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Short Course 12

Advances in diagnostic electron microscopy

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Electron microscopy in analysis of skin diseases

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The advent and success of immunohistochemical and immunocytochemical techniques and, most recently, of biomolecular methods in the diagnosis of skin disorders might seem to render conventional transmission electron microscopical (CTEM) examinations of dermatological specimens to be old-fashioned. Assuming that 95% of dermatological diagnostic work is done by dermatohistopathologists correlating the patients' clinical appearance with the morphology of histological sections, the value of CTEM seems to be drastically reduced and the technique is continuously being replaced by modern ones such as immunoelectron microscopy (IEM) and/or biomolelcular techniques. Moreover, questions such as "CTEM, – and even EM – is it obsolete?" have been raised many times in the past years and most recently by leading scientists and experts in those techniques. Despite croaking about "EM is dead – Is EM dead?" EM and CTEM have several applications and certainly are very useful in the diagnosis and management of many dermatological entities, provided communication between pathologists, dermatologists and dermatohistopathologists defines exactly when and how one can make the maximum use of a diagnostic tool such as electron microscopy.

The short course "Electron Microscopy in Analysis of Skin Diseases" focuses mainly on tissue specimens either from the operating theater or sent in by practicing dermatologists and processed for CTEM in our hospital. The course covers methods of obtaining optimal specimens (including pitfalls); transport of specimens to the laboratory; rapid as well as classical specimen processing into resins; making and examining large area semithin resin sections, including applications of reliable polychromatic diagnostic staining; reembedding of paraffin sections or paraffinized specimen blocks into resin for CTEM; a range of entities of skin diseases where CTEM can be helpful, e.g., cases of bullous epidermolysis, some of which give a very uncommon result; screening for mucosis fungoides (Sezary cells) inepidermal infiltrates or peripheral blood specimens ("buffy coats"); Langerhan's cell histiocytosis (distinction between histiocytosis X and non histiocytosis X, *vs.* nevi); suspected tumor entities *e.g.,* malignant amelanotic melanoma; storage diseases, such as argyria, neuronal ceroid lipofuscinosis as manifested in skin specimens; viral diseases, *e.g.,* orf, purpura fulminans (disseminated intravascular coagulation, destruction of microvasculature by bacteria) and others. Due to time limitation, the topics cannot be covered as a whole nor given detailed descriptions and the intention is to present only a synopsis of the activities in our area. If time permits, hints on subsequent special immunoelectron microscopic methods, as well as some notes on useful internet links concerning literature search and EM image galleries in dermatopathology, will be given and discussed. Also, printed handouts concerning the matters discussed will be available at the lecture site.

The contemporary role of electron microscopy in the diagnosis of mitochondrial encephalomyopathies

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Introduction

Mitochondrial diseases exhibit a wide spectrum of clinical phenotypes, which include pure myopathies as well as multisystem disorders involving major organs such as the brain, skeletal muscle, heart, kidney and liver (1, 2). The age of disease onset ranges from birth to old age and because of the frequent involvement of the central nervous system and muscles, the term mitochondrial encephalomyopathies (MEs) is often used to describe these disorders.

Historically the concept of a mitochondrial myopathy was first introduced by Luft *et al.* (3). Currently, the term mitochondrial disorder is used to describe diseases that are associated with defects in the oxidative phosphorylation system, particularly the ones that are caused by mutations in mitochondrial DNA (mtDNA).

During the 1960s, much diagnostic emphasis was given to the presence of "ragged red fibers" (RRF), visualized with the modified Gomori trichrome stain (4). RRF indicate proliferation of mitochondna, which usually appear as subsarcolemmal aggregates in cryostat sections of muscle fibers, It should be noted that RAE are not specific for primary MEs but can be found in various other neuromuscular disorders, as well as in old age (5). In spite of these limitations, the presence of ARE is still considered the histological hallmark of mitochondrial disorders. Subsequently, electron microscopy of muscle biopsies led to the recognition of different patterns of mitochondrial changes. Shy and Gonatas (6) first identified a case of "pleoconial myopathy", which was characterized by excessive proliferation of normal-looking mitochondria. This was followed by the description of "megaconial myopathy" in which greatly enlarged mitochondria with disoriented cristae were found in the biopsies of affected muscle fibers (7). Other ultrastructural

abnormalities have since been described in mitochondria in association with MEs (8, 9).

In addition to the morphological studies, biochemical analysis of muscle resulted in the identification of specific biochemical defects in respiratory chain enzymes, causing mitochondrial dysfunction. These findings led to a biochemical classification of MEs (10) as well as to the development of histochemical enzyme assays. The most useful of these histochemical reactions, are succinate dehydrogenase (SDH) and cytochrome *c* oxidase (COX), which are routinely used in screening for mitochondrial myopathies.

Mitochondrial DNA analysis, *in situ* hybridization and immunocytochemistry against specific components of the respiratory chain proteins, are new and exciting diagnostic techniques but are largely limited to research centers (1, 11, 12). In routine clinical practice, the diagnosis of MEs still relies heavily on the use of morphological techniques and histochemistry (13,14). Among the morphological methods, the usefulness of electron microscopy has been questioned (2, 14). In this context the main aim of this study is to evaluate the relative role of electron microscopy, compared with histological and histochemical investigations, in diagnosing MEs.

Patients and methods

In the last 3 years, 20 patients were identified who fulfilled the criteria proposed by Walker *et at.* (15) for MEs. This set of criteria uses clinical as well as laboratory data to classify suspected cases of MEs as definite, probable or possible. The need for such criteria arises from the lack of a laboratory "gold standard' diagnostic test. The 20 patients included in the present study were definite or probable MEs and were separated into two broad categories, depending on the age at the time of biopsy. The first group consisted of nine pediatric patients, aged 6 days to 10 years. Their principal clinical symptoms included generalized hypotonia, accompanied by psychomotor retardation and respiratory distress. The second group comprised 11 adult patients with an age range of 19-75 years. Most of these patients presented with myalgias and/or fatigue. Of the 20 patients investigated nine were male and 11 female.

All patients underwent an open muscle biopsy, under local or generalized anesthesia. From each biopsy the bulk of muscle tissue was snap frozen in isopentane, cooled by liquid nitrogen. This tissue was stored in liquid nitrogen and used to obtain cryostat sections, which were cut at 8 m and stained with hematoxylin and eosin and modified Gomori trichrome. In addition, histochemistry for SDH and COX were performed.

At the I~ht microscopic level the diagnostic criteria were as follows: more than 2% RRF or RRF equivalents (RRFe) on the modified Gomori trichrome and SDH respectively were considered significant. If aged less than 30 years, any RRF or RRFe was considered significant. In COX histochemistry more than 5% COX deficient fibers or a generalized reduction were both significant.

Small rectangular pieces of muscle, measuring 1 x 2 mm were fixed in 2.5% glutaraldehyde, in 0.1 M phosphate buffer at pH 7.2, for a minimum of 4 h at 4 **oo.** The tissue was rinsed in phosphate buffer, post fixed in 1% osmium tetroxide, dehydrated in alcohols of increasing strength and cleared in propylene oxide before being embedded in an Epon/Araldite resin mixture. Resin blocks of muscle were orientated in such a manner as to allow sectioning in both longitudinal and transverse directions. From each patient semithin sections were obtained from four separate resin blocks, two of which were longitudinal and two transverse. In turn, these were surveyed by light microscopy and two blocks, one longitudinal and one transverse, were selected from each biopsy for subsequent ultrathin sectioning.

At the ultrastructural level, the diagnostic criteria used were based on finding either mitochondrial aggregates, consisting of at least five layers of mitochondria, or on the presence of structurally abnormal or enlarged mitochondria. A minimum number of three aggregates had to be found, in three different muscle fibers in each case, to be of diagnostic significance. Mitochondria were considered pathologically enlarged if their length was greater than 1 m and their width greater than 0.4 m, as previously defined (6).

Results

The results are presented separately for the pediatric and adult groups.

Pediatric grouo

Light microscopy was positive in seven out of the nine cases. In one patient RRFe were present in SDH, while in the remaining six patients abnormalities in the COX enzyme stain were detected. Three patients showed a diffuse moderate reduction in COX, two had a severely diffuse reduction in COX and the remaining patient exhibited COX deficient fibers. In two out of the nine cases histology and histochemisry were normal. No RRF were detected in this patient group.

Electron microscopy was abnormal in four out of the nine patients. In one patient, electron microscopy revealed the presence of mitochondrial aggregates consisting of pleomorphic mitochondna, many of which exhibited simplified cristae. Three patients exhibited abnormally enlarged mitochondria, with an electron dense matrix and other structural changes, such as simplified or vesicular cristae. Five patients were classified as normal by electron microscopy, although two contained small mitochondrial clusters, two to three layers thick, which did not fulfill our minimal diagnostic criteria (Table 1).

Adult group

In the adult group, light microscopy was abnormal in all 11 patients, displaying a combination of RRF seen in four patients, RRFe seen in seven and abnormalities in COX seen in 11 patients (Table 2).

Careful examination of semithin sections, revealed the presence of excess lipid and glycogen in seven patients; in addition, subsarcolemmal abnormalities suggestive of mitochondrial proliferation were noted in three cases.

Electron microscopy was positive in 10 out of the 11 patients. It is noteworthy that in two patients no mitochondrial proliferation was detected by light microscopy but was present on ultrastructural examination. Detailed ultrastructural findings were as follows: mitochondrial aggregates were seen in 10 patients and in five of these abnormal mitochondrial structure was also present. Mitochondrial aggregates were more frequent under the subsarcolemma but were also present between myofibrils. It should be noted that the mitochondrial aggregates were often more than five layers thick, in most of the cases examined. In the remaining case, careful search revealed the presence of only one mitochondrial aggregate, which was an insufficient diagnostic criterion. In addition, abnormally shaped mitochondria were another prominent feature of the adult patients. Mitochondrial dysmorphology was exemplified by the presence of branched or ameboid mitochondria, as well as by the presence of structural abnormalities of cristae. The latter included the presence of lamellar, densely packed or vesicular cristae. Mitochon-

Table 1. Pediatric group. Skeletal muscle morphological findings.

Patient	1	2	3	4	5	6	7	8	9
Age	21/2 months	5 months	5 years	10 days	6 days	4 months	5 years	11 years	10 years
Sex	M	F	M	F	F	F	M	F	F
Modified Gomori trichrome	N	N	N	N	N	N	N	N	N
Succinate dehydrogenase	N	N	N	N	N	N	N	N	RRFe
Cyfochrome c oxidase	MGR	MGR	ODE	N	MGR	SGR	SGR	N	N
Electron microscope mitochondrial aggregates	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs	P
Mitochondrial structure/size	N	Abn	N	Abn	N	N	N	N	N

N = normal; RRFe = ragged red fibres equivalent; MGR = moderate generalized reduction; CDF = cytochrome c oxidase deficient fibres; SGR = severe generalized reduction; Abs = absent; P = present; Abn = abnormal.

Table 2. Adult group. Skeletal muscle morphological findings.

Patient	1	2	3	4	5	6	7	8	9	10	11
Age in years	48	22	75	42	19	58	19	38	39	42	42
Sex	F	M	F	M	F	F	M	F	M	M	M
Modified Gomori trichrome	RRF	RRF	RRF	N	N	RRF	N	N	N	N	N
Succinate dehydrogenase	550	RRFe	RRFe	N	RRFe	RRFe	BREe	RRFe	RRFe	550	N
Cytochrome *c* oxidase	550	SSC	COF	CDF	CDF	COF	CDF	ssC	CDF	SSC	CDF
Electron microscope mitochondrial aggregates	P	P	P	P	P	P	P	P	Abs	P	P
Mitochondrial structure/size	N	N	Abn	Abn	Abn	Abn crystals	Abn	N	N	N	N

RRF = ragged red fibres equivalent; N = normal; SSC = subtle subsarcolemmal collections; RRFe ragged red fiber equivalent; CDF = cytochrome c oxidase deficient fibres; Abs = absent; P = present; N normal; Abn = abnormal.

dna with an electron dense mitochondrial matrix were also seen in some patients, together with the presence of prominent electron dense granules. Crystalline inclusions were seen in only one patient. In most patients the above mitochondrial changes were present in association with increased amounts of lipid and glycogen, which confirmed the observations from the semithin sections.

Discussion

Accurate diagnosis of MEs relies on careful clinical evaluation and laboratory data from various disciplines, including histology, histochemistry, electron microscopy, biochemistry and, more recently, mtDNA studies. Despite the introduction of newand innovative methods for diagnosing and understanding the pathophysiology of MEs, morphological techniques still form the backbone for the evaluation of muscle biopsies. Electron microscopy has been credited with little diagnostic value among the morphological methods (1, 15).

In this study we attempted to reevaluate the role of electron microscopy in relation to other morphological methods. We separated our patients into two groups since it has been suggested that mitochondrial proliferation, the main morphological indicator of MEs, is partly age dependent (1). In addition, patients included in the study were carefully selected and their biopsies were screened by light microscopy, in order to eliminate disease entities, such as neurogenic atrophy, polymyositis, Duchenne muscular dystrophy and others, which could produce secondary mitochondrial abnormalities (15). Our results demonstrate that, at least in the pediatric age group, electron microscopy is important in identifying mitochondrial abnormalities, not visualized by light microscopy. In three out of nine patients, in which there was no evidence of mitochondrial proliferation by light microscopy, abnormal mitochondria were seen. This has also been reported by others, especially in patients with Leigh syndrome due to partial COX deficiency (16, 17). Our study suggests that mitochondrial aggregates may be an uncommon phenomenon in pediatric cases, which might explain the inability of the modified Gomori trichrome and SDH stains to show any abnormal fibers. A generalized reduction in COX was the most frequent light microscopical abnormality. Therefore, in the diagnosis of pediatric Mes, the two most useful and independent morphological methods are COX histochemistry and electron microscopy.

In the adult group of 11 patients the results demonstrate that the use of the modified Gomori trichrome, in combination with enzyme histochemistry for SDH and COX, are highly sensitive diagnostic tools. Ultrastructural examination showed the presence of mitochondrial aggregates in 10 patients, two of which did not demonstrate RRF, RRFe or subtle sarcolemmal collections. These two patients did, however, have COX deficient fibers. Therefore, in the majority of adult patients, electron microscopy simply added confirmatory evidence but did not contribute significantly to the diagnostic yield of morphological methods. However, in our experience, in patients with borderline mitochondrial proliferation on light microscopy, it is useful to have independently derived ultrastructural evidence of mitochondrial abnormalities before making a diagnosis.

In conclusion, this study clearly demonstrates that electron microscopy remains essential in the diagnosis of pediatric MEs but is of lesser value in adult patients . In the future, greater emphasis on electron microscopic enzyme cytochemistry for COX and other respiratory enzymes will be instrumental in further elucidating the pathogenesis of MEs.

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Electron microscopy in the diagnosis of inherited connective tissue diseases

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Martan syndrome is a dominantly inherited disorder characterized by Cardiovascular, ocular and skeletal abnormalities. The major cardiovascular complications of ascending aorta dilatation and dissection often lead to premature death in the absence of treatment and justify the requirement of relevant diagnostic criteria in the early years of life. The pathogenetic role of fibrillin has been confirmed by the identification of mutations in the fibrillin gene FBN1 in patients with classical Martan syndrome. Immunochemical and immunohistochemical analysis converged to demonstrate defective synthesis, secretion and extracellular matrix formation of fibrillin by cultured dermal fibroblasts. The skin biopsy thus appeared as a noninvasive Clinical *requirement* allowing *immunochemical,* immunohistoChemical and genetic assessments of fibrillin defiCiency in early Marfan syndrome diagnosis.

Fibrillin is a large 35OkDa matrix protein associated with one ot the major subgroups of microfibrils morphologically characterized by 10-13 nm diameter, a hollow cross-section and a beaded appearance with some periodicity. Considered as integral components of elastic elements, these microfibrils are constituents of three types of fibers, namely oxytalan, elaunein and elastic. Oxytalan fibers consist mostly of microfibrils arranged in an orderly manner, although they contain some elastin. Elaunein fibers are present beneath the oxytalan fibers and contain both microfibrils and amorphous elastin. In all the probands the arborescent distribution of the elastic and elaunein fibers was lost and the oxytalan fibrils were scarce, with variable patterns of microfibril alteration.

These patterns of microfibril alteration extended to the elaunein and elastic fibers in which the peripheral microfibrilar apparatus appeared either condensed with vanishing of the microtubular structure or scarce with preservation of ultrastructure. In any case, the close interconnection between the elastic fibers and the collagen fiber bundles supported by microfibrils appeared ruptured or slender, thus emphasizing the discontinuity between the tensile elastic and architectural collagenic frameworks of the dermis in the Marfan patients. The dermal microvasculature was variably affected in relation to the decreased density of microfibrils anchoring the elastic network to the perivascular basement membrane, and displayed gradual constrictive features.

While fibrillin immunodetection in cultured skin fibroblasts allows pathologists to appreciate the level of fibrillin expression deficiency, ultrastructural tissular immunolabeling renders account of the pleomorphism of the pathological implications in the dermal connective matrix and the microvasculature.

Electron microscopy of melanin-synthesizing tumors

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With the availability of relatively new antibodies such as HMB-45, HMB-50 or MART-i (Melan-A), the identification of melanocytic phenotype in an undifferentiated neoplasm has become much easier. These markers, however, are often negative in many (and sometimes in all) of the cells in a given tumor. In addition, there are situations in which the identification of melanin-synthesizing cells is not enough to reach a correct diagnosis. Electron microscopy may play a contributory role in many of these cases. In this part of the course, the application of electron microscopy in four specific settings will be reviewed, namely: i) poorly differentiated tumors, either primary or metastatic, in which malignant melanoma enters the differential diagnosis; ii) soft tissue tumors with melanocytic differentiation; iii) pigmented tumors of the central nervous system (mostly arising in the meninges); and iv) complex neoplasms with aberrant