Understanding the role of the PACE/Furin site and Apoptosis in terms of Factor 8 production in regards to Hemophilia A Therapy

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**Introduction**

Hemophilia A is a X-linked genetic disease that affects 1 in 5000 individuals characterized by a delayed or incomplete ability to successfully clot a blood vessel that has sustained a lesion. This is due to a clotting protein cofactor called Factor VIII (F8) deficiency within the bloodstream. The responsibility of FVIII includes recruitment of various coagulation factors which eventually result in proper blood clotting. Prior to F8 secretion into the blood, F8 undergoes many obstacles such as strong holds by chaperone proteins such as BiP, protein misfolding, and low expression of F8 mRNA which collectively reduce the much needed secretion of this product in hemophilia A patients. (Dorner).

Understanding of the process behind the regulation and secretion of F8 aids in the understanding of why current treatment therapy of Hemophilia A revolves around the creation of more efficient processing of F8. The following information is adapted from learning the work of Greene. Once f8 is synthesized, it is immediately translocated to the Lumen of the ER. Then, a single chain peptide is cleaved to become f8 protein. Modifications occur in the ER such as the addition of residues within the B domain of F8 among other things. This all allows for better interactions between f8 and chaperone proteins of the ER called immunoglobulin binding proteins, or BiP. The importance of BiP is that a fraction of f8 molecules will bind to the protein BiP, and dissociation cannot occur unless much ATP is used. It is important to note that too much accumulation of F8 within an endothelial cell that is unable to properly release it can lead to apoptosis. The remaining amount of F8 that does make it through the ER travels to the Golgi, where F8 undergoes more regulation, processing and cleavage. Finally, lysis within the aforementioned B domain occurs through the paired amino acid cleavage enzyme (PACE/furin). Now, secretion from the cell occurs.

All of these steps leads to an even more minute yield in Hemophilia A patients as compared to normal individuals. Because of this, enhancing the secretion of F8 has been a major focus. Currently, treatment of Hemophilia A involves plasma injection into patients of recombinant F8. However, complications from this arise in the form of high cost to patients, short half-life of protein, and the 20-30% possible chance of the development of F8 inhibitors. To get around this, an abundance of research has gone into effect. In 2009, f8 secretion was first improved by deletion of the B domain in F8, called hFVIII-BDD (Sabitino). However, it was soon noted that deletion of the B domain did not lengthen the half life of fVIII to any great extents, nor did it increase secretion by much. Also, it lead to the same percentage of individuals who developed inhibitors during treatment. Next, A study was done involving a point mutation in the A1 domain of f8, leading to a decrease in Bip binding to f8, which resulted in more secretion. (Swaroop). The, creation of inactivation resistant f8, named IR8, was done in an attempt to see any differences in comparison to Wild type f8 in mice. Wild type f8 gets inactivated once it helps prothrombin be cleaved into thrombin, while IR8 does not go inactive for around 4 hours. Results from this did not show much difference between the 2. (Pipe).

The most recent study done in an attempt to increase F8 production in 2014 involved the deletion of the 4 amino acids of the PACE/Furin site in the Golgi Body of canine models. Human’s contain a PACE/Furin site made up of (RHQR) while canine’s contain a PACE/Furin site made up of (HHQR), a difference of only one amino acid (Greene). All results from this experiment were measured solely on a mechanistic level. What was found was that A 3 fold increase in activity occurred in terms of the amount of f8 secreted (Siner). Injection of K9 f8 with PACE/Furin site deleted corrected the hemophilia coagulopathy in 2 HA dogs. Prior to this experiment, it was believed that Canine Factor VIII (cFVIII) may have increased stability because it is predominantly expressed as a single chain, likely involving the R1645H substitution at a conserved PACE/furin site, which is not present in FVIII species other than dog (Greene).

Though a 3 fold increase in f8 secretion is quite preferential for Hemophilia A patients, apoptosis of endothelial cells still occurring will render this study to a halt. Because of this, the question is now posed; can the deletion of the PACE/Furin site subsequently aid in lowering the amount of apoptosis?

**Experiment**

Entrusting the previous experiment conducted by Siner, there would be no reason to determine levels of f8 after PACE/Furin deletion. However, using ELISA as described in Sabatino will help with that portion of this study (Sabatino 2).

 Prior to recording those findings, construct cloning must to be performed in order to create an endothelial cell with PACE/Furin deletion. This process is adapted from the process used by Greene. Essentially, a gene of interest is expressed in a vector that is easily purchased. In the case of this experiment, the construct of the Ubiquitin protein was of interest for the design of the promoter sequence. This was of interest seeing as it is found in all eukaryotic cells. Canine Factor VIII (cBF8) and cBF8 with PACE/Furin deletion are the two vectors being used. The goal of this portion of the experiment was to conduct restriction enzyme mapping using restriction digest in attempt to create a FVIII variant where the PACE/Furin site at residues 1645-1648 was deleted (Siner). Restriction digest enzymes are able to open up a vector in certain places that they can bind to. An enzyme was able to open up the vector by generating an overhang. This means that rather than the enzyme creating a blunt cut across both strands of DNA, it is cut in a way that leaves overhang on one of the strands of DNA, better allowing for re-annealing of the base pairs. The idea behind this is that the hydrogen bonds of an overhang DNA will be more chemically favorable to re-anneal as opposed to phosphodiester bonds attempting to reanneal after a blunt cleavage. Re-annealing is crucial following the deletion of the 4 amino acids of the PACE/Furin site.

After the overhangs are produced, ligation is the next step in the experiment that must occur in order for both sides of the vector to reanneal. After this is complete, these newly created construct will be transformed into bacterial cells. After plating this bacteria, every colony formed on an Agar plate must be picked and must undergo sequencing to test to see if PACE/Furin Site has been deleted. Once inserted into canine, the next steps may occur.

 In order to measure the degree of apoptosis, Terminal deoxynucleotidyl transferase (dUTP) nick-end labeling (TUNEL) must be used. This technique involves measuring for breaks in DNA strands, indicating severe DNA damage as a result of apoptosis (Gavrieli).

 TUNEL begins with collection of cells and embedding them into paraffin and then washed (Gavrieli). Following that centrifugation occurs, accompanied by another washing. The solution is then resuspended in DNA labeling solution of terminal deoxynucleotide transferase (TdT) enzyme and is incubated. After incubation, cells are rinsed with a buffer and resuspended in staining solution which will mark damaged DNA. Then each sample undergoes flow cytometry to read for the amount of cells undergoing apoptosis (Greene).

**Discussion**

 The results from this finding may harbor many possibilities. One disappointing finding may be that although release of F8 is increased due to lack of PACE/Furin, the cofactors being released may not have gone through proper regulation due to the deletion, resulting in secretion of many F8 proteins that will play no real role in coagulation.

The most favorable finding involves the discovery that lacking PACE/Furin allows for F8 that would usually build up and cause apoptosis to actually be released, subsequently preserving the endothelial cell from apoptosis.

Preservation of endothelial cells and increased secretion of F8 by Hemophilia A patients will affect society remarkably. Increasing the efficacy of f8 would lead to Hemophilia A patients to need less medical care, lower the cost of treatment due to less need of recombinant F8 injection, and improve the quality of life for those suffering with this genetic disease.

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