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Evaluating general ubiquitination techniques to find a method effective for AChR autoantibodies as a potential treatment of Myasthenia Gravis

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Abstract

Myasthenia gravis (MG), a chronic autoimmune disease, affects about 20 out of every 100,000 thousand people globally (Medline Plus, 2020). Currently there is no cure, but the disease can be managed with a variety of methods, the most common options being immunosuppressant medications and surgeries. MG occurs when AChR, or acetylcholine receptors, are targeted by autoantibodies produced by the bodies' immune system. These receptors are membrane proteins found on skeletal muscles which are crucial in muscle contraction, and the damage to them causes weakness of muscles among other symptoms (Chaio, 2015). This novel literature review aimed to explore a treatment option that could target the unique autoantibodies that attack the patient's AChR specifically, rather than the entire immune system as immunosuppressants do. Synthetic ubiquitin tagging was researched, and studies related to ubiquitination were found and reviewed. The practicability of synthetically tagging desired proteins with ubiquitin to initiate proteolysis as a potential treatment for Myasthenia Gravis was then explored. A practical method was found to be able to implement in AChR autoantibodies for MG treatment.

Key words: Myasthenia Gravis, auto-antibodies, immunosuppressants, ubiquitin, proteolysis

Introduction

Overview of Myasthenia Gravis

Myasthenia Gravis is characterized as a chronic autoimmune disease. The most common symptoms include muscle weakness and fatigue (Mayo Clinic Staff, 2021). The disease is caused by autoantibody formation against the nicotinic acetylcholine postsynaptic receptors (abbreviated AChR) at the neuromuscular junction of skeletal muscles (Jowkar et al., 2018). The antibodies bind to the AChR and assemble a complex. An increased amount of calcium flows through this complex, called the membrane attack complex (MAC), and causes damage to the membrane of the receptor. This results in a less effective response to acetylcholine, a neurotransmitter that enables muscle contraction. This influx of calcium also causes damage to sodium channels, which increases the threshold that is required of muscle action potential (Phillips & Vincent, 2016). Essentially, the damage to these receptors results in the rapid fatigue of any muscles under one's voluntary control (Mayo Clinic Staff, 2021). The muscle fatigue varies over time, usually worsening with activity and improving in repose. A patient may experience symptoms such as drooping of the eyelids (ptosis), unusual facial expressions, difficulty holding up their head, speech impairment (dysarthria), chewing and swallowing problems (dysphagia), trouble lifting objects, trouble rising from a seated position, and breathing problems stemming from weakness in

chest wall muscles and the muscle separating the abdomen from the diaphragm, among other symptoms (Medline Plus, 2020).

Current Treatments

Treatment of Myasthenia Gravis currently includes medications, surgery, and other therapies. Treatment plans differ with each patient and depend on age, severity of disease, which muscles are affected, and presence of other medical problems (Conquer MG, 2019). This study focuses on the medicinal aspect of treatment for Myasthenia Gravis. There are currently two types of medications used: anticholinesterases and immunosuppressants (Conquer MG, 2019). Anticholinesterases are drugs that inhibit acetylcholinesterase and butyrylcholinesterase (enzymes that break down acetylcholine), thus prolonging the existence of acetylcholine after it is released from nerve endings in the neuromuscular junction. Anticholinesterases give acetylcholine a competitive advantage over the autoantibodies that block the AChRs by allowing more time for rebinding to the receptors and increasing the amount of acetylcholine released (Nair et al., 2004). However, anticholinesterases only temporarily relieve symptoms as they do not directly address the abnormal immune system attack. In contrast, immunosuppressants suppress the entire immune system to prevent the formation of the AChR autoantibodies in the first place. Prednisone is a commonly prescribed immunosuppressant for many patients with Myasthenia Gravis, which acts by resembling natural hormones produced by the cortex of the human adrenal gland. Other commonly used immunosuppressants include azathioprine (Imuran), mycophenylate mofetil (CellCept), tacrolimus (Prograf), methotrexate, cyclosporine (Sandimmune, Neoral), and cyclophosphamide (Cytoxan, Neosar) (Conquer MG, 2019).

Disadvantages of Current Treatments

Anticholinesterases interfere with the normal functioning of the central nervous system (CNS) and the peripheral nervous system (PNS) through the inhibition of acetylcholinesterase, which hydrolyzes acetylcholine (National Research Council (US) Panel on Anticholinergic Chemicals, 1982). Excess acetylcholine in the synapse can lead to adverse side effects such as muscle twitching, muscle cramps, and sweatiness (Conquer MG, 2019). Immunosuppressants can also have extremely serious side effects. Immunosuppressive drugs increase the risk of infection because they suppress the entire immune system rather than targeting the specific problem, resulting in suppression of both harmful and helpful antibody creation. The immune system is weakened and cannot fight off disease as efficiently (Conquer MG, 2019). The authors of one recent study, published in Jama Open Network, focused on the relation between severe COVID-19 symptoms or hospitalization and patients' use of drug-induced immunosuppression by medicines such as Prednisone. The study found that drug-induced immunosuppression could elevate the effects of COVID-19 if an immunocompromised patient was infected (Kenney, 2021). COVID-19 is just one disease of many that drug-induced immunosuppression leaves patients compromised to. In addition, Prednisone carries the risk of a multitude of possible side effects: insomnia, mood changes, weight gain, fluid retention, reduced resistance to infection, increased susceptibility to diabetes, high blood pressure, osteoporosis, glaucoma, cataracts, and stomach ulcers, along with other less common side effects (Conquer MG, 2019).

Rationale of Exploring Ubiquitin Tagging

This article aims to identify a method of treatment for MG that could specifically target the unique autoantibodies rather than the entire immune system. Ubiquitination of AChR autoantibodies, a type of protein, is a potentially effective method to

treat Myasthenia Gravis to specifically target the immune system attack. Targeted proteolysis was chosen as the method of protein degradation for this project because of the ability to target specific proteins. Ubiquitination is involved in various fundamental cellular processes including protein degradation, gene transcription, DNA repair and replication, intracellular trafficking and virus particle budding. Protein ubiquitination is classified as a type of post-translational modification. Enzymes regulate the conjugation of ubiquitin, a small regulatory protein (Smith, 2018), to lysine residues of a target protein. Ubiquitin-activating E1 enzyme activity is subsequently followed by ubiquitin-conjugating E2 enzyme activity, which is then followed by ubiquitin-ligating E3 enzyme activity to initiate the ubiquitin-proteasome pathway. The system targets 80% of the proteins in a given eukaryotic cell for degradation (Chen et al., 2014). The hydrolyzation of ubiquitin-tagged proteins to their amino acids is catalyzed by the 26S proteasome, which is found in the nucleus and cytosol of all cells and constitutes approximately 1-2% of cell mass. The proteasome binds to the target protein by recognizing the ubiquitin tag (Lecker et al., 2006). The specificity of this system, by the tagging of the individual proteins, is what allows for the potential of a targeted treatment for the AChR autoantibodies.

Research

General Research in the Field of Ubiquitination

There have been multiple studies that have led to the creation of a synthetic ubiquitinated protein, however no research was found on evaluating the feasibility of creating a ubiquitinated AChR autoantibody. Therefore, multiple studies were analyzed to evaluate the effects of these experiments on other proteins, and then used to evaluate the practicability of using the same methods on an AChR autoantibody.

The following studies were analyzed to determine the process of ubiquitination, the type of protein ubiquitinated, and the results of the experiment.

Studies

Study 1: Structure–Activity Analysis of Semisynthetic Nucleosomes: Mechanistic Insights into the Stimulation of Dot1L by Ubiquitylated Histone H2B (McGinty et al., 2008).

1. Process: A study published in *Nature* aimed to examine the effects of a chemically ubiquitinated histone H2B on hDot1L-mediated intranucleosomal methylation (McGinty et al., 2008). A peptide containing the residues 117-125 of histone H2B, including an A117C substitution, was synthesized. Recombinant ubiquitin (1–75)-alpha-thioester was also synthesized, and the two were linked using a ligation auxiliary consisting of two orthogonal amino-thiol handles. Photolysis was used to remove the ligation auxiliary. The protein was then linked to residues 1-116 of recombinant H2B. Another desulphurization reaction was used to convert cysteine to alanine in the product. This led to the creation of a mono-ubiquitinated histone H2B via lysine 120 (McGinty et al., 2008).
2. Protein Used: Histone H2B
3. Results: Tens of millions of grams of ubiquitylated protein were routinely generated. The product created included a G76A mutation, however found that it was indistinguishable from the native protein (McGinty et al., 2008).

Study 2: Highly Efficient and Chemoselective Peptide Ubiquitylation (Kumar et al., 2009).

1. Researchers were able to modify a lysine amino acid by adding thiol group directly on the delta carbon of the side chain (Drahl, 2009). The modified lysine, called δ-mercaptolysine, was then used to modify lysine 6 on a model 17-residue peptide alpha-synuclein. The peptide was then synthesized and a protective group was removed. Following this ligation step, a desulphurization reaction occurred,

allowing for the thiol at the δ carbon to be removed and converted to the unmodified lysine (Kumar et al., 2009).
2. Protein Used: alpha-synuclein
3. Results: The temporary thiol-handle using the δ-mercaptolysine allowed for the ubiquitination of the 17-residue peptide with a 78% yield of isolated product (Kumar et al., 2009).

Study 3: Chemically ubiquitylated PCNA as a probe for eukaryotic translesion DNA synthesis (J. Chen et al., 2010).

1. Process: Researchers used chemically ubiquitylated PCNA (proliferating cell nuclear antigen; a homotrimeric, toroid-shaped protein) as a probe for eukaryotic DNA synthesis. The PCNA they aimed to attach a ubiquitin to was much larger than the histone H2B that McGinty et al. successfully ubiquitinated. In order to accomplish ubiquitination of the PCNA, the researchers created a mutant version of the PCNA (K164C PCNA) that had a cysteine in place of lysine in position 164, which retained normal activity of the wild-type PCNA. They activated the ubiquitin C-terminal carboxylate and then created a modified ubiquitin by introducing a unique thiol at the C-terminus. The team treated it with a water-soluble reagent (5,5′-dithiobis-(2-nitrobenzoic acid)), thus facilitating the formation of a bond between the cysteine in the K164C PCNA and the modified ubiquitin. (J. Chen et al., 2010).
2. Protein Used: PCNA
3. Results: 4.5 mg off chemically-ubiquitinated PCNA was created with a yield of 80%, although not by a native isopeptide bond.

Study 4: A Pyrrolysine Analogue for Site-Specific Protein Ubiquitination (X. Li et al., 2009).

1. Process: By using a pyrrolysine analogue, X. Li et al. were able to demonstrate that it is possible to create a ubiquitinated protein in one ligation step from two genetically encoded segments (X. Li et al., 2009). They used a cysteine-containing analogue of pyrrolysine and incorporated it into position 21 of a calmodulin protein. Subsequently, they reacted that protein with a modified ubiquitin to create a semisynthetic ubiquitinated protein (H. Chen et al., 2018).
2. Protein Used: Calmodulin (CaM)
3. Results: 30% yield of recombinant CaM was ubiquitylated.

Study 5: Traceless and Site-Specific Ubiquitination of Recombinant Proteins (Virdee et al., 2011).

1. Process: Researchers were successful in forming a completely natural isopeptide bond between ubiquitin and a SUMO protein using site-specific incorporation of δ-thiol-l-lysine and δ-hydroxy-l-lysine into recombinant SUMO proteins. They used a pyrrolysyl-tRNA synthetase to link the ubiquitin to a specific lysine that they created in the recombinant protein. They used a series of protection and deprotection steps to create this isopeptide bond (Virdee et al., 2011).
2. Protein Used: SUMO Protein
3. Results: Formed completely natural isopeptide bond in mono-ubiquitinated SUMO proteins.

TABLE 1: Summary of Application of Study Methods to AChR Autoantibodies

Study	1 (McGinty et al., 2008)	2 (Kumar et al., 2009)	3 (J. Chen et al., 2010)	4 (X. Li et al., 2009)	5 (Virdee et al., 2011)
Practical for MG Treatment	No	Yes	No	No	No
Reason	Multistep Synthesis; AChR autoantibodies contain native cysteine	Efficient; Scalable; Can use protection on native cysteines using this method; Can be used for large proteins	Multistep synthesis; unstable bond between ubiquitin and protein	Incorporation of unnatural amino acid; Hard to scale, Protein used in experiment is significantly different than AChR autoantibody	Multistep synthesis, poorly soluble

Discussion

Each study was successful in creating a ubiquitinated protein. In analyzing the data, it can be concluded if the method could be applied to ubiquitination of AChR autoantibodies. The discussion is as follows:

Study 1: This study used a multistep synthesis process that may be hard to recreate in labs. This poses an issue for the use of this method to produce ubiquitinated AChR autoantibodies- the process would need to be scaled up significantly to treat MG in a patient, and the multi-step synthesis could make that difficult. This experiment also relied on the fact that the histone H2B had no native cysteines (Faggiano & Pastore, 2014). However, AChR autoantibodies do contain cysteine, according to their FASTA sequence (NCBI). Since this study provides no method to ubiquitinate proteins containing native cysteines, this method of ubiquitination is not practical for use in MG treatment.

Study 2: This study provides a model that, in principle, could be used in sequential ligation for the synthesis of ubiquitylated proteins. It is highly efficient, which is necessary in order to be able to scale up to treatment. However, the study has two downfalls. One is that it uses a 17-residue model peptide, and claims that in order to achieve synthesis of a ubiquitin to a full-length protein, there will need to be a second ligation step to attach the ubiquitin-ligated peptide with the rest of the peptide. In doing this, one must use a protective group to protect the N-terminal cysteine or the δ-mercaptolysine. The second downfall is that the method works without the presence of other cysteine residues, while the AChR autoantibody contains cysteine residues. However, this problem could be overcome by using temporary protection of the native cysteine, therefore rendering this method a possible effective process to use to ubiquitinate AChR autoantibodies.

Study 3: This protein was similar to the AChR autoantibody in that it is large, and the lysine site of ligation was distant from both ends of the peptide. However, this process requires multistep synthesis and is hard to accomplish, therefore making it difficult to scale up in treatment. One benefit to this treatment is that the PCNA contained native cysteines, similar to the AChR autoantibodies, that were mutated to serines. Yet, in the presence of reducing agents, the ligation is cleaved very quickly, within 10 minutes, therefore making this method very unstable.

Study 4: Incorporating an unnatural amino acid worked in this experiment, however without testing it is not possible to know if incorporating an unnatural amino acid will alter the ability of proteases to recognize a ubiquitin tag on an AChR autoantibody. It is also difficult to synthesize the amount of the unnatural amino acid that would be needed for effective treatment of a patient. CaM is also a significantly different protein than the AChR autoantibody. The ubiquitin doesn't mark CaM for degradation, but rather helps monitor its regulatory activities. Although this study provided a method to synthetically attach ubiquitin to a desired protein, it will not be effective for our purposes.

Study 5: This method requires multistep synthesis, including multiple protection and deprotection steps, which can become difficult while scaling up to creating many ubiquitinated proteins, which would be necessary in using ubiquitination techniques to target AChR autoantibodies. However, Virdee et al.'s method of producing a native peptide bond eliminated the use of denaturing buffer conditions, which allowed for the bonds to hold for longer. However, due to the fact that the bond is poorly soluble, it becomes difficult to break down.

Conclusion

By analyzing the studies, it is clear that a few problems arise when trying to apply current ubiquitination methods to AChR autoantibodies. Three major ones are that first, AChR autoantibodies contain native cysteines, which interferes with many of the methods proposed. Second, to be able to implement ubiquitinated AChR autoantibodies, many of these processes would need to be scalable to create enough product to treat disease in a human patient. Third, AChR autoantibodies are much larger than the majority of proteins used in these experiments. The only study that addresses all of these concerns is Study 2: Highly Efficient and Chemoselective Peptide Ubiquitylation (Kumar et al., 2009). It is scalable due to the efficiency of the method that significantly reduces the amount of time needed. It also proposes a solution for ubiquitinating proteins with native cysteine residues, such as the AChR autoantibody, by proposing the use of a temporary protection auxiliary for the native cysteines while the ligation occurs. In terms of size of the protein, the study indicated that this method could be used in two ligation steps to be able to ubiquitinate a full-length protein. Therefore, this method for ubiquitination of target AChR autoantibodies could be used to create a potential treatment for MG.

It is necessary for this method to be tested in a laboratory to assess the methodology. However, by narrowing down the methods that are appropriate for testing in MG treatment, this article aims to improve on current treatments and provide a clear pathway for the future of AChR autoantibody degradation in MG treatment.

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