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Meeting report

5th Annual Dysferlin Conference 11–14 July 2011, Chicago, Illinois, USA

Douglas E. Albrecht, Laura E. Rufibach, Bradley A. Williams, Nilah Monnier, Esther Hwang, Plavi Mittal *

Jain Foundation Inc., 2310 130th Ave. NE, Suite B101, Bellevue, WA, USA

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1. Introduction

The fifth Annual Dysferlin Conference, sponsored and organized by the Jain Foundation, was held from July 11 to 14, 2011 in Chicago, Illinois. Participants included 34 speakers, 31 poster presenters, 14 dysferlinopathy patients and family members, 5 representatives from other muscular dystrophy foundations, and 12 members of the Jain Foundation team. The objective of the dysferlin conference is to discuss progress towards developing therapies for the dysferlinopathies, Limb Girdle Muscular Dystrophy 2B (LGMD2B) and Miyoshi Myopathy (MMD1). These diseases manifest progressive muscle loss, usually beginning in the late teenage years, and are caused by mutations in the gene encoding dysferlin. The dysferlin protein is required for effective repair of muscle fiber membranes, but little is known about how this defect leads to the muscle loss experienced by patients, or if dysferlin is involved in other cellular processes that contribute to the pathology. The meeting addressed these and other issues specific to dysferlin deficiency, including the best tools for studying dysferlin and possible causes and interventions for the disease. Four dysferlinopathy patients from the Jain Foundation's patient registry also shared their experiences with this disease, including the risk of misdiagnosis with polymyositis and the damaging outcome of prednisone treatment.

2. Session I: How do we compensate for dysferlin's absence?

This session focused on potential treatments that may ameliorate the pathology of dysferlin deficiency without replacing the gene or protein.

Robert Bloch (USA) described how diltiazem (an L-type calcium channel blocker) ameliorates transverse (T-) tubule disruption and muscle damage following injury in dysferlin-deficient A/J mice. Previous studies have suggested that dysferlin is located in the T-tubules. T-tubules are disrupted in the muscles of A/J mice subjected to large strain injury *in vivo*, and in myofibers isolated from A/J mice subjected to osmotic shock *in vitro*. *In vitro*, A/J myofibers show a slower diffusion of a cell-impermeant dye from T-tubules than controls after osmotic shock, suggesting that dye is trapped more when the T-tubule system is damaged in the absence of dysferlin. In solutions containing low calcium concentrations, dye diffusion from A/J fibers accelerates to control rates, suggesting that calcium contributes to the aberrant change in T-tubule structure observed in dysferlin deficiency. Diltiazem, which blocks calcium flux through the L-type calcium channels without inhibiting excitation–contraction coupling in skeletal muscle, protects A/J fibers from osmotic shock injury similarly. Treatment of A/J mice with diltiazem also protects muscle from the effects of large strain injury, improving contractile function and reducing necrosis and macrophage infiltration. These results indicate that diltiazem ameliorates at least the short term pathology following injury in dysferlin-deficient muscle.

Jeffrey Molkentin (USA) described the effect of the mitogen-activated protein kinases (MAPK) in muscular dystrophies. He showed that activating select pathways in the MAPK cascade could induce disease in skeletal muscle, while inhibition of these same pathways by genetic deletion in the mouse or with pharmacologic inhibitors improved pathology in both mdx and delta sarcoglycan-deficient skeletal muscle. Furthermore, an inhibitor of MAPK reduced pathology in A/J mice, collectively suggesting that

* Corresponding author.
E-mail address: plavimittal@jain-foundation.org (P. Mittal).

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MAPK could be an attractive target for affecting dysferlin deficiency or other MDs in humans.

Noah Weisleder (USA) presented studies on the ability of MG53 protein to enhance membrane repair. AAV8 mediated gene transfer to produce overexpression of MG53 improved muscle and cardiac pathology in dystrophic hamsters. MG53 also improves muscle repair when delivered extracellularly, suggesting that MG53 may also have potential as a circulating protein therapy. Based on gene transfer experiments, MG53 increases dysferlin expression in normal muscle and facilitates its targeting to the cell membrane in a caveolin-3 dependent manner. These data indicate the possible therapeutic potential of the use of MG53 in the treatment of dysferlinopathy.

Simone Spuler (Germany) presented data on dysferlin–myostatin double knockout mice. Myostatin is a negative regulator of muscle growth, and inhibition of myostatin to enhance muscle growth is being explored as a therapeutic approach for muscular dystrophies. The myostatin/dysferlin double knockout mice have increased muscle mass compared to the dysferlin deficient controls, however, this did not lead to improved muscle function. In fact, the DKO mice had reduced running distance compared to BLA/J. This result suggests that myostatin inhibition may not be a promising approach for treating dysferlinopathy.

Hannele Ruohola-Baker (USA) presented her discovery that sphingosine-1-phosphate (S1P) modulates muscle pathology in a drosophila model of dystrophinopathy. S1P is a bioactive lipid whose importance to muscle function was revealed by a screen for modifier genes in a drosophila model of dystrophinopathy. Reduction of the *wunen* gene, which is a negative regulator of the bioactive sphingolipid, S1P rescued the drosophila dystrophic phenotype. Increased S1P levels can be achieved genetically or by direct injection of S1P, or by systemic delivery of an inhibitor of S1P lyase, such as THI. Consumption of THI improved the drosophila phenotype and also the mdx mouse phenotype. Experiments to test if S1P treatment will reduce symptoms of dysferlinopathy in mice are underway.

3. Session II: What goes wrong when dysferlin is absent?

This session focused on characterizing in detail the pathological features of dysferlin deficiency.

Miranda Grounds (Australia) discussed the role of oxidative stress in the development of dysferlinopathy. Oxidative stress is thought to contribute to muscle wasting, necrosis, and inflammation in many muscular dystrophies [1,2]. The team of Prof. Grounds calculated the levels of oxidative stress in A/J dysferlin null and A/J control mice at various ages, initially by measuring the amount of thiol oxidation [2]. Thiol oxidation measures the amount of oxidation of sulfhydryl (SH) groups to disulfide bonds caused by exposure to reactive oxygen species (ROS). This kind of oxidation can compromise protein function and affect many aspects of cell function and signaling. A/J dysferlin null mice showed increased thiol oxidation, especially in the psoas and quadriceps muscles; the oxidation levels increased with age. The level of thiol oxidation correlated with the severity of the histopathology in the different muscles tested. These data demonstrate elevated thiol oxidation in dysferlin-deficient muscles, which supports the possibility of using drugs that specifically target protein thiol oxidation as potential therapies for dysferlinopathy.

Gillian Butler-Browne (France) presented data on the study of cell lines expressing mutant caveolin 3 and dysferlin and how these cells respond when subjected to osmotic shock or mechanical stress [3]. This work was obtained in collaboration with Christophe Lamaza. In wild-type cells, mechanical stress flattens out caveolae in the plasma membrane to relieve membrane tension and prevent bursting. Caveolin 3, dysferlin, or dystrophin mutant cell lines have fewer caveolae, decreasing the buffering capacity of the membrane to changes in tension and making them more fragile. These mutant cell lines were also more susceptible to hypo-osmotic stress as indicated by their sensitivity to rupture. These data show that caveolae are an integral part of how a muscle cell handles changes in membrane stress and that dysferlin deficiency impairs this function.

Katie Maguire (USA) described specially bred dysferlin-deficient mice that allow for the monitoring of regeneration, non-invasively, using luciferase. Her data show that there is significantly more regeneration in the dysferlin-deficient mice compared to wild-type as demonstrated by exponential dramatic increase in luciferase signal from 3 to 12 months of age indicating a gradual progression of the disease. There was no significant difference between male and female mice, and proximal muscles showed significantly more disease activity than distal muscles after 6 months of age. The non-invasively obtained luciferase levels correlated nicely with histological evidence of regeneration as detected using classical biomarkers, such as centrally-nucleated fibers and those expressing embryonic myosin heavy chain. The number of myofibers expressing luciferase also increased in dysferlin-deficient mice over time. These 'regeneration-reporter' mice will be a valuable tool for understanding disease progression, as well as investigating therapeutic interventions for dysferlinopathy. These mice are available to the dysferlin research community by contacting Tom Rando.

Elizabeth McNally and **Peter Pytel** (USA) discussed the evaluation of dysferlin/myoferlin double deficient mice (Fer mice) compared to the singly deficient mice of each strain. Dr. McNally showed that both dysferlin deficient and Fer mice had a more severe muscle pathology compared to myoferlin deficient mice, and both displayed progressive muscle damage with myofiber necrosis, centrally nucleated fibers, and at older ages, chronic remodeling. These features were most prominent in the proximal hindlimb, paraspinal, and abdominal muscles. Dr. Pytel expanded on the characterization of these mice by looking at the formation of tubular aggregates when these ferlin proteins were missing. He showed that tubular aggregates

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are most frequently seen in Fer mice, found in low numbers in myoferlin deficient mice, and are absent in dysferlin deficient mice. The hypothesis is that loss of multiple ferlin family members exacerbates the myopathic process. These three mouse models could be useful in studying the different functions of the ferlin family of proteins.

François-Jérôme Authier (France) analyzed the effect of different kinds of exercise on dysferlin deficient muscles. He subjected Bla/J mice to either chronic eccentric exercise (forced downhill running) or chronic concentric exercise (swimming). Analysis of grip strength and histology demonstrated that eccentric exercise had a detrimental effect on muscle; whereas concentric exercise had a beneficial effect on muscle strength. He went on to show that eccentric exercise leads to age dependent delayed myonecrosis and (by using electron microscopy) that the delayed necrosis was due to the accumulation of membrane damage after eccentric exercise. These results could partly explain the phenotypic variability seen in dysferlinopathy and could indicate potential benefits to patients from concentric exercise.

4. Session 3: What does dysferlin look like?

This session focused on understanding the structure and organization of the dysferlin protein.

R. Bryan Sutton (USA) is studying the atomic-level structure of individual domains of dysferlin. He presented a crystal structure of the dysferlin C2A domain at last year's dysferlin conference and has now obtained a preliminary structure of the C2A domain containing the alternate first exon of dysferlin (V1-C2A). Despite very different crystallization conditions, this alternate form of the C2A domain is arranged into two equilateral triangle-shaped trimers in the asymmetric unit, just as was observed for the original C2A domain. This similarity lends support to the hypothesis that these trimers could reflect true inter- or intra-molecular conformations adopted by the multiple C2 domains of the full dysferlin protein. He also demonstrated that a known pathogenic mutation in the C2A domain of dysferlin (mutation V67D) causes misfolding and transforms the domain into a "molten globule"-like state that may be prone to forming amyloid deposits such as those previously observed in some dysferlin patients [4]. He is continuing to pursue the structures of the other dysferlin domains.

Renzhi Han (USA) presented evidence that endogenous dysferlin exists primarily as a dimer. On a non-denaturing gel, dysferlin from muscle homogenates forms a smear at around 460 kDa, twice the size of a single dysferlin molecule. A fluorescence resonant energy transfer (FRET) signal was observed between the EYFP and ECFP tagged dysferlin, indicating that dysferlin molecules are in close proximity to each other, presumably forming oligomers. A linear rather than exponential relationship was observed between EYFP-dysferlin and ECFP-dysferlin fluorescence during photobleaching, consistent with dysferlin being a dimer. He also performed the FRET experiments with individual domains of dysferlin and found that all of the individual C2 domains oligomerize except for C2A.

5. Session 4: What does dysferlin do?

This session explored the potential functions of the dysferlin protein.

Andrew Ziman (USA) used a pH sensitive variant of GFP fused to dysferlin to confirm the location and orientation of the dysferlin protein in T-tubules. Because the interior of the T-tubule is open to the extracellular environment, changing the extracellular pH rapidly changed the emission signal of the pH sensitive GFP, confirming that c-terminus of dysferlin is in the lumen of the T-tubule. The localization of dysferlin to the T-tubules suggests a role for dysferlin in maintaining T-tubule Ca^{2+}-signaling. This hypothesis was tested by pre-loading muscle fibers with sulforhodamine and subsequently measuring dye diffusion rates on washout before and after an osmotic shock injury (OSI). Following OSI, the dye became trapped in the T-tubule system suggesting that the T-tubules had become disconnected from the extracellular environment. Monitoring Ca^{2+} concentration during cycles of electrical stimulation pre- and post-OSI revealed that peak calcium transients are repressed in dysferlin deficient muscle fibers after OSI, and cytosolic calcium levels fail to return to basal levels, leading to an accumulation of cytoplasmic Ca^{2+} in dysferlin deficient muscle fibers. Addition of the calcium channel blocker, diltiazem, restores calcium handling to pre-injury levels and also returns sulforhodamine diffusion to pre-injury levels following OSI, suggesting that T-tubule disruption/recovery is calcium dependent. Since calcium influx in this scenario is likely a combination of channel activation and membrane injury, these findings suggest that diltiazem blocks calcium influx through membrane disruptions, as well as channels, in response to OSI.

Kevin Sonnemann (USA) presented data demonstrating that dysferlin promotes vesicle fusion and directly binds lipids. The vesicle fusion assay used two different fluorescently labeled vesicle pools, one of which contained recombinant dysferlin. The addition of calcium promoted fusion of the distinctly labeled vesicle populations, suggesting that dysferlin is promoting membrane fusion. In membrane binding assays, dysferlin demonstrated both calcium-dependent and calcium-independent lipid binding. Dysferlin bound phosphatidylserine (PS)-containing liposomes only in the presence of calcium. However, in the absence of calcium, dysferlin was able to bind liposomes composed of phosphatidylinositol 4,5-bisphosphate (PIP2). The calcium dependent lipid specificities are consistent with other C2 domain containing proteins that are involved in membrane fusion, supporting a role for dysferlin in vesicle fusion. The role of dysferlin in vesicle fusion supports its involvement in membrane repair. In preliminary laser wounding studies, recombinant TAT-dysferlin decreased FM1-43 entry into muscle fibers, suggesting that exogenous

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recombinant dysferlin treatment may have therapeutic value for dysferlinopathy.

William Duddy (USA) compared the transcriptome of myoblasts and myotubes from wild-type and dysferlin null mice. The goal was to assess transcriptional changes resulting from the absence of dysferlin, both in fully differentiated myotubes and during the differentiation process. Using a 10-fold change criterion, microarray analysis identified 291 genes differently altered during differentiation, including calcium and potassium channels, receptors and adhesion factors. The comparison of wild-type and dysferlin null differentiated myotubes showed expression changes in 118 genes (using a 4-fold change criteria). These included genes encoding the collagen VI heterotrimer, which were upregulated. This is of interest in the context of previous observations [5], which show thickening of the extra-cellular matrix and aggregation of vesicles at the sarcolemmal membrane in dysferlin null tissue. In related work, data from dystrophin deficient cells show an over-secretion of numerous proteins that is associated with altered regulation of vesicle trafficking, suggesting an as yet unexplored aspect of muscular dystrophies.

Isabelle Richard (France) presented data from her yeast two-hybrid screen which used three different regions of the dysferlin protein as bait: the N-terminal three C2 domains, the internal FER/DysF domain, and the last four C2 domains. Using a multi-tiered analysis system, she found 155 protein–protein interactions with these pieces of dysferlin, 36 of which scored well for biological significance. Of these, 19 were identified previously by de Morrée et al. [6]. A broad set of biological functions and pathways were implicated from the observed Y2H interacting proteins, including intracellular transport, ubiquitin mediated proteolysis, and protein translation.

Rumaisa Bashir (UK) presented data on Anoctamin 5 (ANO5) and the similarity of the clinical presentation of patients that have mutations in ANO5 and dysferlin. ANO5 is linked with a non-dysferlin related form of Myoshi Myopathy (MMD3). Some members of the anoctamin family are calcium activated chloride channels, however there is no evidence that ANO5 has similar properties. Recently ANO6, the closest relative to ANO5, was demonstrated to have a lipid/membrane scramblase function. To further study ANO5 function, N-terminal and C-terminal ANO5 antibodies were generated and preliminary data suggests that there are a variety of ANO5 bands on western blots, suggesting extensive alternative splicing or proteolytic processing. In addition, ANO5 deficient cell lines were developed to study ANO5 function and validate the specificity of the antibody. Dysferlin expression in the absence of ANO5 is increased, as is the expression of some isoforms of ANO5 when dysferlin is decreased, suggesting some coordinated regulation of the two proteins.

Pascal Bernatchez (Canada) presented his continuing studies on ferlin proteins in vascular endothelial cells. Myoferlin is critical for VEGFR-2 stability and function in vascular endothelial cells, likely due to myoferlin's involvement in endocytosis and plasma-bound vesicles [7,8]. Dysferlin is also present in vascular endothelia but does not change VEGF responsiveness *in vitro*. However, the absence of dysferlin attenuates VEGF induced angiogenesis, suggesting that dysferlin deficiency is still somehow involved in vascular homeostasis. Further *in vitro* studies found that dysferlin deficient cells have a much higher rate of senescence than wild type cells, as well as an adhesion defect *in vitro*. Furthermore, these phenotypes correlate with a reduction in PCAM-1 expression. Forced expression of PCAM-1 in dysferlin deficient cells partially rescued the adhesion defect. Because vascular endothelial cells are critical for maintaining an appropriate blood supply to working muscles, and are also a regulatory step in the infiltration of inflammatory cells during injury or disease, it is possible that dysferlin's absence from vascular endothelial cells may contribute to the muscle pathology and inflammation seen in dysferlinopathy.

Conrad Weihl (USA) described the involvement of VCP and UBXD1 in caveolin trafficking and lysosomal degradation. Disruption of this pathway through mutations in VCP can lead to muscle pathology, likely through the mislocalization of caveolin 3. The work is significant to dysferlinopathy because the localization of dysferlin is also altered in VCP mutants, suggesting that there may be a connection between proper dysferlin trafficking and maintenance of muscle health. However, it is unclear whether the change in dysferlin distribution is due to the mislocalization of caveolin 3, which is known to associate with dysferlin, or if dysferlin's localization is altered because of some other consequence of the VCP mutations.

6. Session V: Can we find new ways to study dysferlinopathy?

This session presented exploratory studies with the goal of finding better ways to monitor disease progression and subsequent interventions in animal or cellular models of dysferlin deficiency.

Robert Bloch (USA) described the similarities and differences between four different dysferlin-deficient mouse models (A/J, BlaJ, SJL, B10.SJL) and how they respond to a large strain muscle injury (LSI). Baseline analysis of the four strains at 3 months revealed ongoing muscle pathology and measureable weakness in the tibialis anterior of SJL and B10.SJL mice, while the A/J and BlaJ showed no signs of active dystrophy. A time course of histological and functional changes following large strain injury found that all of the lines have increased levels of muscle fiber necrosis and inflammation compared to their respective dysferlin positive controls following LSI. Torque recovery 3 days after injury is reduced in A/J animals, highly variable in BlaJ, and not significantly different than controls in the SJL and B.10SJL. The rapid recovery of SJL and B.10SJL animals may relate to the fact that they are already myopathic at the age tested, and thus primed to recover from the injury. The differences in the response of the animals suggests that A/J mice are especially useful

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for studying acute changes in dysferlinopathic muscle, whereas Bl10.SJL mice are more suited for studying the longer term changes that occur.

Terence Partridge (USA) described a variety of work from his laboratory investigating muscle growth and regeneration. The talk focused on the role of myoblasts/satellite cells in the process of muscle growth, and how that differs from muscle regeneration following injury or disease. By carefully tracking myonuclear number and actin content of individual muscle fibers, he discovered that muscle fiber growth in mice occurs in two very distinct phases – nuclear addition through satellite cell proliferation and fusion early in life, followed by cytoplasmic expansion. The pattern of nuclear addition and cytoplasmic expansion is altered in mdx mice (a mouse model of Duchenne muscular dystrophy), even before the onset of muscle pathology. The second part of the talk discussed challenges in extrapolating the mouse results to humans, where muscle growth is not as well studied, but clearly follows a different pattern, and does not have the same distinct hyperplastic and hypertrophic phases that are observed in mice.

Louis Kunkel (USA) described muscle abnormalities caused by transiently reducing dysferlin expression in zebrafish by morpholino injection. The results suggest that muscle degeneration caused by dysferlin deficiency in zebrafish can be easily monitored, and that a dysferlin null zebrafish model could be useful for screening libraries of compounds in the hope of finding new therapeutics for dysferlin deficiency. He then described progress toward making a line of permanently dysferlin deficient zebrafish using zinc finger technology. These fish can be used for drug screening, based on similar screens in the dystrophin deficient "sapje" zebrafish line.

Isabelle Richard (France) described the analysis methods she uses when studying dysferlin-deficient mice. Histological analysis shows various dystrophic features in many muscles. Notably, the psoas and gluteus muscles tend to show a markedly higher level of pathology than the quadriceps or distal hind limb muscles. She then described a method for assessing the level of muscle fiber necrosis that occurs following an acute injury in dysferlin deficient mice. The method involves injuring the TA muscle with several large strain eccentric contractions, as developed by Dr. Bloch, followed by monitoring the number of necrotic fibers by injecting Evan's blue dye 48 h after the injury, and measuring the numbers of dye positive fibers 24 h later. Using this method, a dramatic increase in Evan's blue positive fibers, relative to injured control animals, is seen, and the increase can be attenuated in mice pre-treated with a dysferlin dual AAV gene therapy strategy. Treadmill running followed by Evan's blue dye administration is also being tried as a method of exacerbating/monitoring muscle pathology in dysferlin deficient mice. Initial results look promising, particularly in forelimb muscles such as the biceps and deltoid, which have large increases in Evan's blue dye uptake compared to wild type animals although the number of necrotic fibers is low.

Joshua Zimmerberg (USA) described ongoing efforts to measure the resealing rate of wounded muscle fibers. Initially some of the flaws of monitoring membrane resealing with FM dyes were discussed, including how the membrane content can influence the dye, and how the light absorption properties may alter membrane wounding with lasers. These issues drove the need to develop new methods for measuring membrane resealing. Initially, the Zimmerberg lab used Ca^{2+} sensitive dyes to monitor Ca^{2+} entry through the wound. This method works, but also has some caveats, including a sample rate in the 10 s of seconds, and difficulties related to monitoring changes in Ca^{2+}, an ion important in many aspects of muscle cell biology. An alternative approach is to use modern electrophysiology techniques to monitor membrane resistance, which should change as a membrane wound reseals. He described several methods that they have pursued toward monitoring membrane resealing using electrophysiology, as well as some of the hurdles they have found due to the unique properties of muscle fibers.

7. Session VI: What is the role of inflammation in dysferlinopathy?

This session addressed the role of the immune system, particularly the inflammatory response to injury, in dysferlinopathy.

Tatiana Cohen (USA) compared the effect of secreted factors from different types of macrophages on myogenesis. Macrophages of different types are generated by different factors activating the precursor cells. M1 macrophages are pro-inflammatory, while M2a are "wound healing" and M2b have a regulatory role. The different populations of macrophages appear in a temporal sequence that helps coordinate the recovery from a muscle injury. Macrophages and myoblasts were co-cultured, to avoid direct contact, but to allow the myogenesis process to be influenced by soluble factors released by the macrophages. The M1 and M2b macrophages inhibited myogenesis of both wild-type and dysferlin-deficient (A/J) myoblasts, while M2a macrophages promoted myogenesis in dysferlin positive and negative myoblasts. Therefore, modulation of macrophage types present in the muscle could be beneficial in dysferlinopathy.

Kanneboyina Nagaraju (USA) discussed the role of toll-like receptors in muscle inflammation. Toll-like receptors (TLR) are located on the cell membrane, and can be activated by tissue damage or microbial invasion. Activation of the TLRs activates the NFkB pathway, leading to activation of inflammatory cells such as macrophages and mast cells. TLR-4 causes IL-1β release in muscle cells, and both SJL and mdx mice have elevated IL-1β. To determine the effect of blocking the TLR pathway, A/J mice were crossed with mice that lack Myd88, a protein important for activation of NFkB following TLR signaling. Results indicated that inhibiting the TLR pathway may be a possible drug target for reducing pathology in dysferlinopathy.

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François-Jérôme Authier (France) discussed the role of complement factors in the activation of the membrane attack (MAC) complex. Cell necrosis by MAC is observed in dysferlin-deficient muscle biopsies. The complement C5b-9 is associated with recent damage in muscle fibers. Intravenous immunoglobulin (IVIg) prevents lysis of myogenic cells in the presence of C5. Using isolated muscle from dysferlin-deficient and wild type animals, he showed that dysferlin-deficient muscle cells are more susceptible to complement attack. Although regulatory proteins of the complement system are reduced in dysferlin deficiency, this is not the cause of the increased susceptibility to complement attack. The inhibiting effect of IVIg on C5 supports studies suggesting that IVIg has a beneficial effect on pathology.

8. Session VII: What is dysferlin's role in membrane repair?

This session focused on the process of membrane repair, and the roles of dysferlin, its interaction partners, and related proteins in this process.

Paul Blank (USA) presented studies comparing laser wounding in dysferlin positive and dysferlin negative muscle fibers. The fibers were labeled with two calcium indicator dyes, Fluo-4-AM and Fura Red AM, whose fluorescence ratio was used to determine the calcium response to wounding. Dysferlin-deficient fibers are repair competent, and do not have a significantly lower damage threshold, but have a greater calcium response compared to wild type. This last finding indicates that calcium handling is altered in dysferlin deficiency.

Renzhi Han (USA) presented studies comparing recovery of tetanic force between dysferlin-deficient and wild type isolated mouse muscles following saponin exposure (50 μg/ml for 5 min). No difference was seen in recovery between the dysferlin-negative and dysferlin-positive muscles. However, a difference was noted between different muscle types, with the soleus muscle (predominantly slow fibers) having better recovery than the extensor digitorum longus (EDL) muscle, which contains mainly fast fibers.

Jyoti Jaiswal (USA) discussed the role of annexin A2 in healing muscle injury. Annexin A2 is a cytoplasmic protein that responds to cell injury by translocating, within seconds after injury, to the cell membrane and to the site of the injury. Annexin A2 interacts with dysferlin and absence of annexin A2 or blocking translocation of annexin A2 to the cell surface in cultured muscle cells result in poor cell membrane repair as well as reduced translocation of dysferlin to the plasma membrane. Annexin A2 expression is high in dysferlinopathic muscles and its level in healthy muscle increases following injury. Mice lacking annexin A2 show reduced muscle strength with no overt inflammation.

Alain Brisson (France) presented studies of annexin-A5's role in membrane repair. Annexin-A5 is the smallest annexin and has the shortest N-terminal segment in the annexin family, with no known binding sites for any annexin-associated protein. Annexin-A5 can form two-dimensional arrays on membrane surfaces containing negatively charged phospholipids in the presence of calcium, with salt bridges joining the molecules together. Cells that lack annexin-A5 show a membrane repair defect, which is restored by adding extracellular wild-type annexin-A5 but not by a mutant annexin-A5 that is unable to form 2D arrays. Annexin-A5 localizes to the area of the cell membrane surrounding a membrane lesion site. It appears that annexin-A5 reinforces the membrane around a lesion, preventing the membrane hole from enlarging [9].

Hua Zhu (USA) presented studies on the role of the caveloae-resident protein PTRF in membrane repair. The muscle repair protein MG53 translocates to sites of injury to enable membrane repair. This repair process is dependent on cholesterol, but MG53 does not interact with cholesterol. MG53 mediated repair is observed in many cell types, but not in HepG2 cells (derived from liver), which do not express PTRF. Restoration of PTRF to HepG2 cells produced MG53-mediated repair. Conversely, knockdown of PTRF in muscle fibers inhibited membrane repair. It appears that PTRF recruits MG53 to the sites of membrane damage by interaction with exposed cholesterol. The repair process can be inhibited by reducing cholesterol, and enhanced by increasing it. In dysferlin-deficient muscle fibers, overexpression of PTFR improves membrane repair, but PTRF overexpression does not improve repair in MG53 deficient fibers. This indicates that MG53, but not dysferlin, is a necessary interaction partner for PTRF to facilitate membrane repair.

Katsuya Miyake (Japan) discussed the role of the F-Actin disassembly factor MICAL in membrane repair. Fluorescently-labeled MICAL, MG53, and dysferlin were expressed in cells subject to laser or mechanical wounding. MICAL accumulates in the vicinity of the wound site before MG53 or dysferlin. After MICAL accumulates, F-actin in the vicinity of the wound site is depolymerized. Both of these processes occur on a second time scale. It is hypothesized that MICAL is needed to depolymerize the cytoskeleton, which forms a physical barrier to the membrane at the wound site. After depolymerization is complete, the membrane repair process involving dysferlin and MG53 can occur.

9. Session VIII: How do we put dysferlin back?

This session discussed methods for restoring expression of the dysferlin protein *in vivo*.

Matt Hirsch (USA) presented a comparison of AAV large gene delivery strategies. He compared split gene and fragment AAV vectors and demonstrated *in vivo* that fragment AAV is more efficient in recombining the dysferlin gene product in mouse muscle, especially at lower doses. He also discussed additional hurdles that must be cleared for gene therapy to be successful, such as efficient muscle transduction, de-targeting liver transduction and development of effective and safe administrative routes (e.g. limb perfusion).

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Eduard Gallardo (Spain) tested the effect of vitamin D3 on the expression of dysferlin protein in muscle and blood monocytes *in vitro*. He demonstrated a dose dependent increase in dysferlin protein levels after treatment of HL60 cells with vitamin D3, as well as an increase in dysferlin protein expression in vitamin D3 treated human monocytes and muscle primary cultures derived from normal and dysferlin mutation carriers.

Dr. Jordi Diaz-Manera (Spain) followed the above work by testing the effect of vitamin D3 on dysferlin expression *in vivo*. He tested people carrying only one dysferlin mutation (i.e. dysferlin carriers) with lower dysferlin protein expression than normal. Twenty-five carriers that met the inclusion/exclusion criteria were included in the trial and divided into a 3:1 ratio of treated vs. untreated. Vitamin D3 was given once per week for 1 year and dysferlin protein levels were evaluated in blood monocytes at various time points throughout the year. He saw a significant increase in dysferlin protein levels in the majority of carriers. Therefore, the use of vitamin D3 treatment in combination with other molecular therapies such as exon skipping, stop codon read through, or gene therapy could increase the efficacy of the molecular strategy by increasing the amount of dysferlin protein produced.

Marisa Karow (USA) discussed her analysis of the use of adipose derived mesenchymal cells (AD-MSC) for stem cell therapy. She showed that while AD-MSCs are easily obtained and transfected, they have a limited lifespan once transfected, which makes clonal expansion impossible. In addition, AD-MSCs were unable to differentiate into myogenic progenitors *in vitro* when co-cultured with C2C12 cells and they did not engraft into injured mouse muscle *in vivo*. Therefore, AD-MSCs were not an appropriate stem cell choice for this cell therapy approach and likely are not a cell type that will work well, if at all, in muscle.

Isabelle Richard (France) presented her data on a dose effect study of a mini-dysferlin construct that can rescue sarcolemmal repair *in vitro*. Expression of the dysferlin minigene at levels greater that endogenous dysferlin resulted in increased central-nucleation in both wildtype and dysferlin deficient (Bla/J) mice. This observation indicates that mini-dysferlin is toxic at high expression levels, much like full length dysferlin, emphasizing the need to carefully control the expression levels of exogenously delivered dysferlin.

Note: Please visit the Jain Foundation website (www.jain-foundation.org) for more details on the 5th Annual Dysferlin Conference proceedings.

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