Neuromuscular Biology and Disease
Histopathology/Pathophysiology overview

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MUSCLE TISSUE PROCESSING & STAINS

• Tissue blocks of skeletal muscle, **frozen** in isopentane cooled in liquid nitrogen. 12 μm thick sections are cut using a cryostat.
• The following **routine** stains are done:
• **Basic histopathological** stains: H & E and Gomori trichrome
• **Special Stains**: oil red O, PAS, Congo red.
• **Enzyme Histochemistry**: NADH, SDH, COX, and ATPase, at pH 9.4, 4.6, 4.2. (Myophosphorylase, MAD, acid phosphatase if needed)
• **Immune staining**: carried out if needed
  – CD3, CD4, CD8, CD20 and CD68 cell markers, MAC
  – dystrophin (dys 1, 2, 3), sarcoglycans (α, β, γ, δ), dystroglycans (α, β), dysferlin, caveolin 3, laminin alpha 2 (merosin), utrophin, spectrin, collagen VI
  – specific antibodies for protein aggregates

• EM piece placed in glutaraldehyde for further processing
• A separate piece of muscle frozen for biochemical/genetic studies
H&E and Gomori Trichrome

Give wide range of information for general pathological reactions:

- Necrosis
- Regeneration
- Fiber size – atrophy/hypertrophy
- Inflammation
- Fibrosis
- Structural changes
- Organelle changes
Pathogenesis of DMD

• 1987 the DMD gene was cloned
  - Opened up new avenues for potential treatment

  The largest gene in human genome — 2.6 m bp
  - a critical obstacle for molecular manipulation

• Encodes a protein with 4 domains:

  24 Spectrin-like repeats + 4-hinges in rod domain
Immune stains: Dystrophinopathies

DMD
Exon 55-63 duplication

BMD
Exon 19-29 duplication

Dys 3
Exon 19-29 duplication

Dys 1
Exon 55-63 duplication

Dys 2
Exon 55-63 duplication

Dys 3
Exon 19-29 duplication

Dys 1
Exon 55-63 duplication

Dys 2
Exon 19-29 duplication
Twelve y/o boy with BMD who has exons 10-44 and promoters Dp260 and Dp240 in-frame deletion
Consequences of Absent Dystrophin

Reduced or absent dystrophin

Mechanically weakened plasma membrane, prone to focal tears during contractile activity

Massive influx of extracellular calcium, activation of proteolytic enzymes

Segmental necrosis in muscle cell
Landmark Article:
Duchenne dystrophy: Electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber
Bahram Mokri, MD; and Andrew G. Engel, MD
Neurology 1975;25:1111-1120
These findings suggested that the membrane lesions were an ineffective barrier to the ingress of extracellular fluid, and we confirmed this by demonstrating the focal penetration of peroxidase-labeled extracellular fluid into the “delta lesions.”

“We concluded our report by stating that “... if the cause of the structural defects resided in the membrane itself (as it very well might), it could be caused by an abnormal lipid component or by a defective structural protein in the membrane. Further studies directed at the molecular architecture of the muscle fiber plasma membrane will clarify these questions.”
Intracellular calcium accumulation in Duchenne dystrophy and other myopathies: A study of 67,000 muscle fibers in 114 biopsies

JOHN B. BODENSTEINER, M.D., and ANDREW G. ENGEL, M.D.
NEUROLOGY 28:439-446, May 1978

EM studies have shown plasmalemmal defects in a proportion of non-necrotic muscle fibers in DMD, suggesting that intracellular Ca++ overloading may be an important mechanism of muscle fiber degeneration. To investigate this the localization of calcium with the von Kossa method, with alizarin red, and with GBHA were done in serial, fresh-frozen sections from biopsy specimens

- In DMD, non-necrotic calcium-positive fibers occurred with a mean frequency of 4.83 %. For all other groups, the corresponding value was 0.57 % [0.21 % in normal to 1.76 % in scleroderma, p < 0.001].
- 43% of the fibers were calcium-positive in DMD, whereas calcium-positive large-dark fibers were extremely rare in the other cases.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Cases</th>
<th>Fibers analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duchenne dystrophy</td>
<td>24</td>
<td>122,348</td>
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<tr>
<td>Duchenne dystrophy carrier</td>
<td>2</td>
<td>9,861</td>
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<tr>
<td>Becker dystrophy</td>
<td>2</td>
<td>8,114</td>
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<tr>
<td>Myotonic dystrophy</td>
<td>18</td>
<td>50,456</td>
</tr>
<tr>
<td>Limb-girdle dystrophy</td>
<td>4</td>
<td>20,458</td>
</tr>
<tr>
<td>Ocular-limb muscle dystrophy</td>
<td>2</td>
<td>1,537</td>
</tr>
<tr>
<td>Facioscapulohumeral dystrophy</td>
<td>1</td>
<td>10,859</td>
</tr>
<tr>
<td>Polymyositis</td>
<td>32</td>
<td>160,239</td>
</tr>
<tr>
<td>Dermatomyositis</td>
<td>8</td>
<td>78,217</td>
</tr>
<tr>
<td>Unclassified collagen vascular disease</td>
<td>5</td>
<td>28,907</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>2</td>
<td>7,424</td>
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<tr>
<td>Phosphorylase deficiency</td>
<td>3</td>
<td>11,682</td>
</tr>
<tr>
<td>Normal controls</td>
<td>11</td>
<td>57,451</td>
</tr>
<tr>
<td>Total</td>
<td>114</td>
<td>567,553</td>
</tr>
</tbody>
</table>
Relevance of genetic animal models of muscular dystrophy to human muscular dystrophies.
Mendell JR, Higgins R, Sahenk Z, Cosmos E.

**Syrian Hamster model**
(δ-sarcoglycan-deficient)

HRP-leak into delta lesions in 1 µm Thick sections

Calcium Stains

Spectrum of changes in the soleus muscle; trichrome
Diffuse alizarin red staining for calcium

“...the percentage and the pattern of leakage of both calcium and HRP suggested that the dystrophic process in the mouse may more closely simulate a kind of neuromuscular disease(s) other than Duchenne human dystrophy.”

Extensive HRP leakage across the entire cross-sectional diameter of the fibers

Dystrophica Muscularis mouse model (dy/dy and dy^2J/dy^2J) (genetic defects in laminin α2-chain)
Animal models for muscular dystrophy show different patterns of sarcolemmal disruption. Straub V, Rafael JA, Chamberlain JS, Campbell KP

- Loss of Sarcolemmal Integrity in the \textit{Mdx} Mice
- Negligible membrane defect in \textit{dy/dy} mice

Uptake of EBD into hind leg muscles 6 h after injection
Duchenne Muscular Dystrophy: Functional Ischemia Reproduces Its Characteristic Lesions
Author(s): J. R. Mendell, W. King Engel and E. C. Derrer

Abstract. The highly characteristic early and midstage histological lesions of Duchenne dystrophy were reproduced experimentally in the rat by the combination of a vascular abnormality, aortic ligation, which does not affect the structure of the intramuscular blood vessels, and the humoral vasoactive substance 5-hydroxytryptamine. Neither ligation nor injection of 5-hydroxytryptamine alone causes changes in the muscle fibers. This result establishes the possibility of a similar combined mechanism for a nonstructural ischemia pathogenesis in Duchenne dystrophy. The proposed pathogenesis is contrary to the generally held idea that the cause is an intrinsic abnormality of muscle fiber metabolism.
The role of different isoforms of nNOS, namely nNOSμ and nNOSβ in skeletal muscle, is illustrated. Two binding sites of nNOSμ can be seen: at the dystrophin rod domain and at α-syntrophin (a member of dystrophin-glycoprotein complex). NO derived from nNOSμ enhances blood flow to muscle during activity by preventing vasoconstriction of nearby blood vessels during muscle contraction; the increased blood flow is a source of oxygen supply to the muscle during exercise. In contrast, nNOSβ signaling at the Golgi complex regulates force generation during and after exercise generating cGMP dependent protein kinase G (PKG). (Adapted with permission of the American Society for Clinical Investigation from Percival et al. 2010 [117]). Abbreviations: nNOS- nitric oxide synthase; BV-blood vessel; aDG-alpha-dystroglycan; b-DG- beta-dystroglycan; NO- nitric oxide; Syn–syntrophin; GTP- guanosine triphosphate; sGC- soluble guanylyl cyclase; cGMP- cyclic guanosine monophosphate.

Necrosis

Factors triggering necrosis in muscle cells:

- Lengthening contractions
  - dystrophic muscle particularly vulnerable
- Ischemia
  - dermatomyositis
- Energy deprivation
  - Glycolytic defects
- Toxic agents
  - Cardiotoxin, neutoxin, statins

In the course of necrosis:

- Plasma membrane becomes permeable
  -- Ca$^{++}$ entry, activation of phospholipases, proteases (calpains)
- Some DAG complex- lost early; by 24 hrs dys lost
- Activation of compliment cascade, diffuse cytoplasmic appearance of lytic C5-9 (MAC) within muscle

**Segmental Necrosis**
Phagocytosis

- Starts ~ 6 to 8 hrs after the fiber passed the “point of no return”
  - sarcolemmal and myonuclear dissolution (earliest change), followed by gradual dissolution of contractile elements
  - what is not destroyed: Basal Lamina & Satellite Cells

- In surviving stumps - T tubule dilatation
- Abundant macrophages within endomysium

Acid phosphatase
Temporal sequence of inflammatory and regenerative events following muscle injury:

- **Neutrophils**
- **Inflammatory macrophages CD68+**
- **Anti-inflammatory macrophages CD163+**
- **Satellite cells activation, proliferation and fusion**
- **Embryonic MyHC**
- **NMJ formation**
- **Adult fast MyHC**
- **Slow MyHC**

Myofiber growth and embryonic MyHC expression in regenerating skeletal muscle

**Satellite Cells**

- Muscle specific stem cells located beneath the basal lamina of the myofiber
- Pax7, N-CAM, m-cadherin, CD34 useful marker for quiescent SCs
- Prevalence = r S/M
- Major role in
  - Natural growth
  - Muscle maintenance, work hypertrophy
  - Regeneration
- Proliferative/differentiating processes lead transformation into myoblast/myotubes in necrotic segments
- Limit of their mitotic cycles?

Model for satellite cell self-renewal and differentiation

Activated satellite cells in necrotic fibers
IDEAL MUSCLE FIBER REGENERATION

1. Surviving segment → Necrotic segment → Surviving segment
   - Satellite cell
   - Macrophage
   - Myoblast (activated satellite cells)
   - Basal lamina
   - Plasma membrane

2. Regenerating Myotubes

3. Full restoration of the normal fibre calibre

Karpati, G; 2008
Histological Features of Regenerating Muscle

• Basophilic cytoplasm, reflecting high content of ribosomes
• Nuclei tend to be pale and large
• Relative excess of glycogen and mitochondria (early)
• Emb & Neo forms of myCH
• Diffuse cytoplasmic desmin stain

Satellite cell
Desmin IF

COX
Muscle Fiber Regeneration
ABERRATIONS OF MUSCLE FIBER REGENERATION

1. Regenerated segment is of smaller caliber than the rest of the fiber

2. Forked fibers due to incomplete lateral fusion of myotubes

3. Multiple independent fibers due to lack of fusion of myotubes with the surviving stump

4. Empty basement membrane sleeve due to lack of regeneration
Histopathological spectrum of dystrophic process

Early stage
Prominent necrosis/regeneration, inflammation
Minimal fibrosis

Late stage
Prominent fibrosis
Minimal necrosis/regeneration, inflammation

• Endomysial fibrosis is an end-stage consequence of muscle fiber loss
  - often explained by a putative failure of muscle fiber regeneration that requires a stepwise process: activation, of SCs with proliferation & differentiation followed by lateral fusion of myotubes with each other and with surviving stumps

• Muscle-specific miRs play important posttranscriptional regulatory roles in this process
  - miR-1, miR-206 facilitate SC differentiation
  - down-regulation or inhibition of miR-1, miR-206 enhances SC proliferation and increases Pax7 protein levels in vivo
microRNA-206 promotes skeletal muscle regeneration and delays progression of Duchenne muscular dystrophy in mice.
Liu N, Williams AH, Maxeiner JM, Bezprozvannaya S, Shelton JM, Richardson JA, Bassel-Duby R, Olson EN.

- Genetic deletion of miR-206 in mice substantially delayed regeneration induced by cardiotoxin injury.

- Loss of miR-206 accelerated and exacerbated the dystrophic phenotype in a mouse model of Duchenne muscular dystrophy.

- miR-206 acts to promote satellite cell differentiation and fusion into muscle fibers through suppressing a collection of negative regulators of myogenesis.

- These findings reveal an essential role for miR-206 in satellite cell differentiation during skeletal muscle regeneration and indicate that miR-206 slows progression of Duchenne muscular dystrophy.
LGMD2A:
- caused by mutations in the \textit{CAPN3}, encoding \textit{Ca}^{2+} - activated cysteine protease
- role in sarcomere assembly, turnover and maintenance
- in Calpainopathy there is a good correlation between age, duration of symptoms and degree of fibrosis
- microRNA dysregulation leads to inability of Pax7-positive SCs to transit from proliferation to differentiation resulting in impaired regeneration and fibrosis in LGMD 2A

\textit{Rosales et al., Muscle & Nerve, 2013}
Satellite Cells in Dystrophic Process (calpainopathy)

A

<table>
<thead>
<tr>
<th>Biopsies</th>
<th>n</th>
<th>Age</th>
<th>DD</th>
<th>FG</th>
<th>SC/type1</th>
<th>SC/type2</th>
<th>SC/fiber</th>
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</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>1</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>0.117</td>
<td>0.196</td>
<td>0.147</td>
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<tr>
<td>Group 2</td>
<td>3</td>
<td>19.7 ± 2.7</td>
<td>7.7 ± 3.3</td>
<td>1 ± 0.0</td>
<td>0.134 ± 0.032</td>
<td>0.210 ± 0.076</td>
<td>0.168 ± 0.051</td>
</tr>
<tr>
<td>Group 3</td>
<td>9</td>
<td>37.8 ± 4.8</td>
<td>19.7 ± 3.6</td>
<td>3.1 ± 0.2</td>
<td>0.189 ± 0.054</td>
<td>0.298 ± 0.087</td>
<td>0.205 ± 0.052</td>
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<tr>
<td>Control</td>
<td>3</td>
<td>45.7 ± 5.0</td>
<td></td>
<td></td>
<td>0.081 ± 0.001</td>
<td>0.056 ± 0.010</td>
<td>0.065 ± 0.006</td>
</tr>
</tbody>
</table>

B

Satellite Cell Number in Calpainopathy Muscle

C

Satellite Cell Number in Lobulated and non-Lobulated Biopsies

D

E

a

b

II

c

II

b

c
Expression Levels of miR-1, miR-133a and miR-206 in Muscle of LGMD2A
Elevated satellite cell number in Duchenne muscular dystrophy
Michael Kottlors & Janbernd Kirschner
Cell Tissue Research, 2010

Pax7-positive nuclei number per muscle cell of DMD and control muscle at the age from 2 to 9 years showing the higher number of Pax7-positive nuclei in DMD muscle in comparison with control muscle.

Pax7-positive nuclei number per muscle cell of DMD and control muscle plotted against desmin-positive area showing the increased Pax7-positive nuclei numbers in DMD, despite decreasing desmin-positive area (increasing fibrosis).

- Pax7-positive SC number is elevated in DMD in comparison to controls, even in advanced stage of dystrophy
- The expression of myogenin is not correlated with fibrosis or age suggesting variable factors influencing the differentiation of satellite cells
DMD Pathophysiology Overview

Jill A. Rafael-Fortney, Ph.D.
Associate Professor
Dept. Molecular and Cellular Biochemistry
(Physiology & Cell Biology, Cardiology)
Skeletal muscle pathology

- Muscle weakness
- Myofiber degeneration; regeneration; focal inflammation
- Regenerated muscle fibers display a large variation in diameter and have centrally located nuclei
- Gradual replacement of fibers with adipose and connective tissue (fibrosis)

18 month-old DMD biopsy

late stage DMD

(L. Zhou; neuromuscular.wustl.edu)
Dystrophin and the muscle membrane

- Dystrophin
- Sarcoglycans
- DGC
- Integrins
- Sarcospan
- F-actin
- α7, β1
- Dystroglycans
- Syntrophins/dystrobrevins/nNOS
Dystrophin and the muscle membrane

Integrins

basal lamina

Dystroglycans

Sarcoglycans

Sarcospan

Syntrophins/dystrobrevins/nNOS

DGC

F-actin

Dystrophin

Class 4
DMD and the muscle membrane

Integrins

basal lamina

Membrane damage

Loss of actin-ECM connection

F-actin

Free radicals

Ca^{2+}

nNOS

Activation of proteases
Mitochondrial dysfunction
Downstream signaling changes

(Williams, J.Physiol, 1993)
Mouse models

**mdx**
Dystrophin-deficient
- normal mouse lifespan (2 yrs)
- mild skeletal muscle fibrosis
- mild cardiomyopathy

**Het**
Dystrophin-deficient; missing 1 copy of utrophin
- normal mouse lifespan (2 yrs)
- severe skeletal muscle fibrosis
- Cardiomyopathy progression more similar to DMD patients

**dko**
Dystrophin/utrophin-deficient
- Dies 10-12 weeks-of-age
- mild skeletal muscle fibrosis
- severe cardiomyopathy
Dystrophic histopathology

10 week-old *mdx* diaphragm

24 month-old *mdx* diaphragm

Membrane damage

Class 8

Inflammation

Class 10

Regeneration

Class 12

Fibrosis
DMD and utrophin/dystrophin-deficient mouse hearts show the same pattern of scarring with NORMAL ejection fraction.

(Delfin, NMD, 2012)
Dystrophic hearts show common indicators of heart failure

- Step 1: Cardiac contractile dysfunction / reduced β-adrenergic response
- Step 2: Cardiomyocyte damage
- Step 3: Unregulated MMP remodeling
- Step 4: Collagen scarring

**ALL PRESENT WHILE NORMAL EJECTION FRACTION IS PRESERVED**
Understanding the pathogenesis provides therapeutic opportunities…
Dystrophin Replacement

- Dystrophin gene therapy
- Exon skipping
- Stop codon readthrough

Class 13
Upregulation of compensatory linkages

- Upregulation of utrophin
Upregulation of compensatory linkages

- Upregulation of biglycan
Upregulation of compensatory linkages

- Upregulation of integrins / laminins
Pathophysiology and therapeutic approaches

Integrins

F-actin

basal lamina

Loss of actin-ECM connection

Membrane damage

Membrane stabilization

Inflammation

Anti-inflammatory

Class 9

Muscle growth
Stimulate regeneration
Improve muscle differentiation

Class 10

Class 12

Antifibrotics

Fibrosis

Class 11

Glucocorticoids – clinically delays disease progression with side-effects
- NFκB inhibition - effects on inflammatory cells and muscle
Pathophysiology and therapeutic approaches

- Basal lamina
- F-actin
- Integrins (α7β1)
- Calcium handling
  - Activation of proteases
  - Mitochondrial dysfunction
  - Signaling pathway changes
- Oxidative stress
- Protease inhibitors
- Class 11: Free radicals, Ca^{2+}

(Class 11)
DMD and the muscle membrane

Integrins

α7 β1

basal lamina

F-actin

nNOS

Multifactorial effects on:
Microvasculature
Signaling / muscle strength?

PDE5 inhibitors

Class 11
Translation to DMD patients

- Vast majority of pathophysiology is from animal models
  - Histopathology verified in patients
- Improvements in which parameters in mice translate to clinical improvements in longevity or quality of life?
- What percentage improvement in mice is therapeutic for patients?
  - Statistically significant improvements are often small
- Need for useful patient biomarkers
Questions?
DMD and utrophin/dystrophin-deficient mouse hearts show the same pattern of scarring with NORMAL ejection fraction.