Wake Forest™
School of Medicine

Department of Neurobiology and Anatomy

Project title: Administration of Hsp70 maintains muscle innervation in the SOD1 mouse- a new therapeutic approach?
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Progress Report for First Two-Years of Study
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Summary
Researchers at Wake Forest School of Medicine have found a new treatment that may delay the onset of symptoms and increase the lifespan for those who are afflicted with ALS, or Lou Gehrig's disease. The researchers have determined that injections of a particular type of protein called heat shock protein (Hsp) 70 may benefit ALS suffers. The study was conducted in the mutant SOD1 mouse model of ALS. When the mice were given treatments of Hsp70, their survival was increased. This study focused on protecting the motor neurons in the mice that had ALS. The injected protein was not detected in the nervous system of the mice. Rather, this treatment appeared to work where the neurons and muscles contact each other. When the neurons and muscles loose contact, muscle weakness occurs, the prominent symptom of ALS. In a second study, the group found that the contact between neurons and muscle was maintained much longer in treated mice as compared to untreated mice. While these studies have shown positive signs, researchers caution that many more studies are needed before they can begin to conduct clinical trials in people. The group has a smaller fragment of the protein that also shown positive effects and is currently being more intensely tested. The group is determining the best way to efficiently produce the proteins to assure they will function properly. Wake Forrest School of Medicine is the only medical center that is engaged in researching this potential ALS treatment. Should this approach be successful in ALS, it is anticipated that it may be beneficial for neuropathies and other neuromuscular disorders.

Moving from research to care
ALS is a devastating process that rapidly damages the central nervous system. It causes motor neurons, the nerve cells that control muscles, to die. It is fatal, with no recognized cure or effective treatment; average survival after diagnosis is less than three years. Wake Forest Baptist Medical Center is home to one of the first ALS Association's certified, multidisciplinary ALS Clinics. The clinical group, under the direction of Dr. James Caress has extensive experience conducting clinical trials. Additionally, the ALS Center, that includes the Clinic and Translational Science Units, is a member of the Northeast ALS Consortium (NEALS). NEALS functions as an academic research consortium of medical institutions equipped to perform clinical trials in Amyotrophic Lateral Sclerosis (ALS) and motor neuron diseases. Despite the name, The Northeast ALS Consortium (NEALS) is an international group with over 100 member sites in North America, Ireland, and Israel. With the support of the Blazeman Foundation, our group is

poised to move forward effectively from the research laboratory—or the "bench"—into clinical use benefiting patients.
Goal: To produce sufficient recombinant protein and complete preclinical studies to obtain United States Food and Drug Administration (FDA) Investigational New Drug application to move forward with a Phase 1/2 clinical trial, and ultimately a Phase 3 trial.

Return
There is no treatment for ALS and patients die on average 3 years after diagnosis. Current estimates are that 30,000 individuals in the U.S. have ALS. ALS is considered an orphan disease, but ALS has a similar incidence as Multiple Sclerosis. An estimated 2.3 million individuals live with MS. An effective therapeutic would effectively increase the number of individuals living with ALS while improving quality of life.

BACKGROUND
The etiological event triggering muscle denervation and other pathological events remains undetermined, although several hallmark pathological features have been identified including: increased oxidative stress and reactive oxygen species (ROS) production, glutamate excitotoxicity, protein aggregation, and axonal transport deficits. Each of these proposed mechanisms creates a stressful environment, compromising the cell's functionality and survival. A common cellular mechanism following stress is the heat shock response, characterized by an increase in the transcription of a subset of genes resulting in the production of inducible heat shock proteins (Hsps), notably Hsp70. We have previously published studies that indicate:

- MNs do not increase expression of Hsp70 following several stress paradigms (Robinson et al., 2005 J. Neuroscience 25(42): 9735-9745; 2007 Dev. Neurobiol. 68: 1–17).

- Hsp70 is secreted by muscle and astrocytes, cells known to help MNs survive (Robinson et al., 2005; Taylor et al., 2008 Dev. Neurobiol. 67(13): 1815-1829).

- Administration of Hsp70 to MNs in culture deprived of trophic support, subjected to oxidative stress, subjected to glutamate toxicity or deprived of glucose can promote MN survival (Robinson et al.,2005, 2007).

- We can produce functional recombinant human Hsc70 (Robinson et al., 2007).

- Muscle extract, a potent source of trophic support contains Hsp70 and when Hsp70 is removed, the survival promoting activity is greatly reduced (Robinson et al., 2005).

- Administration of Hsp70 to chick embryos during the period of naturally occurring MN cell death during development inhibits MN death (Robinson et al., 2005).

- Administration of rhHsp70 to SOD1G93A mice delays symptom onset, improves motor behavior and extends survival (Gifondorwa et al., 2007 J. Neurosci. 27(48): 13173-13180; 2012 Neurol Res Int. 2012: 170426).

- rhHsp70 is localized to peripheral tissues, including muscle, but not CNS (Gifondorwa et al., 2007).

- Administration of rhHsp70 significantly delays denervation of neuromuscular junctions and improves motor performance (Gifondorwa et al, 2007, 2012).

ORIGINAL AIMS OF PROJECT

While MN degeneration is a late stage event in the ALS, muscle denervation occurs significantly earlier in the disease and is presumably the cause of muscle weakness, a prominent clinical symptom. Strategies to prevent denervation may improve quality of life by maintaining muscle control and slowing disease progression. We reported that administration of recombinant human hsp70 (rhHsp70) delayed symptom onset and increased lifespan in SOD1G93A mice. rhHsp70 was localized to the muscle and not CNS suggesting it modulates peripheral pathophysiology. In a second study rhHsp70 administration appeared to arrest denervation. These results are encouraging and suggest that rhHsp70 may be therapeutic strategy in ALS. Furthermore, the FDA has granted orphan drug status for Hsp70. However, current commercially available recombinant protein is prohibitably expensive, and of variable stability and function making further investigation of rhHsp70's therapeutic potential not possible. The goals of this project were as follows:

1. To develop the protocol to efficiently make the recombinant protein that will facilitate GMP large-scale production strategies. We were also to develop the protocol to produce the substrate binding domain fragment of Hsp70 (SBD). We showed previously that this derivativet was effective at promoting motoneuron survival in vitro. We also had hoped to develop new, smaller derivatives of the protein to generate fresh intellectual property (IP). With fresh IP, commercial investment in the project would be possible.

2. To test the full-length Hsp70, SBD and smaller derivatives of the protein in the SOD1 mouse model of ALS to confirm efficacy.

3. To perform a dose response study to determine the optimal dose of the protein and derivatives.

4. To have an independent lab confirm our results.

With successful completion of these goals, we intend to apply for an Investigational New Drug status for recombinant Hsp70 and move toward clinical trials.

PROGRESS

Aim 1: *Develop protocols to produce rhHsp70 (constitutive form) and derivatives for large-scale and GMP production of the proteins.*

The protocol developed over the past two years has resulted in a 95% purity recombinant protein and endotoxin levels that are below the FDA threshold for injection into the nervous system. Additionally, the proteins can be generated with good yields and the full-length

protein demonstrates full function. The system employed has taken advantage of a multistep chromatographic process that includes affinity chromatography and ion exchange.

Production of recombinant proteins is not a trivial process. In the cases of large proteins, such as Hsp70, reliable and reproducible production protocols are difficult to attain, because we must insure that the functional protein is made, modified appropriately and secreted in sufficient quantities for successful purification. We encountered and resolved several major hurdles over the past two years. These hurdles included determining 1) the appropriate technical and biochemical approaches to obtain high purity and yield of the protein, 2) removing endotoxin to levels well below FDA threshold, and 3) insure functionality of the protein. These first two hurdles required 18 months of work; however, the investment was valuable as we now have purified, functional full-length protein that is currently being test in the animal model. The function of the full-length protein was confirmed in two distinct assays that measured ATP activity, substrate binding and co-chaperone interactions. In addition to the full-length protein, we also have produced the substrate-binding domain fragment. The purpose for creating the substrate domain is three-fold: 1) it is believed the substrate domain is the work horse of the molecule and is responsible for the beneficial effects of the protein, 2) in earlier motoneuron tissue culture experiments the SBD protein was more effective at promoting cell survival than the full-length protein (Robinson et al., 2007), and 3) smaller proteins are easier to produce and result in greater yield.

Note on derivatives: We had originally planned to generate smaller derivatives of the protein. Smaller sized proteins will be easier to produce, can be modified to extend half-life, and are easier to administer. Additionally, there was an anticipation of generating fresh intellectual property (IP) associated with these new derivatives. Fresh IP would be beneficial in terms of obtaining investment dollars to help move toward large-scale production and clinical trial. While we were optimistic that smaller fragments could be generated, our analysis of the protein structure failed to yield a smaller fragment that would have a scientific rationale for being biologically active. For this reason, we made the decision to focus our efforts and resources on moving forward with the larger full-length and SBD proteins. If we confirm the survival-promoting activity of the recombinant protein, and are successful in early clinical studies, work to determine if a smaller, yet equally effective fragment will be justified.

Aim 2: *Test the full-length Hsp70, SBD and smaller derivatives of the protein in the SOD1 mouse model of ALS to confirm efficacy.*

The first study to confirm our original results to demonstrate the recombinant full-length protein and the SBD can delay NMJ denervation is underway. Unfortunately, these studies did not proceed on schedule as originally planned. The hurdles to overcome in protein production necessitated a delay in the animal studies. We were further delayed when our breeding colony did not produce as hoped. These challenges are common in biomedical research and can be overcome. Unfortunately, they do extend the time necessary to complete the study. Mice are treated beginning postnatal day 30 through postnatal day 75 (45 days). Following treatment, muscle is analyzed to determine the effect of treatment on maintaining NMJ innervation. 14-16 age and gender matched mice are treated in each group (saline, full-length and SBD). We are currently treating the last cohort of mice with the first dose of full-length and SBD proteins

(1 mg/kg). Processing of tissue to determine innervation is also underway. We will begin analysis of the tissue shortly. All material is coded so the technician treating the mice and processing tissue do not know which treatment was delivered. Because this is a blinded-study, we will not know results until all groups have been completed.

Third year and beyond

During the next year, we will be completing Aims 3 and 4. There are three critical issues to be considered as we move forward. The first is to determine the optimal dose to achieve the protective effect of recombinant Hsp70 (Aim 3). We are currently repeating our original experiments with a dose of 1 mg/kg. At the time of the original study, because of the extraordinary cost of the commercially available protein, we could not obtain sufficient quantities of protein to conduct a dose-response study. With the ability to produce the recombinant protein in-house, we can now accomplish this goal. The importance of a dose-response study is two-fold. First and foremost, it predicts the dosage that will confer the greatest effects in clinical trial. Secondly, knowing the appropriate dose helps to more efficiently and effectively plan future clinical studies thereby helping to minimize costs of production of protein. Breeding cages are currently underway to generate the additional animals necessary for the experiments. We are proposing to test at least 3 doses, and 16-20 animals are needed for each dose. While we do not anticipate difficulty with these experiments, the time necessary to complete proposed experiments may be extended because of Limited numbers of animals at any one time.

Independent confirmation of our results will be critical moving forward for an IND application from the FDA (Aim 4). As originally planned, once the optimal dose is determined, we will work with Dr. Terry Heiman-Patterson to have her lab confirm our results. We will send her the recombinant protein(s) and controls at appropriate doses. The Heiman-Patterson lab will be blinded as to the contents of each sample.

Finally, we must develop an assay to reliably detect if recombinant Hsc70 and/or SBD proteins are present in muscle of treated animals. “How are we going to determine if the protein gets to “site of action” (i.e., muscle) in patients?” This is a critical question to answer if we are able to proceed to clinical trial. We hypothesize that Hsp70 acts at the NMJ. It will be important to demonstrate localization of the protein. We are proposing to treat with recombinant human protein. Because this is a protein normally expressed, we do not expect to have adverse effects in patients. However, this raises a significant issue with regard to detection because it can be difficult to distinguish the recombinant protein from the naturally produced protein. This is not a trivial question to answer, but essential to answer. Many previous preclinical and clinical trials in ALS have failed to address this issue and therefore it is impossible to attribute negative results to failure of the therapeutic or failure of appropriate delivery. We will begin to develop a reliable method to distinguish between the two. Our initial approach will utilize the His-Hsc70 protein. Mice will be treated with different doses of protein. At 1, 6, 12, 24, and 48 hours muscles will be collected and protein isolated. The isolated muscle protein will be run over a Nickel column to which the His-tag will bind. Bound protein will be eluted and assayed by Western Blot, and 6xHis and human Hsc 70 ELISA to measure concentration of Hsc70. An important aspect of these experiments is that we will determine if we can measure actual, full-

length protein and not simply freed or metabolized His tag. Additionally, these experiments will allow us to determine half-life of the protein.

Our goal in this project is to conduct sufficient pre-clinical studies whose results, together with those already published will serve as the foundation for an investigational new drug (IND) application from the FDA. While there is no guarantee of immediate FDA approval, we are proceeding with a reasonable expectation that approval will be granted when we can demonstrate efficacy in the mutant SOD1 ALS mouse model, no adverse effects and delivery to expected site of action. Other issues that may contribute to FDA approval are as follows. The protein to be used in treatment is an endogenously expressed protein in human subjects, therefore, significant adverse effects are not expected as might be observed with novel drugs or molecules not usually expressed. Additionally, while we have not yet determined the specific half-life of the protein (the development of the ELISA assay described above will help us determine half-life), like all proteins, ours will be degraded and therefore have a limited time of exposure. If the IND application is approved, the basic science contributions to this project will lessen and the clinical expertise of Dr. Caress and the ALS Clinic will take on a greater role.