University of Nevada, Reno

The Role of Utrophin, Sarcospan, and Glycosyltransferase Activity in the Pathogenesis of Duchenne Muscular Dystrophy and a Representative Case Study

A thesis submitted in partial fulfillment
of the requirements for the degree of

Bachelor of Science in Biochemistry & Molecular Biology

by

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May, 2013

UNIVERSITY OF NEVADA RENO

THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

Susan T. Alaei

entitled

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be accepted in partial fulfillment of the
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May 2013

Abstract

Duchenne Muscular Dystrophy is a degenerative muscle disease that is characterized by the breakdown of skeletal muscle as a result of membrane instability. A mutation in the dystrophin gene, one of the largest gene in the human genome, results in a complete lack of dystrophin in the membrane of skeletal muscle cells. Damaged muscle fibers result in necrosis and promote the formation of fatty, connective tissue. There is presently no cure for the disease and treatment primarily consists of long-term steroid therapy to slow its progression. Current research involves examining the various mechanisms involved in stabilizing membrane proteins in the absence of dystrophin. The interactions of the proteins sarcospan and utrophin as well as related glycosyltransferase activity are further examined in depth.

A case study of a 5 year old patient is discussed in order to shed light on the clinical manifestations of the disease in its early stages.

Acknowledgement

A number of people helped make this thesis possible with their continual support. I would first like to thank Dr. Josh Baker for taking the time to give thoughtful feedback throughout the entire writing process. He has been a constant foundation of support since when this thesis was little more than an idea. Furthermore, I would like to thank Kira Brooks, MSIV, for her invaluable contribution in taking the time to assist me in creating this case study. Her knowledge and experience allowed me to write an in-depth case and I am honored to have been able to work with her.

I would also like to thank Jessica Kim and Justin Yeung for their valued feedback while writing this thesis.

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Chapter I: History, Pathophysiology, and Current Research

Introduction

Duchenne Muscular Dystrophy (DMD) is a degenerative X-linked recessive disease that affects 1 in 3600 male infants. DMD results from mutations in the *Xp21* gene, a gene which codes for the protein dystrophin. Dystrophin is a cytoplasmic protein that provides structural support to muscle cells by stabilizing the dystrophin-associated glycoprotein complex (DAGC), a transmembrane complex consisting of integral and peripheral membrane proteins which are responsible for connecting the cytoskeleton of muscle fibers to the extracellular matrix, thereby maintaining the integrity of muscle cells. Frameshift mutations within the dystrophin gene result in a complete lack of dystrophin in DMD. The absence of dystrophin compromises the integrity of the DAGC which results in cell death and severe progressive myopathy.

Symptomology and Epidemiology

Symptoms of DMD usually appear before the age of 6; they may appear as early as 3 years of age. Many infants are found to be unable to crawl or display movement of their limbs characteristic of normal muscle development in children. Patients display a difficulty in getting up or lying down, as muscle weakness in the hips, thighs, calf muscles and pelvic area is representative of the early stages of DMD. Diminished control of fine motor skills is also indicative of the disease. A further progression may see pseudohypertrophy of calf and shoulder muscles resulting from massive scar tissue. DMD patients usually lose their ability to walk and are wheelchair-bound by the age of 12 (Jennekens, 1991). Deterioration of the back muscles can consequently lead to acute scoliosis. A small percentage of patients also display evidence of cognitive impairment which result in learning disabilities and an average IQ of 80-85 on the Wechsler Children Intelligence Scale (Anderson, 2002). The rate of prevalence of mental impairment in DMD patients is approximately 33%. These cognitive deficiencies, however, are not of a progressive nature. Breathing difficulties and cardiomyopathy arise in the advance stages of DMD when the disease spreads to cardiac and pulmonary tissues, resulting in the possible need of assisted ventilation and pacemakers. The average life expectancy of DMD patients is 25 years of age. Respiratory or cardiac complications are the eventual causes of death.

Another form of muscular dystrophy, Becker Muscular Dystrophy (BMD), results from in-frame mutations within the dystrophin gene. It is a less severe form of muscular dystrophy in which degeneration progresses at a slower rate with a later onset of the symptoms. The nature of the mutation does not lead to a complete lack of dystrophin;

rather, the normal physiological levels of dystrophin are reduced or the dystrophin produced is shown to have an irregular structure (England, 1990). Patients with BMD typically live twice as long as those with DMD and may even have a normal lifespan.

Diagnosis

Creatine Phosphokinase (CK) is an enzyme that catalyzes the reversible conversion of creatine to phosphocreatine with the consumption of one ATP. The MM-CK isoenzyme is specifically abundant in skeletal and cardiac muscle. Elevated CK levels are indicative of muscle damage. The creatine phosphokinase test is used to diagnose DMD in patients, as there are usually elevated levels of stress in skeletal or cardiac muscles (Mendell, 2012). A muscle biopsy stained with dye can further reveal the presence, or lack of, dystrophin. An electromyography can also be run to determine muscle health and the extent of nerve control of muscles.

Pathophysiology

The dystrophin gene is the largest in the human genome. Comprising of approximately 2.4 million base-pairs, the gene consists of 79 exons and takes over 16 hours to be transcribed and spliced (Koenig *et al,* 1987). Although DMD primarily arises from large insertions or deletions that cause massive frameshift mutations, small point mutations can also contribute to the pathology of the disease. Insertions and deletions contribute to approximately 60% of DMD cases while 40% are attributed to smaller point mutations (Hoffman, 2001). The massive size of the dystrophin gene complex gives opportunity for multiple mutations to occur. The severity of the mutation results in a

complete lack of dystrophin and a diminished number of DAGC proteins as well, compromising the structural stability provided by the cytoskeleton and extracellular matrix (Fig 1).

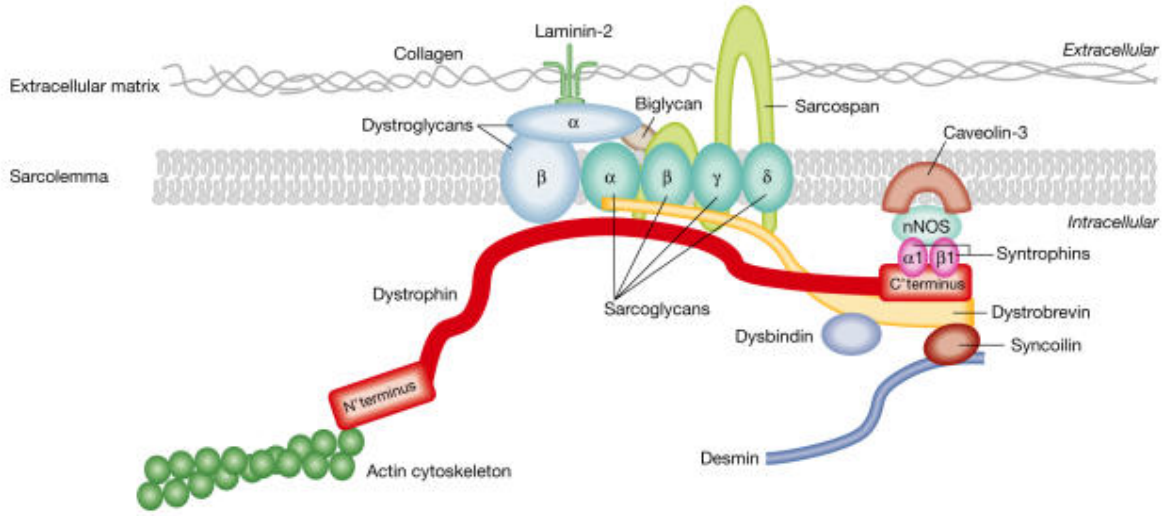

Figure 1. The dystrophin-associated glycoprotein complex of a normal muscle cell. Dystrophin provides the necessary support by connecting the cytoskeleton to the rest of the complex. Absence of dystrophin affects several other proteins within the complex as well as their binding capabilities to other proteins and the extracellular matrix (Original source: Nowak, 2004).

The dystrophin protein has several tissue-specific isoforms that arise from promoters along the dystrophin gene— the brain, muscle, and Purkinje promoters (Makover, 1991).The brain promoter codes for dystrophin expression in the hippocampus and cortical neurons while the Pukinje promoter directs dystrophin expression in cerebellar Purkinje cells (Górecki, 1992). The muscle promoter drives the primary

expression of dystrophin in skeletal muscle and cardiac cells, although the brain promoter is also responsible for certain amounts of dystrophin in cardiac muscle (Muntoni, 1995).

The absence of dystrophin results in the disruption of the DAGC which consists of proteins such as dystroglycans, sarcoglycans, integrins and caveolin. Furthermore, the lack of dystrophin has shown evidence of a diminished number of other membrane-associated proteins in the complex that leads to further instability and the disruption of cell signaling. A loss of certain surface glycoproteins makes the sarcolemma more susceptible to micro-rupturing. The level of sarcalumenin, a luminal Ca^{2+} binding protein in skeletal muscles, is significantly lowered in DMD patients. Sarcalumenin is responsible for calcium buffering within the sarcoplasmic reticulum (SR). Compared to normal muscles cells, the expression of sarcalumenin is approximately 70% lower in diseased muscle fibers (Dowling, 2004). Abnormal clustering of calsequestrin, a calcium-binding protein responsible for storing calcium in the SR, contributes to the maintenance of the Ca^{2+}concentration gradient. The structural instability disrupts the homeostasis of ion activity, leading to increased cytosolic Ca^{2+} levels as well as damaged luminal Ca^{2+} buffering (Dowling, 2003) as seen in figure 2. The structural instability allows for Ca^{2+} to leak across the membrane to disrupt the extensive signaling pathway within the mitochondria, affecting the mechanism of muscle contraction and damaging the muscle cells in an induced necrosis. The attempt to repair this damage leads to the formation of scar and connective tissue which contributes to the characteristic pseudohypertrophy in the calf and shoulder muscles seen in patients.

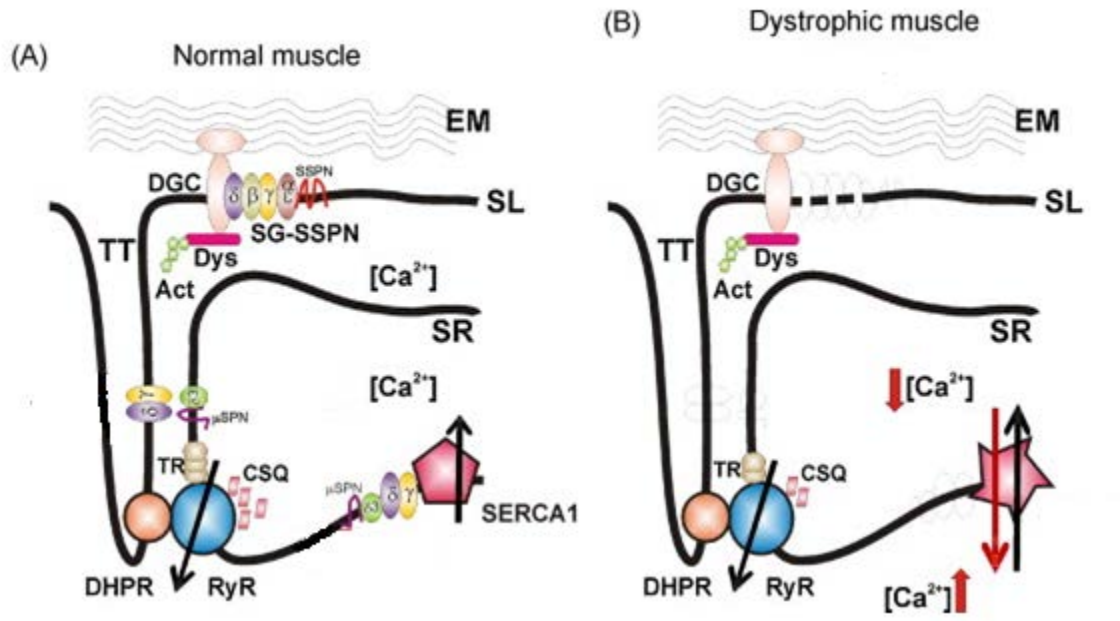

Figure 2. Comparison of Ca^{2+} signaling in normal and dystrophin muscle cells. The image on the left shows normal calcium concentrations both inside the SR and in the cytosol. The image on the right shows calcium concentrations in dystrophic muscle. The SERCA1, C^{2+}-ATPase is assisted by calsequestrin to ease the pumping gradient. Lack of calsequestrin in dystrophic muscle interferes with the ATPase activity and causes a higher concentration of Ca^{2+} in the cytosol than in the SR. (Original Source: Solares-Pérez *et al*, 2010)

Cognitive impairment associated with DMD results from an absence of dystrophin is the brain. The cerebral cortex of the brain plays a prominent role in memory, language, thought, in awareness. The deficiency of the protein in the cerebral cortex is thought be the primary cause for the development of learning disabilities in patients. Additionally, a deficiency in the cerebellum is thought to contribute to the pulmonary issues that arise in the later stages of the disease. As stated previously, the multiple mutations within the dystrophin gene are thought to contribute to deficit of other

proteins involved in maintaining cell structure integrity. A deficit of glycosyltransferase-like protein LARGE1 is common in DMD patients. The protein is responsible for the glycosylation of the α-dystroglycan complex which promotes the binding of the complex to laminin-2 and the extracellular matrix (Brockington *et al*, 2005). The LARGE protein is known to have a high expression in the brain. A lack of LARGE is suspected of diminishing the binding capability of α-dystroglycan to laminin and causing white matter changes and structural abnormalities in the brain, leading to cognitive impairment (Longman *et al*, 2003).

Sarcospan, a transmembrane protein, directly associates with utrophin and maintains the integrity of the sarcoglycan subcomplex. Spanning the membrane several times, it is an anchor for the subcomplex and plays a key role in stabilizing the binding to the extracellular matrix and does so by elevating the expression of utrophin (Peter, 2008). Utrophin, ubiquitous dystrophin, is a protein found at the neuromuscular junctions of normal muscle cells. Its structure is homologous to dystrophin and is important in maintaining structural integrity and plays a role in stabilizing the acetylcholine receptor clusters (Huh, 2002). During fetal development, utrophin is found in abundance in the sarcolemma during muscle differentiation. When the fetus begins to express dystrophin, utrophin is replaced and disappears from most all areas except at the aforementioned neuromuscular synapses. Utrophin expression is considerably increased in patients with DMD, perhaps to compensate for the lack of dystrophin in an attempt to maintain structural integrity.

Advanced stages of DMD patients show signs of respiratory and cardiac failure. Degeneration spreads to smooth muscle cells of the diaphragm, decreasing respiratory

function. This deterioration affects muscles used in coughing, allowing for bacterial and viral growth in the lungs because of an inability to clear lung secretions. Progressive cell death and replacement of conductive tissue by thick, fibrous tissue can result in heart disease known as X-linked dilated cardiomyopathy, although this specific cardiomyopathy is not very prevalent. Mutations in the muscle isoform of dystrophin affect expression in specifically the heart which contributes to the onset of cardiomyopathy (Ferlini, 2012). This onset can be exacerbated by the physical inactivity of a patient who is wheelchair bound.

Treatment

There is currently no cure for DMD. The most successful course of treatment is management of the disease with corticosteroids. Prednisone is the primary steroid used to slow the progression of the disease and rebuild muscle strength. It has been shown to slow the progression of the disease and increase the rate of repair and regeneration. The use of corticosteroids has also been shown to enhance lung and cardiac function as well as reduce the need for scoliosis surgery (Beytía, 2012). Prednisone, however, is associated with a number of side effects including weight gain, hypertension, diabetes and skeletal fractures. Deflazacort, a derivative of Prednisone, has diminished side effects; however, the corticosteroid is associated with cataracts and is currently unavailable in the United States.

Physical therapy has shown to be effective in managing muscle strength and function. Physical therapy can reduce muscle tightening and built muscle strength. Physical inactivity can contribute to the worsening of the disease, especially its

progression towards pulmonary and cardiac issues. Cardiomyopathy in DMD usually is not associated with heart failure. Even so, with the development of cardiomyopathy, a 24-hour Holter ECG record is used to continuously monitor heart activity. Ventilation support is common when pulmonary issues arise.

Current Research

There are many avenues of research being pursued to reach an effective cure. Gene replacement therapy of missing dystrophin has yielded unsuccessful results. Transduction of cells to induce gene expression of dystrophin yields only localized effects; while the injection therapy does successfully induce functioning dystrophin, it only does so for muscle cells near the injection site (O'Hara, 2001). Effective results would require massive amounts as well as continual, perhaps life-time, injections to transduce cells of a whole muscle to express dystrophin. In response, viral vectors are being studied as a systemic delivery method.

Other promising research avenues involve the upregulation of certain proteins within the DAGC, such as integrin, dystroglycan, utrophin, or sarcospan. Overexpression of utrophin may compensate for the lack of dystrophin based on their similarities. An upregulation of specific glycosyltransferases may also prove effective in strengthening binding to the extracellular matrix by increasing glycosylation activity of certain proteins. Increasing expression of these proteins may compensate for the structural instability induced by a lack of dystrophin. A therapy that involves overexpression of several of these proteins delivered via viral vector may lead to a more effective and promising therapy.

Chapter II: Stabilization of the Dystrophin-Associated Glycoprotein Complex through the Interaction of Sarcospan and Utrophin and the Enhancement of Intra-complex Binding via Galgt2 Glycosyltransferase Activity

The dystrophin-associated glycoprotein complex (DAGC) is a transmembrane complex made up of integral and peripheral membrane proteins. The complex is responsible for connecting the cytoskeleton of muscle fibers to the extracellular matrix. The lack of dystrophin in DMD patients disrupts the structural stability of the DAGC, interrupting protein binding interactions and leading to impaired laminin binding to the extracellular matrix, causing the complex to fall apart. It is thought that the lack of dystrophin also affects the number of proteins present at the membrane as well as their interactions with one another, contributing to an overall weakening of the complex in DMD patients. Evidence has shown that expression of utrophin, a protein homologous to dystrophin, is upregulated in patients with DMD, perhaps to compensate for the lack of dystrophin. Sarcospan, a transmembrane protein, has shown to function as an anchor for the complex, interacting directly with utrophin and several sarcoglycans. Its presence is integral in stabilizing the dystrogylcan subunits of the complex that directly interact with the extracellular matrix via laminin. The strength of this binding interaction is enhanced by glycosylation of the dystroglycan subunits. Stabilization of the dystroglycan subunit and its interactions with the extracellular matrix can be accomplished by studying the role of the utrophin-specific glycosyltransferase activity of Galgt2 in intra-complex and laminin binding and by understanding the mechanisms by which sarcospan interacts with both utrophin and sarcoglycans.

Utrophin

Utrophin, ubiquitous dystrophin, is found at the neuromuscular junctions of normal muscle cells. It plays a key role in stabilizing the utrophin-associated glycoprotein complex. Although utrophin is normally replaced with dystrophin during fetal development, its expression is altered in DMD. Its presence expands beyond its normal place in the neuromuscular junctions and takes the place of dystrophin to reestablish structural stability (Matsumura, 1992). In patients with DMD, these elevated utrophin levels in absence of dystrophin form a complex analogous to the DAGC, known as the utrophin-associated glycoprotein complex (UAGC). However, the naturally elevated levels of utrophin in DMD patients cannot halt the pathology of the disease alone.

Sarcospan

Utrophin's ability to stabilize the protein complex is dependent upon the protein sarcospan. Sarcospan is a transmembrane protein that is a vital part of the UAGC. It is a major component in coordinating adhesion proteins within the complex to the extracellular matrix. Sarcospan directly associates with both utrophin and the sarcoglycan subcomplex and is important in regulating utrophin levels (Peter, 2008). Sarcospan possesses a homology to tetraspanin proteins; it spans the membrane several times to anchor the complex to the membrane. It contains four transmembrane domains as well as a large extracellular loop (Maecker, 1997). Studies have shown that although the absence of dystrophin reduces the expression of sarcospan, the presence of utrophin in the neuromuscular junctions retains the expression this anchor protein. Furthermore, the

upregulation of utrophin beyond its normal location promotes the elevated expression of sarcospan as well (Crosbie *et al*, 1999).

The sarcoglycan (SG) subcomplex is composed of four transmembrane glycoproteins that each pass through the membrane once. These protein subunits are referred to as α-, β-, γ-, and δ-SG (Lim and Cambell, 1998). The alpha-dystroglycan (α-DG) protein, an extracellular cell-surface protein, is another core component of the glycoprotein complex. α-DG is a receptor which directly interacts with the laminin-2 component of the extracellular matrix and plays a critical role in binding (Cabrera *et al*, 2012). It is associated with the integral membrane protein β-dystroglycan (β-DG) via non-covalent interactions. The COOH-terminus of β-DG directly associates with the COOH-terminus domain of either dystrophin or utrophin (Jung, 1995). The SGs are responsible for coordinating the attachment of α-DG to the cell membrane through their direct interactions with β-DG. A mutation in any of these protein subunits can lead to an autosomal form of muscular dystrophy (Crosbie *et al*, 1999). Without sarcospan, the stability of the SGs is compromised, affecting their interactions with the DG complex and impairing their role in laminin binding.

The expression of sarcospan and its effects on the integrity of the UAGC correlate with the level of utrophin expression. This expression is not due to an increase in utrophin transcription; in studies of dystrophic mice treated with sarcospan, the levels of utrophin mRNA were relatively the same as dystrophic mice not treated with sarcospan, despite a measured increase in utrophin levels (Peter *et al*, 2008). It is thought that utrophin transportation through the endoplasmic reticulum and Golgi apparatus is augmented by sarcospan, which enhances utrophin expression at the membrane.

Furthermore, the tetraspanin nature of sarcospan suggests that its scaffolding ability is vital in utrophin stabilization, suggesting that elevated utrophin levels may be due to efficient utrophin utilization by sarcospan at the cell membrane. It is interesting to note that the addition of sarcospan increases utrophin expression but does not affect dystrophin expression, suggesting that the interaction between utrophin and sarcospan is quite specific. A deficiency of sarcospan within muscle fibers has shown to have also diminished utrophin expression, further fortifying the specificity of their association.

Research and Treatment: The Association between Sarcospan and Utrophin

An increase in utrophin or dystrophin expression would seem to be the most effective treatment for DMD, and traditionally these proteins have been the main target of potential DMD therapies. However, the genetic nature of these proteins proves to be a problem in creating viral vectors. The utrophin gene is 900kbp long while dystrophin is coded by one of the largest human genes in the genome at 2.4Mbp long (Helliwell, 1992). The massive size of these genes makes it difficult to insert them within a viral vector and has often resulted in creating shortened or “mini dystrophin” cDNA as a substitute (Alameddine, 1994). Although these viral vectors have been shown to alleviate DMD pathology, the process is a labored and expensive one. Sarcospan, in contrast, is coded by a 1.0 kbp long gene. Its small size gives it an advantage, as it can be more easily packaged into viral vectors and introduced within the target cell without the need for size modification. Sarcospan’s effect on utrophin expression can be used to provide structural stability to the UAGC and slow, if not reverse, the progression of DMD. Although utrophin is found to have expanded expression outside the neuromuscular junction in

DMD, the upregulation of sarcospan extends the effects of utrophin beyond its normal reach and can be detected all throughout the sarcolemma (Peter, 2008). Overexpression of sarcospan delivered via viral vectors would in turn increase utrophin transportation within muscle cells and its expression near the cell surface where the absent dystrophin should have been.

Although elevated expression of sarcospan could prove to be an effective treatment, the magnitude of this upregulation must be carefully controlled for several reasons. As seen in the diagram of the UAGC (fig 3), sarcospan is firmly embedded within the plasma membrane. It is also very closely associated with the sarcoglycan subcomplex. A 10-fold overexpression of sarcospan could lead to sarcospan clustering into insoluble protein groups at the membrane site (Peter *et al*, 2007). This physical abnormality is shown to disturb the assembly of the UAGC at the membrane and further destabilize binding to the extracellular matrix. However, a milder upregulation of sarcospan, approximately a 3-fold overexpression, found that levels of proteins within the UAGC could be increased without disrupting the structure and binding function of the complex (Peter *et al*, 2007). As such, increased expression of sarcospan through transduction with viral vectors must be carefully regulated to prevent and further aggravation of DMD pathology.

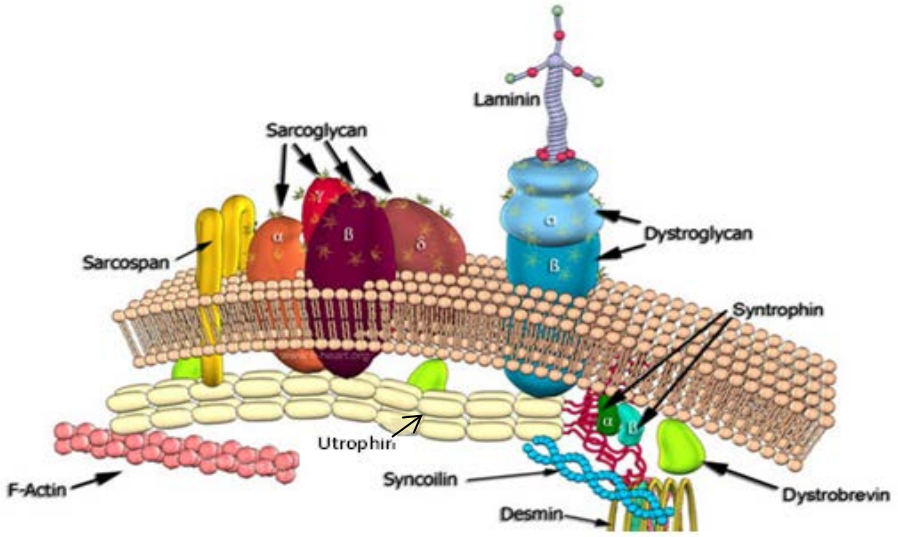

Figure 3. The utrophin-associated glycoprotein complex. The diagram shows the tetraspanin-like sarcospan associated with both sarcoglycans and utrophin. Utrophin is shown binding to both the actin cytoskeleton and the β-dystroglycan unit. α-dystroglycan associates with laminin which in turn associates with the extracellular matrix (not shown) (Original Source: e-heart.org, 2009).

The Role of Glycosylation Events

The role of α-dystroglycan in binding to the extracellular matrix via laminin could also be a target path for treatment. Binding to the extracellular matrix is affected by cell surface glycosylation of the proteins within the complex. The glycosylation of either dystroglycan subunit is modified by genetic changes to that individual subunit. Binding of β-DG to utrophin is associated with specific glycosylation events unique to utrophin itself. The utilization of utrophin, rather than dystrophin, correlates with specific glycosylation changes on α-DG (Yoon, 2009). Because α-DG can be differentially glycosylated, genetically altering these subunit-associated genes can lead to increased glycosylation, an event that will promote binding to either laminin or utrophin and

increase stability of the UAGC. An aspect of DMD that contributes to the worsening of the disease is the damage caused by muscle contractions. Affected muscle fibers with damaged DAGCs and impaired binding are continually weakened by the force of each contraction. Better binding will promote increased utrophin and laminin usage by these dystroglycan subunits, which will further stabilize the UAGC and reverse the progression of DMD pathology.

The study of the specificity of glycosyltransferase activity identified Galgt2 as the glycosyltransferase preferential for the modification of DG binding to utrophin (Nguyen, 2002). Galgt2 is an *N*-acetylgalactosamine (GalNAc) saccharide glycosyltransferase. Its activity is such that it adds a GalNAc to a premade trisaccharide to make a tetrasaccharide-modified α-DG subunit (Cabrera, 2012). This tetrasaccharide modification is specific to proteins localized in the neuromuscular junctions and, in turn, directly related to utrophin. Because the proteins and assembly of the UAGC have shown to be interconnected, overexpression of Galgt2 has shown to not only to increase utrophin and laminin binding, but has also contributed to the stability of the complex by increasing the amounts of other proteins such as α- and β-DG as well as α-, β-, and γ-SG (Xu, 2009). The increase of certain SGs serves to provide additional support in anchoring β-DG within the membrane. Furthermore, overexpression of Galgt2 has shown to normalize the force of muscle contractions by relieving the strain caused by poor binding within the UAGC, increasing and reinforcing the interactions between these proteins and the extracellular matrix (Martin, 2009). The specificity of this glycosylation event suggests the possibility of an integration of multiple upregulation therapies unique to utrophin in order to strengthen the UAGC.

Integrated Therapy

The role of the proteins involved in maintaining muscle fiber structural integrity provides the opportunity to incorporate multiple aspects of the UGAC in creating a therapy to compensate for the lack of dystrophin. A careful regulation of sarcospan overexpression coupled with upregulation of glycosylation activity could rebuild the structural integrity of the complex by specifically targeting utrophin interactions and binding activity. In terms of tissue specificity, Galgt2 is known to be widely expressed in a variety of tissues, including mucosal tissues (Montiel, 2003). This makes it an ideal target for systemic gene therapy. A therapy that comprises of a combination between highly controlled sarcospan overexpression and Galgt2 glycosyltransferase activity upregulation could potentially alleviate DMD pathology and compensate for possible problems that could arise from too much overexpression of sarcospan. It would also be specific to utrophin-mediated stability, which would eliminate the need for dystrophin-dependent therapies. The studied effects of Galgt2 glycosylation on the elevated levels of sarcoglycans would provide further support of β-DG and, in turn, contribute to stabilizing the α-DG subunit in laminin binding. Using viral vectors to transduce expression of these proteins in muscle cells seems to be a feasible method of delivery because of the small size of both the sarcospan and Galgt2 gene, resolving the obstacle presented by the massive size of both utrophin and dystrophin genes. Creating one or several viral vectors specific to the overexpression of these proteins and transducing muscle cells with them could be a potentially viable treatment option for this devastating disease.

Clinical Case Study

Chief Complaint: Patient “walking clumsily” per mother

HPI: Brandon Stevenson is a 5-year-old white male brought into the clinic by his mother with the chief complaint that he is “clumsy when walking.” His difficulties were first brought to her attention 6 months ago when his preschool teacher mentioned that he was having trouble running around and keeping up with the other kids on the playground. Although his 6-year-old sister never had any of these concerns, she attributed it to his small size and young age. Since then, however, she has noticed that his clumsiness has become more pronounced and he has started to walk somewhat abnormally. He has been climbing stairs in a “strange manner,” always using the wall for support and “using only one leading leg to climb up.” She doesn’t believe he is feeling any pain, and when asked, the patient replied that “nothing hurts” and that his legs are “just tired a lot.” His weakness is described as being limited to his lower body, and his mother has noticed that his calves are “a little larger than normal,” especially in comparison to his sister. He has not fallen or had any head injuries recently. His weakness doesn’t subside with napping or sleeping and remains constant throughout the day. Nothing seems to alleviate his symptoms.

Past Medical History: Brandon was born vaginally with no complications during delivery. His birth weight was 7lbs 2oz. He has shown a normal growth pattern during the past five years, though his weight has always been borderline underweight. His most recent office visit indicates he has had some minor weight loss. He is prone to ear infections, experiencing two in the past year alone. He has not had any surgeries and has never been hospitalized. He is not currently taking any medications and is up to date on his immunizations. He has received the flu shot recently.

Family History: Both Brandon's mother and father are in good health with no medical concerns. Brandon's maternal grandmother has type II diabetes. His paternal grandfather was a heavy smoker and died from lung cancer at age 64. His paternal uncle died from brain cancer at age 22. Brandon's mother mentioned that her maternal uncle died in his early teens from heart complications, but she does not know the exact cause of death. His 6-year-old sister has never had any problems running and has not experienced any of the difficulties Brandon is having.

Social/Sexual History: Brandon lives with his mother, father and 6-year-old sister in their new home. They own one family pet, a dog which they have had for 7 years now. Brandon began attending an all-day preschool six months ago and has previously attended day care since he was two years old. He gets along well with the other children, but prefers to spend his free time indoors reading. He has a good relationship with both his parents and is very close to his older sister. Brandon has never travelled outside the country and has not gone on any trips recently. His father is a full-time high school teacher and his mother is a part-time assistant professor at the local university. Brandon has no sexual history.

Allergies/Medications: Brandon is allergic to penicillin. His allergy results in rashes on his chest and back as well as itchiness. He uses Children's Benadryl to treat his symptoms. He has no other allergies and is not on any medications currently.

Review of Systems

HEENT: (-) cough or sore throat, (-) ear pain

Pulmonary: (-) shortness of breath

Cardiovascular: (-) chest pain

Abdomen: (-) change in bowel habits

Musculoskeletal: (-) joint pain, (+) weakness in lower extremities

Neurological: (-) headaches, (-) dizziness

Physical Exam Results

Temperature	=	98.6˚F, ear
Blood Pressure	=	96/57mmHg
Pulse	=	84/min
Respiration	=	23/min
Height	=	39”
Weight	=	33 lbs.

General: Slightly underweight male appears healthy but tired.

HEENT:

Head: Scalp is smooth and supple with no lesions or areas of tenderness noted.

Face is symmetrical with no physical abnormalities.

Eyes: Pupils are round and equally reactive to light and accommodation.

Ears: Both left and right auditory canals are clear of fluid. The left tympanic membrane is pearly grey and intact. Scarring is present in the right ear.

Nose: Nose is symmetric and septum is midline. Nasal mucosa is pink and moist. No signs of nasal congestion or polyps.

Throat: Neck is supple with no tenderness or stiffness. No lymphadenopathy and no thyromegaly or nodules were noted. Lips, tongue, and mucosa are moist and pink with no lesions. Tonsils are small with no exudates.

Pulmonary: Respirations are normal with no signs or respiratory distress. No cough, wheezes, or rhonchi present with auscultation. No tenderness of the chest wall elicited on exam.

Cardiovascular: Heart rate and rhythm is regular. No murmurs, rubs, or gallops auscultated on exam. Radial and pedal pulses were normal.

Abdomen: No swelling or redness of the abdomen and no tenderness with palpitation. Bowl sounds present in all 4 quadrants. No masses found.

Musculoskeletal: Fingers and hands appear normal with full range of motion. Spine shows signs of lumbar lordosis. Calves appear to be hypertrophied. Patient demonstrates difficulty standing up by exhibiting Gower's sign. Ankle joints appear stiff and he demonstrates an abnormal gait at times by walking on his toes. Feet are cold to the touch. Muscle strength in the upper extremities is 5/5 and a 4/5 strength in his lower extremities.

Neurologic: Patient is alert. He has normal sensation in all extremities and responds to pain and temperature sensations. Deep tendon reflexes are 2+.

Laboratory and Imaging Results

Complete Blood Count with Differential

RBC Count	4.6 $x10^6/\mu L$
Hemoglobin	13.2g/dL
Hematocrit	39.6%
MCV	86.1fL
MCH	28.7pg
MCHC	33.3%
RDW	13.5%
WBC Count	7.56 $x10^3/\mu L$
Neutrophil Count (Absolute)	3.76 $x10^3/\mu L$
Neutrophil Count (Relative)	49.8%
Lymphocyte (Absolute)	3.19$x10^3/\mu L$
Lymphocyte (Relative)	42.2%
Monocyte Count (Absolute)	0.39 $x10^3/\mu L$
Monocyte Count (Relative)	5.2%
Eosinophil Count (Absolute)	0.18 $x10^3/\mu L$
Eosinophil Count (Relative)	2.4%

Basophil Count (Absolute)	0.03 $x10^3/\mu L$
Basophil Count (Relative)	0.4%
Platelet Count	380 $x10^3/\mu L$
MPV	8.6fL

Comprehensive Metabolic Panel

Albumin	4.1g/dL
Alkaline phosphatase	160 IU/L
ALT	186 IU/L
AST	235 IU/L
BUN	9.6 mg/dL
Calcium	9.1 mg/dL
Chloride	102 mmol/L
CO2	24 mmol/L
Creatinine	1.01 mg/dL
Glucose test	86mg/dL
Potassium test	4.1 mmol/L
Sodium	141.4 mmol/L
Total bilirubin	0.9 mg/dL
Total protein	7.1 g/dL

Thyroid Panel

TSH	10.23µg/dL
Free T4	1.2ng/dL
Free T3	532pg/dL

Serum Creatine Kinase

CPK	16,200 IU/L

Electromyography

Proximal muscles biceps brachii and rectus femoris were tested. An abnormal number of small, polyphasic motor unit action potentials were detected. Motor potentials were of shorter duration and low amplitude and area. There is a slight decrease of interference pattern and amplitude size.

Muscle Biopsy

Muscle biopsy was done on a specimen from the left calf muscle. Variable muscle fiber size with some evidence of fiber necrosis. Small amounts of fatty connective tissue present. Immunohistostaining reveals no dystrophin present in any of the muscle fibers. Results indicate evidence of myopathy.

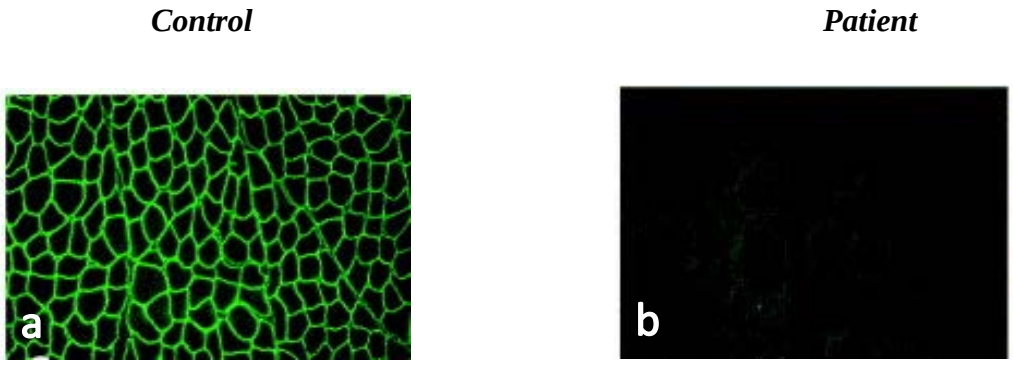

Figure 4. Immunofluorescence staining for dystrophin in normal and diseased muscle. (a) Healthy control muscle with dystrophin present in membrane. (b)Patient muscle stained for dystrophin reveals no dystrophin present in membrane (Original Source: Petrini et al, 2003).

Multiplex Ligation-dependent Probe Amplification Genetic Test

The dystrophin gene was tested to detect any abnormalities within its various loci.

Testing revealed a mutation in the gene with an in-frame deletion of exons 49-51.

Differential Diagnosis: Duchenne Muscular Dystrophy, Becker Muscular Dystrophy, neuropathy, hyperthyroidism, electrolyte imbalance, osteomyelitis, anemia

Assessment

Brandon was brought in with complaints of clumsy walking. Most notably, it was seen that he experiences difficulty standing up by exhibiting Gower's sign and his calves appear to be hypertrophied. These observations were classic enough in their presentation to suspect muscular dystrophy. However, more benign and common causes of lower limb weakness were considered before pursuing a diagnosis of muscular dystrophy. The following tests were order to rule out the likelihood of anemia, osteomyelitis, a possibly electrolyte imbalance, and hyperthyroidism.

Brandon's CBC rules out the possibility of anemia based on normal values for his hemoglobin, hematocrit, MCH, MCV, and erythrocyte distribution width. His WBC and platelet count are normal and do not necessitate further testing for leukemia or an infection, such as osteomyelitis. His comprehensive metabolic panel results revealed normal potassium, sodium, and calcium levels. An electrolyte imbalance as a cause of his weakness is unlikely based on these results. A standard thyroid panel indicated that he has normal levels of TSH, T4, and T3, ruling out hyperthyroidism. His ALT and AST were slightly elevated, which could indicate impaired liver function. However, with Brandon's age and symptoms, further evaluation was not pursued at this time as these findings did not prove to be clinically significant and are a known to be associated with DMD.

In pursuing a possible diagnosis of Duchenne Muscular Dystrophy, a serum creatine kinase test was ordered. Brandon's serum creatine kinase levels were exceptionally elevated, indicating high levels of muscle damage and further supporting the suspicion of muscular dystrophy. An electromyography was ordered to examine the state of his muscle contractions. His results of decreased amplitude and duration of motor unit action potential as well as an increase in polyphasyic action potentials are characteristic of the early phases of DMD. Furthermore, a full interference pattern was detected despite a slight decrease in amplitude. These findings, along with normal deep tendon reflexes, are not characteristic of a neuropathy, making it an unlikely diagnosis. A muscle biopsy of the left calf was done to check the levels of dystrophin. Immunohistostaining for dystrophin in the biopsy revealed a complete lack of dystrophin in his muscle cells. Based on these results, Duchenne muscular dystrophy is strongly suspected as the cause for his symptoms. Because no dystrophin was detected, Becker Muscular Dystrophy seems unlikely. Brandon has had normal developmental milestones until this point. The sudden onset and progression of his symptoms in these last six months from difficulty running around to having difficulty standing up and, upon his most recent physical exam, displaying abnormal gait when walking are significant. The diagnosis of DMD was confirmed with Multiplex Ligation-dependent Probe Amplification genetic testing that revealed an in-frame deletion of exons 49-51 on the dystrophin gene.

Treatment Plan

Brandon symptoms indicate that he is in the early stages of Duchenne Muscular Dystrophy. Research has shown that daily steroid therapy can help slow a decline in muscle wasting and even increase muscle strength. It can help reduce the risk of scoliosis and stabilize pulmonary function as well as reduce the incidence of cardiomyopathy. Brandon is referred to a pediatric neurologist to discuss long-term management of his corticoid steroid therapy. He is started on a steroid therapy of 11.25 mg of Prednisone a day until his referral goes through, and is scheduled to return in 2 weeks for a follow-up on his treatment to assess the effectiveness of the steroid treatment and any side effects he may be experiencing. To establish baseline lung function and monitor his long-term respiratory management, Brandon is also referred to a pulmonologist.

For further evaluation of mobility and possible assistance, physical therapy is recommended. To maintain functional strength and flexibility, Brandon is referred to a physical therapist that has had experience working with DMD patients. Physical therapy should help control limb contractures of his lower extremities. Brandon is also encouraged to take up swimming or walking to help maintain strength.

Brandon's family is also encouraged to make an appointment with a psychologist. They are given the names of a few psychologists who specialize in family therapy. They are also given information on support groups in the community whose families have been affected by DMD.

The family is referred to a geneticist for further genetic testing to assess the risks and possibility of present and future family members developing DMD.

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