeled competitor RNA (Fig. 1B). An equilibrium dissociation constant \( K_d \) of 30 nM, an affinity that was very similar to that of iMG RNA \( K_d \) of 25 nM), was detected for tMG RNA. Moreover, the binding affinity of Peg-tMG RNA \( K_d \) of 75 nM) to mAb198 was only about three-fold less than that of iMG RNA. Thus, both a truncated version of the selected RNA aptamer against mAb198 and its PEG-conjugated form retain high affinity of the aptamer to the antibody. By contrast, Scramble RNA could hardly displace the binding of iMG RNA to mAb198 even at the highest concentration of RNA, indicating that mutation of the internal loop regions disrupts the aptamer’s binding activity to the antibody.

3.2. Inhibition of autoantibody-mediated antigenic modulation of AChR expression by RNA aptamer

Once it was ascertained that the truncated and modified RNA aptamer with PEG bound to mAb198 with high affinity, we next determined if the RNA could protect AChR on human cells from the effects of mAb198. Incubation of human TE671 cells with mAb198 resulted in a dose- and time-dependent degradation of AChR on the cell surface (data not shown) [23], which is referred to as antigenic down-modulation. To assess the modified aptamer’s ability to inhibit antigenic down-modulation induced by the antibody, TE671 cells were incubated with mAb198 in the presence or absence of the RNAs (Fig. 2A). AChR degradation was assessed by measuring the amount of \[^{125}I\]Bgt that could bind the AChRs remaining on the cells after treatment with the antibody, as previously described [14]. While a negative control RNA competitor, such as Scramble RNA, showed little inhibition of the mAb198-mediated AChR down-modulation, fMG RNA showed efficient protection of TE671 cells from the antibody and inhibited AChR down-modulation by up to 65% as previously shown [15]. Moreover, both tMG RNA and Peg-tMG RNA also efficiently blocked TE671 cells from mAb198 and inhibited antigenic down-modulation by up to 62% and 50%, respectively. Furthermore, inhibition of AChR down-modulation with the aptamers was dose-dependent (data not shown). Thus, truncation and PEG conjugation of the RNA aptamer selected against mAb198 showed no significant difference in the protection of cells from the antibody, compared with the originally selected iMG RNA.

We have previously observed that iMG RNA could cross-react with anti-AChR autoantibodies from MG patient sera. This result was probably due to the fact that most pathologically relevant autoantibodies from MG patients appear to bind the AChR’s MIR that was recognized by mAb198 [2,24]. However, the RNA blocked a certain fraction of the antibodies in the MG patient sera from binding to, and hence the down-modulation of, AChR on TE671 cells with patient variability [15]. This is probably due to different immune responses against AChR in the different individuals. Nevertheless, fMG RNA could inhibit the autoantibodies at least in one patient in a dose-dependent manner almost as well as it could protect cells from mAb198. Thus, we wanted to determine if the modified RNA aptamer could inhibit the down-modulation of AChR expression mediated by autoantibodies in the patient sera (Fig. 2B). Addition of iMG RNA, but not control RNA such as Scramble RNA, protected a significant fraction of AChR on TE671 cells from the patient’s autoantibodies, and inhibited the AChR down-modulation by 47%. Truncated tMG RNA and Peg-tMG RNA also efficiently protected a certain fraction of the cells from the autoantibodies, and inhibited the antigenic down-modulation by 43% and 35%, respectively. Thus, truncation and PEG conjugation of the RNA aptamer against mAb198 did not severely affect the aptamer’s ability to block AChR down-modulation mediated by the most inhibited patient sera.

3.3. Inhibition of mAb198-mediated passive transfer of EAMG in rats by RNA aptamer

Once the truncated and modified RNA aptamer with PEG was shown to interact with mAb198 with high affinity and inhibited AChR degradation induced by autoantibodies in cells, we examined whether the modified RNA aptamer could block EAMG generated by mAb198 to evaluate the therapeutic potential of the RNA aptamer (Fig. 3). Severe muscular weakness was observed in 6-week-old rats within 24 h after a
with the antibody alone, confirming the specific protection of animal neuromuscles from AchR loss induced by the antibody with the Peg-tMG RNA aptamer. Thus, it was concluded that Peg-tMG RNA could effectively inhibit mAb198-mediated passive transfer of EAMG in animals.

The pathogenic occurrence of autoimmune responses in MG patients could be potentially modulated with small molecules that bind the combining site of autoantibodies, and hence inhibit their interaction with the MIR of the autoantigen, AchR. Thus, MIR mimotopes, such as short peptides against autoantibody, or anti-AchR Fab fragments have been developed as protectors of cells from the autoantibodies [5–7]. However, difficulties in isolating short peptide binding target proteins with high affinity [25] or possible AchR loss by the Fab fragment in MG patients [6] might limit their clinical applicability. As an alternative anti-MG therapeutic candidate, we have previously isolated a specific RNA aptamer with high avidity against anti-AchR antibody. In this study, we observed that the RNA aptamer could efficiently protect animals from EAMG passively induced by the antibody. For the enhancement of in vivo bioavailability of the RNA aptamer, we truncated and modified the RNA with PEG. This modified RNA aptamer retained both high binding affinity to the antibody and effective bioactivity blocking AchR on human cells from the autoantibodies. Importantly, the modified RNA aptamer alone neither induced EAMG nor showed any loss of AchR in animals. Moreover, short oligonucleotides such as RNA aptamers are intrinsically poor antigens, and they are appropriate for chemical synthesis at a large-scale. These results combined with previous observations [14–16], therefore, strongly suggest that RNA aptamer against anti-AchR antibody could be an attractive lead compound for the development of specific and non-toxic anti-MG drugs. However, heterogeneous populations of anti-AchR autoantibodies present in MG patient sera [26] will probably limit the clinical utility of the RNA. Indeed, the single kind of RNA aptamer against only mAb198 cross-reacted poorly with other anti-MIR antibodies, inhibited bioactivity of autoantibodies only from a limited number of patients at a certain level, [14,15] and hardly protected EAMG actively induced by immunization with AchR in animals (data not shown). Therefore, to be therapeutically valuable, it should be worthwhile to isolate RNA aptamers against additional anti-MIR antibodies, or a pool of various autoantibodies in MG patients. If a combination of such RNAs prevents the binding of a wider variety of the anti-AchR autoantibodies in MG patients, the RNAs can then be applied to specific agents against MG.

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