11. The modern approach to the histopathological diagnosis of muscle diseases

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Abstract. Muscle biopsy is an essential tool in diagnosis of neuromuscular disorders. It can be used for histochemical, immunohistochemical and biochemical tests as well as for genetic studies. Immunohistochemistry has a central role in the evaluation of muscle biopsies and pathological diagnosis of muscular disorders. In order to complement diagnostic procedures obtained by immunohistochemistry with molecular methods, muscle biopsy may be used as a source for RNA and protein isolation. A practical method for the interpretation of muscle biopsies, following so-called algorithm “inside out approach”, involves certain order of procedures in analysis of muscle fibres, muscle connective tissue and blood vessels. Multidisciplinary approach encompassing a variety of techniques is required in order to successfully complete diagnosis of muscular disorders as the most important information for patients and their families.

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Introduction

The assessment of muscle biopsy is an essential component in the diagnosis of neuromuscular disorders. With occasional exceptions, it is an essential element in the assessment of a patient with suspected myopathy (1). Pathological methods applied during the processing of muscle biopsy samples determine quality of the interpretation of pathological findings. Formerly were with limited number of techniques, but the advent of histochemistry, as well as identification of novel protein markers associated with skeletal muscle diseases (especially among sarcomeric proteins) (2-4) and molecular defects has led to the increasing role of immunohistochemistry in routine diagnostics of human and animal neuromuscular disorders (5).

The diagnosis of most neuromuscular diseases rests on careful clinical evaluation of the patient, electromyography, the muscle biopsy, and in some instances, molecular genetic studies. Muscle biopsy, associated with histochemical and immunohistological techniques, plays a key role in diagnosis of many neuromuscular disorders. Moreover, muscle biopsy represents a starting material for genetic studies and enables basic scientists to perform biomedical research. Regular clinical-pathological muscle review meetings are essential for feedback, learning, and diagnosis refinement (6,7).

Routine treatment of muscle biopsy samples

Analysis of fresh sample of muscle tissue is the most important for histopathological diagnosis. After the sample is removed, it should be processed within 15–20 min. Delay of up to 2 h has minimal effect on light microscopy studies, but ultrastructural artifacts may be visible and the results of biochemical studies, such as the activity of respiratory chain enzymes, will be affected (8). Direct immersion of the specimen into liquid nitrogen enables some gaseous nitrogen to surround the specimen and slows the cooling process. More rapid freezing, better preservation of structure, as well as avoiding of ice crystal artifacts are achieved by freezing tissue in isopentane or propane cooled in liquid nitrogen (to −160°C) followed by storage at -80°C or in liquid nitrogen (9).

All histochemical, and most immunohistochemical studies are performed on frozen sections. A suitable section thickness for histology and histochemistry is 8–10 μm, cut in a cryostat at −23 to −25°C. Fixation and paraffin embedding distort fiber architecture and affect enzyme activity, making metabolic histological studies impossible on such material. Some antibodies for immunohistochemistry recognize epitopes in fixed material, but a full panel of tests is not usually possible. Fixation is necessary for
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electron microscopy (EM). Insights into the basic architecture of the muscle
tissue are achieved by good transverse orientation of muscle sample under a
dissecting microscope (8,9).

The shape of the muscle fibers, their size, the presence or absence of
clustering fibers, proliferation of connective tissue and fat, the presence or
absence of necrosis and inflammatory components are the first parameters to be
estimated. In normal muscle, fibers are polygonal in shape and closely packed
with very little endomysial connective tissue between them. Fiber sizes of
normal muscle show a healthy distribution and in adult males range from
approximately 40 to 80 μm, while in adult females from approximately 30 to
70 μm. Most nuclei in normal muscle are subsarcolemmal and some nuclei near
the periphery of fibers are related to capillaries which lie in slight indentations. In
normal muscle at least one capillary is seen adjacent to each fiber. Necrosis and
inflammatory infiltrates are not found in normal muscle (8,9). There are
excellent reviews available in many myology and neuromuscular pathology
textbooks for more comprehensive and detailed information on individual stains
and technique used in muscle pathology (8-10).

Here, we will briefly describe a range of basic histochemical nonenzyme
staining techniques, routinely applied in the practice (Table 1) (8-11). The
first step in the analysis of muscle biopsies is to determine the gross
structural abnormalities. This can be done by hematoxylin and eosin (H&E)
staining in order to reveal the overall structure of all components of the
tissue (8-11). A well-stained H&E section often provides the key diagnostic
information (12,13).

The Gomori trichrome technique (GMT), as modified by Engel and
Cunningham (14), is a capricious stain that reveals similar features as H&E,
but better identifies intermyofibrillar mitochondria seen as fine red dots
throughout the fiber. The higher number of mitochondria is present in type 1
fibers (stained darker) (8,9). GMT is very important in the identification of
rods, structures stained in red (15), membranous myelin-like whorls of
rimmed vacuoles (16,17) and abnormal proliferation of mitochondria (the so
called “Ragged Red Fibers”) (18).

The periodic acid–Schiff technique (PAS) reveals the glycogen content
of fibers. The finding is confirmed by α-amylase digestion (DPAS) of the
glycogen. Only the periphery of each fiber and the capillaries are seen unless
polyglucosan material, resistant to digestion, is present (9).

Enzyme histochemistry (Table 1) has become firmly established as a
link between the morphology and biochemistry of tissues (19). Enzyme
histochemical techniques are essential for revealing the different types of
fibers, structural defects (distribution of mitochondria; abnormal
mitochondria, myofibrillar disruption, cores) and absence of an enzyme.
The skeletal muscle is composed of two main fiber types (type 1 and 2) and the type 2 fibers are further divided into 2A and 2B. The rapid development of immunohistochemical techniques in the field of neuromuscular biopsy in the last ten years has enabled immunohistochemical detection of type of fiber on paraffin or frozen sections (20,21). For example, antibody to skeletal muscle isoform 3 of Z disk protein alpha actinin (alpha-actinin-3) may be used to label fast twitch fibers (22).

**Table 1.** Essential histochemical and enzymohistochemical staining techniques used for muscle biopsy.

<table>
<thead>
<tr>
<th>Histochemical staining techniques</th>
<th>Significance</th>
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<tbody>
<tr>
<td>Haematoxylin and eosin (H&amp;E)</td>
<td>Revealing the overall structure of all components of the tissue</td>
</tr>
<tr>
<td>Gomori trichrome (GMT)</td>
<td>Identify intermyofibrillar mitochondria</td>
</tr>
<tr>
<td>Periodic acid-Schiff</td>
<td>Reveals the glycogen content of fibers</td>
</tr>
<tr>
<td>Verhoeff–van Gieson</td>
<td>Highlights connective tissue and elastin, important for determining the amount of connective tissue</td>
</tr>
<tr>
<td>Oil red and Sudan black</td>
<td>Reveals neutral lipid content as small droplets in the fibers</td>
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</table>

<table>
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<tr>
<th>Enzymohistochemical staining techniques</th>
<th>Significance (8,9,11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced nicotinamide adenine dinucleotidetetrazolium reductase (NADH-TR)</td>
<td>Useful to study the intermiofibrillar network comprising mitochondria and sarcoplasmic reticulum.</td>
</tr>
<tr>
<td>Succinate dehydrogenase (SDH)</td>
<td>Purely mitochondrial enzyme reveals fiber type pattern, distribution of mitochondria</td>
</tr>
<tr>
<td>Cytochrome c oxidase (COX)</td>
<td>Purely mitochondrial enzyme reveals fiber pattern, fibers devoid of activity</td>
</tr>
<tr>
<td>Phosphorilase</td>
<td>Absent in type V glycogenosis</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>High in lysosomal storage disease and vacuolar myopathy</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>High in blood vessels in some inflammatory myopathies</td>
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</table>
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Immunohistochemical labeling of muscle biopsies, as well as, other biopsies with antibodies to specific proteins has an essential role in the pathological diagnosis of disorders. It is very informative in the evaluation of muscle biopsies and in examining protein localization or protein levels (absence, reduction or accumulation) (23). Results obtained with immunohistochemical technique reflect localization of a protein (8-11). In order to measure their relative amounts, present in different samples, Western blot technique can be used. Automated immunohistochemistry reduces workload and day-to-day variability in staining (13). There are numerous mono and polyclonal antibodies commercially available that work on human specimens as well as in several other animal species, and numerous detection systems (3,11). If using secondary antibodies labeled with fluorescent dyes, fluorescent microscope must be used for evaluation of results. Nowadays, some modern laboratories possess confocal microscopes which enables simultaneous detection of several molecular markers due to their labeling with different fluorophores. The major benefit of confocal imaging is a dramatically increased contrast by removal of out-of-focus haze. This is achieved by scanning single focused point of laser illumination (spot or slit) across a sample in the X, Y and Z directions. Signals pass back to a detector through a pinhole aperture, which blocks light from other regions of the specimen. Confocal microscopy is also used for time-lapse imaging when dynamic events in cells are investigated.

Here, we will focus on the use of proteins and RNA, isolated from muscle biopsies, in routine diagnostics of muscular disorders since their analysis requires a sample of the tissue expressing the target biomolecules. Since most of the diagnostic proteins are only expressed in muscle, skeletal muscle biopsy has great importance when diagnostic protein analysis has to be performed. Nowadays, the range of gene products to analyze is increasing, making a muscle biopsy an instrumental tool for the diagnostic protocol (24).

The analysis of protein expression is established as an essential link between underlying genotype and clinical manifestations. It may aid i) accurate diagnosis (to indicate the type of inheritance or start point for searching of gene mutations), ii) prognosis (to indicate type of mutation), iii) location of functionally important regions targeted by mutations (missense mutations associated with a pathological phenotype or in-frame deletions with a mild clinical phenotype). The benefits of protein analysis in diagnostic procedures are numerous, such as improvement of genetic counseling, efficiency of mutation analysis and demonstration of disruption or maintenance of protein synthesis.

Muscle biopsy, as well as cultured primary muscle cells, is a valuable sources for isolation of RNA. The main drawback if RNA is extracted from
frozen muscle tissue is typically small yields of total RNA due to the impaired complete tissue homogenization caused by the presence of connective tissue between muscle fibers. The problem could be solved by using freeze-dried samples stored at −20°C, since freeze drying facilitates RNA isolation from tissues difficult to homogenize (25). Both yield and RNA stability are shown to increase, while homogenization step is facilitated. RNA samples should be stored at −80 °C until required and subsequently used for common gene expression techniques, such as quantitative reverse transcription-PCR (qRT-PCR).

Western blot analysis is used in cell and molecular biology, in order to identify specific proteins from a complex mixture of proteins extracted from cells or tissue. Three types of information about the protein component of a sample mixture could be provided: is it present, what is its size and approximately how much of it is present. The technique is performed in three steps: (1) separation of proteins by size, by SDS polyacrilamide gel electrophoresis (SDS-PAGE), (2) transfer of proteins to a solid support (nitrocellulose or PVDF membranes), and (3) detection of target protein using a specific primary and secondary antibody for visualization. Western blotting is often used for differential diagnosis of muscular dystrophies and other muscle disorders where the disease gene is known. Immunoblot protocols often require the solubilization of a quite large portion of a muscle biopsy (20-100 mg). Cooper and authors have reported a significant improvement of Western blot methodology using only a single biopsy cryosection (8μm tick, 10mm²) as a source for sample preparation (26).

When loading protein extracts on the gel, it is advisable to include a loading control such as myosin heavy chain as indication of how much “muscle” protein is present in each sample, since affected muscles may be fibrotic or contain fat. Usually, only one protein is visualized in a single lane, but there is also a possibility to perform multiple Western blot technique to detect several proteins simultaneously (27).

The intensity of the band after visualization corresponds to the amount of detected protein. The values cannot be expressed in absolute units, but in relation to normal control samples run on the same blot. Processed blots could be scanned and analyzed with, for example, freely available ImageJ software (http://rsbweb.nih.gov).

Cultured primary muscle cells, derived from donor satellite cells (stimulated to re-enter the cell cycle, proliferate and finally terminally differentiate), are used to study muscle cell structure and function in a wide variety of physiological and pathophysiological states. They are an important tool for investigation of the effects of myopathies on skeletal muscle function (28).
Muscle biopsy interpretation

There is a practical approach in the interpretation of muscle biopsies (8,9). During the analysis of altered muscle pathology specific algorithm is applied. It is convenient to analyze biopsy employing the so-called “inside out approach” method (13), in which certain order of procedures is followed: i) muscle fibres, ii) endomysium, perimysium and epimysium and lastly iii) the blood vessels. In order to evaluate abnormalities, structure and appearance of muscle fibres are analyzed following the order listed in Table 2.

Myopathic disorders are those that affect primarily the muscle while neurogenic disorders are caused by damage or disease of the motor neurons or peripheral nerves that innervate skeletal muscle and therefore secondarily affect the muscle. Myopathic lesions are characterized by changes in muscle fibers and their variability is determined by pathological process.

Sewry and authors have presented a simple and practical scheme where muscle fiber components, together with its nerve supply and motor endplate are referred to the disorders that involve particular organelles and structures (the scheme practically represents the organizational plan of the book) (8). Accordingly, architectural and structural changes in diseased muscle are result of many possible defects in proteins of the sarcomere, proteins of the Z-disk, intermediate filaments proteins, mitochondrial structure, sarcoplasmic reticulum and T-tubules structures and cytoplasmic proteins.

Table 2. Structures analyzed during the application of “inside out approach” method.

<table>
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<tr>
<th>I</th>
<th>Muscle fibres</th>
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<td>1.</td>
<td>Changes in fiber shape and size</td>
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<td>2.</td>
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<td>4.</td>
<td>Degeneration and regeneration</td>
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<td>5.</td>
<td>Architectural and structural changes (e.g. rods, cores, abnormal mitochondria, tubular aggregates, vacuoles, accumulation of storage product, e.g. glycogen and intracellular lipid)</td>
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<tr>
<th>II</th>
<th>Endomysium, Perimysium and Epimysium</th>
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<tbody>
<tr>
<td>1.</td>
<td>Fibrosis and adipose tissue</td>
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<td>2.</td>
<td>Cellular inflammatory reactions</td>
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<tr>
<th>III</th>
<th>Blood Vessels</th>
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I. Muscle fibers

1. Changes in fiber shape and size

For the assessment of fibre size, care must be taken to ensure that the biopsy is orientated transversely (8,13). The size and shape of muscle fibers are an important clue to diagnose neuromuscular diseases, since it has been shown that morphometric data can discern very early changes in the distribution pattern of fiber size in muscle biopsy samples (9). In a case of abnormal size, muscle fibers can be small or large. Two distinct populations of small and large fibres are characteristic of denervation or type-specific atrophy, or a congenital myopathy. When the changes are random and diffuse they support myopathy or dystrophy. In myopathic/dystrophic disorders, atrophic and hypertrophic fibers are randomly distributed throughout the sample, in contrast to the grouping seen in neurogenic disorders. It is important to distinguish fiber type predominance from fiber type grouping. If one fiber type is predominant, those of the other type, although they may be few in number, are usually randomly distributed and not grouped, as in denervation. Presence of small angulated fibres and small or large groups of atrophic fibers (Figure 1) are sure signs of denervation, while the presence of perifascicular atrophic fibers is a sign of dermatomyositis in some, but not all, cases. Severely atrophic fibers may be seen as clumps of nuclei and they can occur in long-standing (end stage) neurogenic or myopathic conditions.

Figure 1. Group atrophy of fast fibers in a case of denervation (anti fast myosin x 10).
2. Changes in fiber type patterns

In normal muscle there is a checkerboard, mosaic pattern of type 1 (slow twitch) and type 2 (fast twitch) fibers. It is important to identify the predominance or atrophy/hypotrophy status of a specific fiber type as sign of pathological changes. In neurogenic disorders the groups of atrophic fibres contain both types, while the groups of hypertrophic fibres contain only one type of fibers. Atrophy of type 2 fibers is a nonspecific myopathic finding. When type 2 subtypes are considered, both 2A and 2B may be affected but specific involvement of type 2B fibres is the most common. Selective atrophy of type 1 fibres occurs in several congenital myopathies and myotonic dystrophy. Predominance of type 1 fibers is a common myopathic feature, but since fiber type proportions vary between muscles, it is important to know which muscle has been sampled. Predominance of type 2 fibers is less common but occurs following spinal cord injury and in motor neuron diseases.

3. Changes of muscle nuclei

Muscle fibers are multinucleated syncytial cells and in normal muscle fibers nuclei reside beneath the sarcolemma. Muscle nuclei may have modified position and/or appearance in diseased muscle. Changes in nuclear positioning result from defects in the proteins of specialized complex which mediate interaction between nuclear envelope and nuclear matrix. Nuclei displaced from normal position are a common feature of pathological muscle. They are particularly prominent in some disorders including those caused by defects in genes encoding proteins of the nuclear envelope. It is often stated that more than 3% of internal nuclei in transverse section is abnormal but this is probably an overestimate in pediatric muscle, where even a few internal nuclei probably reflect abnormality. Nuclei in the center of fibers are the pathological hallmark of disorders collectively known as centronuclear myopathies (29). Internal nuclei are often seen along the fibrous septa in split fibres. These nuclei occur in small groups, point towards severe atrophy and are commonly seen in long-standing denervation.

A numerous internal nuclei are special characteristic of muscle diseases with DNA expansions. Most mutations that cause disease affect the coding region of gene, but some muscle disorders result from an unstable expansion of a repeated sequence such as two forms of myotonic dystrophy (DM1 and DM2) and oculopharyngeal muscular dystrophy. DM1 is caused by the expansion of a CTG triplet in the 3’ untranslated region of the DM protein kinase gene on 19q13, while DM2 results from a tetrancleotide repeat
expansion of CCTG segment in the first intron of the zinc finger 9 (ZNF 9) gene on 3q21 (30,31). Molecular techniques for detecting these rearrangements are highly reliable so muscle pathology has a less important role in diagnosis, particularly of DM1, one of the most common inherited disorders of muscle. However, clinical features of DM2 may be less obvious and muscle pathology is then useful.

4. Degeneration and regeneration

Similarly to other tissues, muscle undergoes ischemic injury, necrosis, fibrosis, and atrophy, but it is also able to regenerate. Vesicular nucleus is referred to the state when nucleus becomes swollen and rounded, the nucleoplasm transparent, and the nucleolus very prominent. As their number increase, it is more likely that myopathy will be diagnosed. These vesicular nuclei are frequently associated with basophilic fibres, which are thought to be the evidence of regeneration (Figure 2). In normal interphase nucleus chromatin material is usually finely dispersed, but when it becomes granular and clumped we describe it as tigroid nucleus. Their presence is usually associated with neuropathies rather than myopathies, and they have also been noted in myotonic dystrophy. Necrotic nuclei may become darker upon staining and shrunken or pyknotic.

Necrotic myofibers are swollen; their cytoplasm is paler than the surrounding viable myofibers and has a homogenized appearance without striations (9). Macrophages could be found around necrotic myofibers, removing

Figure 2. Fiber necrosis, infiltration of macrophages and regenerating fibers (arrows) (HE x 20).
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cellular debris. Necrosis may be segmental and affects only part of a fiber. Damaged fibers, prior to necrosis and invasion by macrophages, may lose glycogen and appear as white fibers upon the PAS staining (8), in contrast to the variable pink color of the other fibers. Following necrosis, satellite cells are activated and give rise to regenerating fibers which are, in the early stages of regeneration, basophilic.

5. Architectural and structural changes

Architectural and structural changes arise from the defects in many proteins of the sarcomere, proteins of the Z-disk, intermediate filaments proteins, mitochondrial structure, sarcoplasmic reticulum and T-tubules structures and cytoplasmic proteins (8,9).

The presence of nemaline rods points to nemaline myopathies caused by mutations in genes coding for proteins associated with the muscle thin filaments (32). Defects in proteins of the Z-disk (myotilin, telethonin, ZASP, filamin C, BAG3) cause the development of disorders collectively termed myofibrillar myopathies (33). Affected muscle fibers show myopathic changes, but striking morphological feature is the presence of abnormal intracellular protein inclusions. The inclusions appear as single or multiple accumulations of amorphous material, small granules, spherical or lobulated hyaline structures and/or cytoplasmic bodies (34). Intermediate filaments proteins are a family of proteins of the cytoskeleton. Lamin A/C, desmin and plectin have pathological significance in muscle and their aggregation is an important pathological marker (8,9).

Increase in the number of mitochondria, alterations in their size and distribution, abnormal structure of cristae, or the presence of inclusions are pathological signs. Detection of a mitochondrial defects may require a combination of techniques including histology (presence of ragged red fibers (Figure 3) with abundant structurally abnormal mitochondria), enzyme histochemistry (presence of fibers deficient in cytochrome c oxidase), and electron microscopy (visualization of structurally abnormal cristae or inclusions) (35).

Sarcoplasmic reticulum and T-tubules play an essential role in muscle excitation, contraction and ion flux, particularly calcium. Defects in the RYR1 gene are associated with a wide spectrum of pathological findings such as core lesions and lack of oxidative enzyme activity (Figure 4), often found in congenital myopathy and central core disease (36).

Defects in cytoplasmic proteins such as enzymes (calpain-3, GNE) chaperone proteins (SIL1, αB-crystallin, Kelch proteins) and proteins involved in autophagy (LAMP2, VMA21, VCP, TRIM32) are also
associated with other rare muscle disorders including vacuolar myopathies, such as Danon disease and XMEA or sarcotubular myopathy, the Marinesco–Sjögren syndrome, αB-crystallinopathy, and nemaline myopathy type 6 (8).

**Figure 3.** “Ragged red” fibres in case of mitochondrial myopathy (GMT x 20).

**Figure 4.** Unstained central part of the fibers demonstrate inactivity of oxidative enzymes (SDH x 20).
II. Endomysium, perimysium, and epimysium

Fibrosis and inflammation are the two principal pathological findings in the endomysium, perimysium and epimysium.

1. Fibrosis and adipose tissue

Endomysial and perimysial fibrosis, as well as increased amount of adipose tissue, is always abnormal and dominant feature of muscular dystrophy, especially congenital muscular dystrophy, but may also be seen in end-stage denervation and end-stage myopathy. Perimysial fibrosis is of lesser diagnostic importance, especially if not accompanied by endomysial fibrosis (13).

2. Cellular inflammatory reactions

Dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM) (Figure 3) belong to the heterogeneous group of the inflammatory myopathies characterized by infiltration of inflammatory cells into and around muscle cell. Also, muscle fibers become necrotic and atrophic. Although inflammation could be patchy, immunocytochemistry is crucial in identification of CD8 lymphocytes nearby nonnecrotic fibers. Furthermore, major histocompatibility complex I (MHC-I) expression is not patchy, but widely upregulated even in areas without inflammation. In the case of polymyositis, MHC/CD8 lesions are always present (37). Granulomatous inflammation (Figure 5) suggests either tuberculosis or sarcoidosis (38,39).

Figure 5. Granulomatous myositis with Langerhans giant cells in a case of sarcoidosis.
III. Blood vessels

A diagnosis of vasculitis is easy when classic features of vasculitis are present in an endomysial blood vessel. Whenever a perivascular lymphocytic infiltrate encroaches on a vessel wall, vasculitis must be suspected. Carefully examined H&E stained section is very important but precise diagnosis, using special stains, must be performed (13).

Conclusion

Identification of the disease genes and their protein products, as well as determination of the ‘downstream’ or secondary consequences of particular genetic mutations are requisite for obtaining important new insights into the pathophysiology of inherited muscle diseases, as well as basic muscle biology. Further study of novel muscle genes as disease markers will improve the understanding of the mechanisms of the disease process and reveal potential therapeutic possibilities. Since all neuromuscular diseases are rare, it is extremely important to follow standardized and harmonized procedures in order to obtain accurate diagnosis, when possible. Creation of a national muscle tissue biobank and joining EuroBioBank will help in acceleration of research on these incurable diseases.

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References

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