A Promising Antigen-specific Immunotherapy for the Treatment of Myasthenia Gravis

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ABSTRACT

Myasthenia gravis (MG) is a T cell-dependent, antibody-mediated, autoimmune disorder with well-established antigenic targets at the neuromuscular junction. MG autoantibodies mainly target the nicotinic acetylcholine receptor (AChR) and especially epitopes located in the extracellular domain of the α1 subunit (α1-ECD). Today, most therapeutic regimens for MG are non-specific and not curative, requiring chronic treatments that are associated with significant side effects. We aim to develop an antigen-specific therapeutic approach, based on reestablishing tolerance towards the AChR, the dominant autoantigen in MG. To this end, we used a soluble mutated form of the human α1-ECD, which incorporates a major fraction of MG autoreactive T cell epitopes and examined the therapeutic efficiency of intravenous administration in a rat experimental autoimmune MG model. We found that repeated intravenous administration of α1-ECD for up to 12 days led to a robust amelioration of disease symptoms in a dose and time-dependent manner. The observed therapeutic effect of α1-ECD was significantly better than the effect of two current mainstay drugs for MG treatment. There were no signs of toxicity in α1-ECD-treated animals and further studies are underway to fully elucidate the immunological mechanism underlying the treatment effect. In this review we will summarize and discuss our most recently published findings, which strongly suggest that intravenous administration of α1-ECD may represent an efficacious and safe therapeutic approach to treat MG and thus that α1-ECD represents a potential new first in class drug for clinical application in MG.

Key words: autoimmune disease; myasthenia gravis; acetylcholine receptor; antigen specific immune tolerance; intravenous tolerance.

Abbreviations: MG, myasthenia gravis; AChR, nicotinic acetylcholine receptor; NMJ, neuromuscular junction; α1-ECD, extracellular domain of the α1 subunit of the human acetylcholine receptor; α1-ECDm, mutated form of the α1-ECD; α1-ECDmt, mutated and tagged form of the α1-ECD; EAMG, experimental autoimmune myasthenia gravis.

Introduction

Myasthenia gravis (MG) is a prototype organ-specific autoimmune disorder affecting the structure and function of the neuromuscular junction (NMJ), causing weakness and fatigability of skeletal muscles. It is a T cell-dependent antibody mediated disease, primarily caused by autoantibodies against the acetylcholine receptor (AChR). AChR antibodies are found in approximately 85% of MG patients, termed AChR-MG (1). Fewer patients have autoantibodies against other NMJ proteins, such as muscle specific kinase (MuSK) (~9% of patients) or low-density lipoprotein receptor-related protein 4 (LRP4) (~2% of patients) (2). The AChR is a transmembrane pentameric glycoprotein that along with other proteins (including MuSK and LRP4) forms a clustered complex in the post-synaptic membrane of the NMJ. This complex allows transmission of excitatory signals from the axon terminal of motor neurons to the muscle. The AChR is composed of five subunits with an $(\alpha 1)_2\beta 1\varepsilon\delta$ stoichiometry in adult and $(\alpha 1)_2\beta 1\gamma\delta$ in fetal or denervated muscles (3). Each subunit is composed of an N-terminal extracellular domain (ECD), four transmembrane domains (TM1-TM4) and a largely unstructured intracellular domain between TM3 and TM4. The ECDs contain most of the disease relevant autoantibody epitopes. Although, antibodies against the TM and intracellular domains can be found in MG patient sera, they are probably not clinically significant as they cannot bind to their targets in undamaged muscle membranes (4,5). In particular, the ECD of the AChR α1 subunit (α1-ECD) seems to be targeted by most of AChR-specific autoantibodies. It contains the so-called main immunogenic region (MIR), a group of overlapping MG epitopes with a central core located between amino acids 67 and 76 (6,7). AChR-reactive $CD4^+$ T cells have long been identified in MG patients and are essential for T cell dependent production of high affinity autoantibodies by B cells. Analysis of the basis for the T cell activation has identified T cell reactive peptides, most of which are derived from the α1-ECD (4,8–10). Thus, T and B cell epitopes appear to mainly originate from the α1-ECD, indicating its significance in designing AChR-MG therapeutics based on antigen-specific tolerance induction.

Current MG therapeutics are not curative and not antigen-specific. They mostly attain either symptomatic relief for the patients or work by general immunosuppression, potentially leading to significant

side-effects (11). Mainstay treatment options include acetylcholinesterase inhibitors, corticosteroids, intravenous immunoglobulin, plasmapheresis, and thymectomy (12). More recent treatments targeting molecules of the inflammatory response, such as complement, FcRn , proteasome components, and B cell or plasma cell markers, have also been explored with some positive outcomes (13–17). However, response to therapy may differ depending on autoantibody profile, clinical manifestation, and disease onset. For example, MuSK-MG patients do not usually respond well to acetylcholinesterase inhibitors and thymectomy is beneficial mostly for early onset AChR-MG patients (18). Additionally, complement inhibitors usually work better against AChR-MG, while B cell depleting agents such as rituximab are proposed as second in line options for refractory MuSK-MG (19,20).

An ideal therapeutic strategy would only target the autoreactive components of the immune system without impeding normal responses. Such an approach would focus on the regulation of the immune system and promote tolerance reestablishment against the targeted epitopes, in an antigen-specific manner. Therefore, this targeted approach would limit the risk of side-effects and help prevent disease recurrence (21). In this review, key aspects of intravenous antigen-specific tolerance induction are discussed.

Induction of tolerance as a treatment for MG

Induction of tolerance by administration of autoantigens has been addressed in animal models for several autoimmune diseases. In the context of multiple sclerosis, therapeutic tolerance has been achieved in mouse experimental autoimmune encephalomyelitis (EAE) models. Subcutaneous administration of myelin basic protein (MBP) peptide in escalating doses, either prior to or after disease induction, lead to a dose-dependent therapeutic response (22,23). Furthermore, there is evidence that following a repetitive dosing schedule, by either mucosal or non-mucosal routes, immune homeostasis is restored through immunoregulatory transcriptome alterations (22). A more recent study has shown that intradermal injection of a murine myelin oligodendrocyte glycoprotein conjugate led to antigen-specific T cell anergy and peripheral type-2 myeloid response (24). Clinical trials have also provided encouraging data with autoantigens delivered as a peptide-cocktail or as peptide-loaded dendritic cells, following a repetitive dosing schedule (25,26).

With respect to MG, multiple studies have examined tolerance reestablishment in experimental autoimmune MG (EAMG) animal models by administering AChR domains through mucosal routes (27–30). The mechanism behind the therapeutic effect possibly relies on the regulatory role of tissue-resident immune cells in lymphoid organs. For example, oral treatment with a recombinant α1-ECD prevented or ameliorated ongoing EAMG in rats, characterized by a decrease of Th1 response markers and a shift in auto-antibody IgG isotypes from IgG2 to IgG1. Furthermore, the α1-ECD dose affected the response; oral administration of lower doses led to active suppression of the immune response, while higher doses favored clonal anergy, most likely by limiting the proliferation of the autoantigen-specific T cells (31).

Nasal administration of AChR fragments has also shown positive results. Low doses of recombinant human α1-ECD suppressed ongoing EAMG in rats most probably by mechanisms of active suppression rather than clonal anergy, accompanied by a shift of Th1 to Th2/Th3 AChR-specific response (27). Higher antigen doses were necessary to ameliorate disease when treatment was administered after disease induction compared to preventive administration prior to induction (29). Furthermore, a 10-fold lower dose of α1-ECD was needed to achieve a similar therapeutic effect as oral administration (31).

Some studies have made use of AChR-derived peptides and immunodominant T-cell epitopes to reinstate tolerance, as opposed to whole protein domains. Induction of tolerance was reported after oral or nasal administration of immunodominant T cell epitopes derived from the *Torpedo californica* AChR (T-AChR) α-subunit in mice prior to disease induction. This was accompanied by reduced levels of autoantibodies and proinflammatory cytokines expressed by T-AChR reactive T cells, probably via mechanisms of clonal anergy (30,32). However, in other studies nasal administration of AChR-derived peptides in rats failed to have a significant effect on EAMG disease development, despite the fact that tolerization against those specific AChR epitopes was achieved (33,34). This could be due to an inability of tolerance-spreading over a wider bystander epitope range, or due to significant heterogeneity between dominant B and T cell epitope repertoires. Thus, such studies have highlighted that the use of peptides may not always be optimal for clinical application. On the contrary, the use of proteins comprising the majority of epitopes targeted, would not rely on bystander effects and would allow antigen processing and presentation in a native context, therefore, minimizing such limitations (35).

Intravenous α1-ECD as a promising drug candidate for MG therapy

Intravenous delivery of antigen could take advantage of a natural non-inflammatory path, reaching several organs with resident immune cells involved in the induction and

maintenance of tolerance. This mode of treatment delivery has been reported in other autoimmune diseases with promising results (21). In a clinical trial for relapsing multiple sclerosis, a cocktail of 4 MBP tolerogenic epitopes given in repeated escalating doses over 8 to 32 weeks resulted in a significant decrease in new lesions observed (25). Similarly, nanoparticle coated gliadin induced antigen-specific T cell tolerance in celiac disease patients, which also involved a repeated antigen dosing design (36).

Recently, for the first time, we explored antigen-specific tolerance induction by intravenous drug administration in EAMG rats as a therapeutic strategy for AChR-MG (37). We used human α1-ECD, as it contains the majority of AChR-MG-relevant pathogenic B and T cell epitopes. Our team has also previously described the construction of a recombinant human α1-ECD mutant, in which the Cys-loop has been exchanged with that of the acetylcholine-binding protein (AChBP), a homologous soluble protein from the snail *Lymnaea stagnalis*, to improve its hydrophilicity, and consequently its solubility and stability (38). Compared to the *wild* type protein, this mutant was found to have practically identical binding to autoantibodies from MG patient sera (39). The mutant domain (hα1-ECD_{mt}) was expressed in the yeast *Pichia pastoris* as a glycosylated soluble secreted protein with near-native conformation. It contained a C-terminal 6-HIS-tag to facilitate purification via metal-affinity chromatography. A tag free α1-ECD (hα1-ECD_{m}) mutant was also produced in *E. coli,* where it was present in high quantities in inclusion bodies. Following solubilization in urea the protein was allowed to refold overnight at 4°C before final purification by anion exchange and size-exclusion chromatography.

For the *in vivo* studies of therapeutic efficacy, a Lewis rat EAMG model was used. In most cases EAMG was induced in rats by AChR protein extracted from the electric organ of *T. californica* (40). More recently, we described a robust and reproducible EAMG model in female Lewis rats using hα1-ECD_{mt} in CFA (41). Symptoms usually develop 6-8 weeks after induction and, should the rats be left untreated, persist for several weeks allowing for the long-term evaluation of therapeutic interventions. Since the model is induced with the human sequence of α1-ECD, it is well suited for the study of antigen-specific therapeutic approaches (42).

Using the aforementioned tools, we proceeded to evaluate the therapeutic efficacy of intravenous α1-ECD administration. Importantly, all treatment regimens followed a therapeutic rather than a preventive regimen, treatment was always administered after disease induction (Figure 1). EAMG rats were first treated for twelve consecutive days with 100 μg hα1-ECD_{mt} intravenously (tail vein) or intranasally (droplets in nostrils), at seven days post disease induction. Disease progression was then monitored for at least 120 days. We observed that intravenous administration resulted in a highly significant reduction in the rats' EAMG score, representing a huge improvement in therapeutic effect compared to that obtained in rats treated by intranasal administration or in mock (PBS) treated rats (37). A more detailed assessment of intravenous drug-administration demonstrated that the effect was dose-dependent, with higher protein doses yielding a more profound therapeutic effect. These findings were corroborated, in addition to the EAMG scores, by changes in animal body and decrement of the compound muscle action potential in response to repetitive nerve stimulation.

Since the goal of the proposed strategy is to treat active, ongoing disease, we also examined the therapeutic potential of intravenous α1-ECD_{mt} at later time points, when rats display progressive disease at the molecular and the clinical levels (21 and 40 days after disease induction,

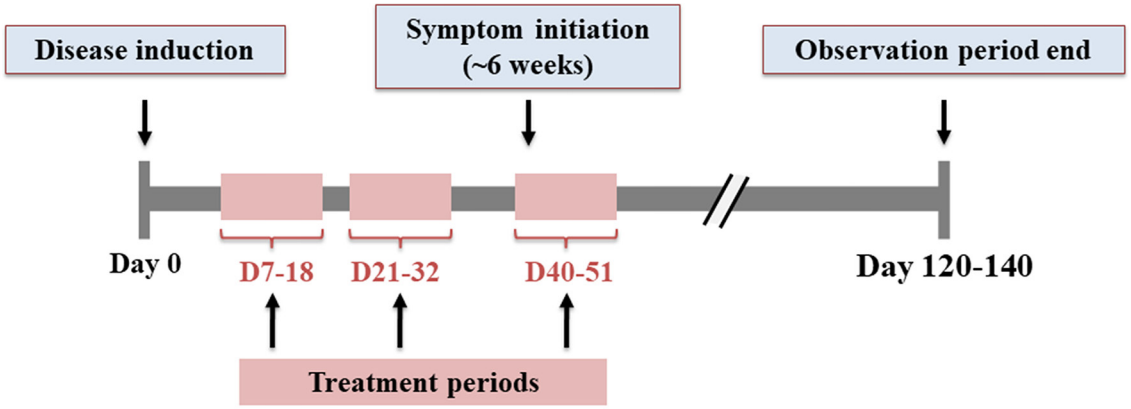

Figure 1. Schematic representation of the treatment regimens implemented in the rat EAMG animal model. Treatments were administered for 12 days starting at different times after disease induction. The animals were followed for at least 120 days after induction of disease to monitor long term effects of treatment.

Table 1: Average EAMG scores at the end of the observation period of rats treated with hα1-ECD$_{mt}$ by intravenous administration initiated at different time points and of their respective control groups. (Derived from data published in ref #31).

Treatment initiation (days after induction)	Treatment regimen (daily doses)	EAMG score (±SEM)
Day 7	PBS (x12)	2.74 (± 0.32)
	5 μg hα1-ECD$_{mt}$ (x12)	2.50 (± 0.72)
	25 μg hα1-ECD$_{mt}$ (x12)	1.05 (± 0.46)
	100 μg hα1-ECD$_{mt}$ (x12)	0.28 (± 0.14)
Day 21	PBS (x12)	3.14 (± 0.40)
	100 μg hα1-ECD$_{mt}$ (x12)	1.52 (± 0.39)
	500 μg hα1-ECD$_{mt}$ (x12)	0.57 (± 0.57)
Day 40	PBS (x12)	2.42 (± 0.49)
	100 μg hα1-ECD$_{mt}$ (x12)	2.06 (± 0.38)
	500 μg hα1-ECD$_{mt}$ (x12)	1.33 (± 0.84)
	1000 μg hα1-ECD$_{mt}$ (x12)	0.33 (± 0.33)

respectively). Treatment initiation at both later time points was found to have a powerful therapeutic effect, lasting at least until day 140 after disease induction. This effect was also dose-dependent (Table 1), where larger overall doses at later time points achieved a similar robust therapeutic effect to smaller doses given at earlier time points. The somewhat larger doses required for effective treatment of active full-blown disease compared to disease prevention could be due to accumulation of damage at the NMJ and/or the establishment of memory cells by the time treatment begins. Interestingly, it appears that overall exposure time was also crucial for optimal response to therapy. Thus, a given total protein amount administered in fewer doses was less effective than the same amount distributed over more frequent administrations. Specifically, daily injections of 100 μg hα1-ECD$_{mt}$ had a more profound effect in EAMG amelioration compared to 400 μg hα1-ECD$_{mt}$thrice (every 4 days) over a 12-day period, even though the total amount of protein administered was the same (1200 μg).

These observations are similar to what has been reported in other EAE models. Intravenous administration of a multi-epitope protein comprised of five different encephalitogenic peptides (75ug per dose for six administrations) offered long-lasting suppression of EAE in mice by downregulating pathogenic T cells and upregulating CD4+ Tregs (43). More recently, Casella et al. showed the therapeutic effect of intravenously injected oligodendrocyte-derived extracellular vesicles containing multiple myelin antigens (such as myelin basic protein, myelin oligodendocyte glycoprotein and myelin proteolipid protein) in EAE mice (44). The suppressive effect involved a mechanisms of autoreactive T cell anergy and apoptosis, rather than T regulatory cell activation. These studies have also utilized a repeated antigen dosing schedule to induce a tolerogenic effect. Indeed, there is evidence from studies in EAE that the dose and administration schedule play a significant role in the observed effect (22).

Investigating the pharmacokinetic properties of α1-ECD$_{mt}$ following intravenous administration revealed a very short plasma half-life (3.6 - 5.5 % of administered protein remained in the circulation 6 hours post injection). This can potentially explain the benefit of repeated dosing, as it prolongs the exposure of relevant cell populations to the protein. Interestingly, the pharmacokinetic profile of α1-ECD$_{mt}$ was not altered by the presence of autoantibodies or the stage of disease development. This has been demonstrated by studies performed in healthy and EAMG rats injected on day 40 after disease induction, when the α1-ECD antibody response is near its peak. As hα1-ECD$_{mt}$ displayed a short plasma half-life, modifications that would

increase its half-life in the circulation may further increase its therapeutic effect. Strategies based on attachment of polyethylene glycol chains (PEG), conjugation to albumin binding domains or an immunoglobulin Fc region and nanoparticle inclusion, have been used extensively by the pharmaceutical industry to improve the pharmacokinetic profile of biotherapeutics (45,46). Such optimization strategies could potentially allow for a dosing strategy with fewer doses. Biodistribution analysis of hα1-ECD$_{mt}$ after 6 hours showed that the majority of the protein was localized in the liver, kidneys and spleen, organs with a known role in tolerance induction and maintenance (47–49). Studies elucidating the involvement of these organs in the therapeutic effect are ongoing and aim to increase our understanding of the mechanism of action. Furthermore, these studies will provide a foundation for the development of next generation therapeutics. In this context, further assessment of immunological mechanisms resulting in EAMG amelioration, such as analysis of cytokine profile and relative frequencies of inflammatory and regulatory T and B cells, are being addressed in ongoing studies.

AChR autoantibodies have been shown to be pathogenic due to their ability to induce EAMG in animal models by passive transfer and because of the clinical improvement of patients after plasmapheresis (50–52). However, AChR antibody titers do not correlate with disease severity in MG patients (53). Furthermore, in our rat model there is poor correlation between EAMG score and rat AChR autoantibody titers, and negligible correlation with α1-ECD antibodies (41). Nonetheless, we sought to examine changes in autoantibody titers in response to treatment. We found that treatment at the earlier time point (day 7) caused a reduction in AChR antibody titers, while administration at the later time points (day 21 or 40) led to an increase in autoantibodies. Similar results were obtained for the α1-ECD antibodies. As mentioned previously, there was no correlation of the autoantibody titers with EAMG scores in rats following treatment. Some previous studies on oral tolerance have also shown an increase in autoantibody titers, despite the fact that disease was ameliorated (54). Therefore, these data underline that disease progression and response to treatment are not correlated to the entire autoantibody pool, but to subsets with specific distinct qualities such as antigen affinity, specificity, antibody isotype, and potential for antigenic modulation or complement activation. To provide insights into the treatment mechanism of action, these characteristics should be addressed to better understand their role in disease manifestation and progression.

Importantly, the potential immunogenicity of the administered protein and its effect on the normal function of the immune system should be investigated. Preliminary non-GLP toxicological studies involving injection of large doses (500 μg) of a1-ECD$_m$ in healthy rats demonstrated that the drug candidate was safe, well tolerated, and no changes in the levels of IL-2, IL-6, IL-10, IFN-γ, TNF-α and C-reactive protein were detected. Furthermore, *in silico* immunotoxicity analyses did not show any increased risk of immunogenicity in humans for a1-ECD$_m$. Nevertheless, further studies, which are underway, are needed to fully elucidate these aspects. The EAMG model in these studies and the therapeutic experimental set up make use of the same protein domain for disease induction and treatment. Studies where the disease is induced with all AChR subunit ECDs or with the torpedo AChR could further elucidate the therapeutic efficacy of α1-ECD$_{mt}$. It should be noted, that in a rat EAMG model induced by α1-ECD immunization, which also included intracellular parts of the receptor, demonstrated significant epitope spreading (55). Furthermore, antibodies against the α1-ECD seem to be the key pathogenic factor in MG. It has been suggested that changes in this class of antibodies is correlated to disease in individual patients, while an increase in antibodies against other subunits did not cause worsening of clinical symptoms (56). This also correlates well with our rat EAMG model in which the α1-ECD is pathogenic while the other AChR subunits weakly induce disease even though they give rise to antibodies (41).

To further establish the value of the novel treatment approach, we compared its efficacy to two commonly used therapies for MG patients in clinical practice, pyridostigmine and methylprednisolone. Pyridostigmine, a cholinesterase inhibitor, was given intraperitoneally (1 mg per rat) and methylprednisolone, a corticosteroid, was given orally (18.5 mg/Kg). Although both doses are higher than what is commonly used for patient treatment, these levels are well tolerated by rats (57). All treatments were initiated 40 days after disease induction. Rats treated with intravenous α1-ECD$_{mt}$ presented with effective reduction of disease symptoms compared to rats treated with the two standard treatments. For comparison, in a study performed by others, rats treated with an experimental anti-rat FcRn monoclonal antibody, a treatment modality recently approved for MG treatment, did not present with reduced disease symptoms compared to rats treated with dexamethasone, another corticosteroid (58). These results underscore the potential of our drug candidate as they demonstrate a superior efficacy of intravenous α1-ECD treatment in our model compared to pyridostigmine and methylprednisolone, two established therapies for MG.

α1-ECD$_{mt}$ contains a 6-HIS-tag which may pose an immunogenicity risk and is thus not ideal for clinical

application. To facilitate the translatability of our approach, we also investigated the therapeutic potential of α1-ECD$_m$, a protein without any tag. Moreover, the α1-ECD$_m$ protein was produced in *E. coli* to allow the potential for manufacturing scale-up purposes. As expected, the two proteins were found to have practically identical therapeutic effect when administered 21 or 40 days after disease induction. Since α1-ECD$_m$ was produced in a prokaryotic expression system, it lacked post-translational modifications, while its yeast counterpart was glycosylated. Their similar efficacy suggests that for our drug candidate glycosylation does not play a major role in its capacity to induce antigen-specific tolerance towards AChR.

Conclusions

Our novel and highly promising drug candidate currently in development, has a strong preclinical foundation as a safe, effective and disease-specific therapeutic option for patients with AChR-MG. It utilizes the organism's own antigen-presenting mechanisms and machinery to skew the autoimmune response towards tolerance without requirement of personalized autoepitopes, since it comprises multiple-epitope presentation in a native context. In our EAMG model, hα1-ECD produces a powerful long-lasting effect in a dose and time-dependent manner, following a short two-week once-daily intravenous dosing regimen. It effectively treated early and late-stage disease, using higher doses for a curative effect in later stages of disease, possibly necessitated by accumulated extensive damage at the NMJ and presence of memory cells. The potential of this antigen-specific tolerance therapy was highlighted by the fact that it greatly surpassed the therapeutic effect of two routinely prescribed treatments for MG. Therefore, it could provide an innovative and alternative route for clinical application with minimal side-effects.

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Conflicts of interest

KL has received research support from Toleranzia AB. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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