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School of Medicine
Department of Neurobiology and Anatomy

Administration of Hsp70 Maintains Muscle Innervation in the SOD1 Mouse – A New Therapeutic Approach?

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Thank you Blazeman Foundation for ALS for your support of Research to understand and find a cure for ALS. Support for our team's research allows us to:

- Continue our work to advance potential therapies for ALS patients.
- Recruit scientists with complementary expertise who can collaborate in developing new therapies.
- Train future scientists to become part of the search for a cure for ALS.

Our lab at Wake Forest has determined that injections of a particular type of protein called heat shock protein (Hsp) 70 may benefit ALS suffers. Our original study was conducted in a mouse model of ALS. When the mice were given treatments of Hsp70, their survival was increased. 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, we found that the contact between neurons and muscle was maintained much longer in treated mice as compared to untreated mice.

We have largely completed the project and summarize our results below. Specific methods and results will be report in a publication that will be shared with Blazeman Warriors.

Protein Production and Purification

We developed the methodology to reliably and reproducibly produce the constitutively expressed iso-form of Hsp70 protein (Hsc70). The use of large biologics, especially proteins, to treat neurodegenerative diseases has been hindered by their excessive cost and variances in their purity or activity between batches. Our lab previously developed a protocol for the purification of rhHsc70 using denaturing conditions (Robinson et al., 2008. Develop. Neurobiol. 68: 1–17). While highly effective at generating protein at high purity, the buffer system we used previously utilized a series of harsh chemicals that lead to rapid protein aggregation during renaturing when attempting to upscale to larger batches of protein. For this reason we developed a non-denaturing purification protocol for rhHsc70. Size exclusion resulted in preparations rich in full-length protein and removal of degradation fragments. Endotoxin levels were considered negative and undetectable at less than 0.6 EU/mL. We conducted mass spectrometry analysis to confirm our protein achieved 76% sequence coverage, a number well within the expected value given the protein concentration of the sample distributed for proteomic analysis. We further confirmed that we purified the 6x-his-tagged human Hsc70 protein expressed by our vector via western blot by double labeling with antibodies specific to

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Hsc70 and 6x-histidine

The recombinant protein has functional activity

In order to refold and release proteins, Hsc70 must utilize and replenish ATP from the nucleotide binding domain to open and close the helical lid of its substrate binding domain. We confirmed ATPase activity of our protein with commercially available recombinant protein. In a side-by-side comparison of commercially available protein and found that our protein had two-fold activity greater than commercially available protein.

Protein activity will inevitably vary between batches, thus before proceeding to administer our protein to animals we chose to normalize our doses to a repeatedly achievable level of ATPase activity to ensure that each treatment provided a consistent amount of active protein. We assigned 1 unit of protein activity equal to 25 pMol of Pi released in our ATPase activity assay and, using this measure, created a dose response curve to analyze the therapeutic viability of exogenously administered rhHsc70 on preserving peripheral muscle innervation. On average, 1 unit of protein activity was equal to 1 ug of protein across batches, such that a 20 unit injection of protein contained approximately 20 ug of protein.

These data suggest that the protocol we've developed for the non-denaturing purification of rhHsc70 is a reliable cost-efficient alternative to purchasing rhHsc70 for investigators who wish to use Hsc70 in experimental models.

Unexpectedly, the protein does not prevent neuromuscular junction denervation in the mouse model of ALS.

Following confirmation of active protein, we next assessed rhHsc70's therapeutic viability in preserving neuromuscular junction innervation in the $SOD1^{G93A}$ mouse model of ALS. Previously, we have shown that systemic administration of recombinant human Hsp70 beginning at postnatal day 30 delays peripheral muscle denervation in this mouse model (Gifondorwa et al., 2012). Animals received injections three times a week, and to reduce liter variability all animals that received rhHsc70 in this study had at least one age-, litter-, and gender-matched, BSA-treated control. Doses were based on protein activity level to normalize the injections against any variability in protein activity between purified batches. We tested four doses of the protein. There was no effect on delaying denervation of neuromuscular junctions. There was no toxic effect of the protein based on gross examination of systemic organs such as liver and no enhanced neuromuscular junction denervation.

We next performed experiments to determine if the protein was reaching muscle, the presumed site of activity (Gifondorwa et al., 2008, 2012). Unlike in out previous experiments, we only detected a fragment of the recombinant protein in liver. We were not able to detect the full-length protein. These results suggested that the recombinant protein was unexpectedly rapidly metabolized.

We are extremely disappointed with these results of this study. In our original studies we used the inducible form of the protein (Hsp70). The proteins are very similar in sequence, structure, and function. Indeed, many have assumed the two proteins to be interchangeable, and we found that both proteins worked similarly in promoting motoneuron survival (Robinson et al. 2008. Develop. Neurobiol. 68: 1–17). Sequence analysis did not reveal differences in potential cleavage sites of the proteins that would result in Hsc70 being more rapidly metabolized than Hsp70.

Future Directions

We are performing a final series of experiments where the protein is being administered by intramuscular injection. The goal of these experiments is determine if administration of the protein to the site of activity will be effective. If we achieve positive results from this study, we can conclude that the protein is indeed effective in the ALS mouse model, but additional protein modifications will be required for it to be therapeutically viable.

While the results of our study are disappointing, it is important to keep in mind that ***contributions to research extend significantly beyond a single project.*** Supporting research allows us to:

- Conduct studies to prove or disprove proposed ideas
- Recruit scientists with complementary expertise, who together can collaborate and develop new potential therapies
- Train the future scientists who will find a cure.

The Blazeman Support of the Hsp70 project is a perfect example. Funding from the foundation allowed us to bring on Mac Robinson, who as a graduate student in our lab developed the first protocol we used to produce recombinant protein. When we received the Blazeman Funding, Mac re-joined us as a junior faculty member and was essential to developing the protocols we now use to produce Hsp70. Interestingly, Mac completed his postdoctoral training in our Human Genomics and Personalized Medicine Center where he studied IL-6 signaling in asthma. Having Mac back in the group led to collaboration with the ALS research group and the Human Genomics group where our team is now exploring a precision medicine approach for treating certain patients with ALS.

There are no cures for ALS, and in the past 22 years, only two FDA approved medications have become available to treat the progression and severity of ALS. Thus, there is an extreme need to identify medicines that can control ALS progression, and thus prolong and preserve a higher quality of life for ALS patients and their families. When new medications are developed for ALS, new insights into how the disease starts and worsens over time can be developed. These new insights could help in developing a cure for ALS.

Interleukin 6 (IL6) is a protein called a cytokine. When the body develops inflammation, for example from an allergic reaction, the body produces high amounts of IL6. Thus, IL6 is one of the most important markers of disease, including ALS. However, high levels of IL6 can have detrimental effects on cells during chronic inflammation, leading the cells to change their size, shape, and function. IL6 does this by combining with a protein called a receptor (IL6 receptor) located on the surface of cells. Normally, only cells that have IL6 receptors can respond to IL6. This type of cell signaling is normal. However, when the IL6 receptor breaks off a cell, it can combine with IL6 and trigger abnormal signaling in cells that do not normally have IL6 receptors. This process is called IL6 transsignaling, and is a process associated with a number of diseases like arthritis and cancers. IL6 has good and bad properties in the body depending on what types of cells are affected by IL6 signaling. In humans, the amount of IL6 transsignaling can be regulated by a mutation in the gene that makes the IL6 receptor. This mutation causes the IL6 receptor to break off of cells at increased levels, thus this mutation makes IL6 transsignaling occur at higher levels in people who inherit this mutation.

We have conducted an initial study whose results suggest that ALS patients that inherit the mutation in the IL6 receptor gene may develop ALS that progresses faster and is more severe. However, this phenomena is difficult to study in patients with ALS because IL6 transsignaling occurs in multiple areas affected by the disease and whether the effect is beneficial or detrimental at a specific area is not known. We hypothesize IL6 transsignaling plays a potential protective role for motoneurons in the periphery, while later when extracellular levels of IL6 increase with increased muscle atrophy and decreased lung function, transsignaling promotes a breakdown in the blood brain barrier that fosters, IL6 transsignaling in the CNS that can promote disease progression through glial activation. Thus we propose to use an existing mouse model of ALS, called the SOD1 mouse that represents patients who do not have the IL6 receptor mutation. To study how patients who have the mutation have worse disease, we will have to build a mouse model of IL6 transsignaling by modifying the IL6 receptor in the mouse, and then breeding this mouse with the mutated IL6 receptor gene ($Il6ra^{Sec}$ mouse) with the SOD1 mouse. By developing this hybrid $Il6ra^{Sec}$ x SOD1 mouse, we will be able to measure the effects of IL6 transsignaling on the progression and severity of ALS by determining where IL6 transsignaling occurs and if plays a role in changing the rates loss of contact between motoneuron and muscle or processes in the spinal cord that make disease worse. Most importantly, we will be able to use this mouse model to determine if medicines that target IL6 transsignaling can help decrease the speed of ALS progression and severity. In turn, we can use this information to develop and test existing and new medicines that target IL6 transsignaling in humans with ALS.

We are preparing the publication of our initial study whose results suggest that ALS patients that inherit the mutation in the IL6 receptor gene may develop ALS that progresses faster and is more severe. This study is the direct result of the Blazeman Foundation. With Blazeman

support we recruited Mac Robinson and through our interactions developed this avenue of research. Furthermore, continued support has allowed two students to join the lab to pursue ALS research. Miles Lyon worked on the Hsp70 project as a Master's student and is continuing in the lab as he pursues his PhD. Marlena Wosiski-Kuhn, a MD/PhD student has continued on the Il6 transsignaling project. Both Miles and Marlena presented their work at Annual Society for Neuroscience Meeting in November (see below).

On behalf of all members of our ALS Center, we continue to express our gratitude to the Blazeman Foundation - because of this support we continue as Jon asked, we choose to "Believe...Pick a stronger word than Hope...Cure"