Diagnostic Transmission Electron Microscopy

The Impact of Pathological Examination of Tissue Ultrastructure

The high resolution and sensitivity of electron microscopy is still a valuable tool, and for some diseases it is the gold standard in pathological diagnosis. Today the sample turnaround time for processing in the lab can be significantly reduced from days to hours by the microwave technology. In urgent clinical cases, microwave-assisted tissue processing, in combination with digital image acquisition, enables a "same-day" diagnosis. Ultrastructural telepathology allows instant and live second opinion retrieval from a remote expert worldwide.

Introduction

Modern evidence-based medicine needs a cytology or tissue-based pathologic diagnosis describing the nature of a lesion and an interpretation of the data providing advice for an individual therapeutic strategy. This is achieved by pathologists mainly by light microscopy (LM) examination of H&E-stained sections (approx. 4 µm thick) of tissue embedded in paraffin wax. Special stains and techniques, like immunohistochemistry (IHC), flow cytometry, cytogenetics, and molecular techniques (gene rearrangement analysis, fluorescence in situ-hybridization (FISH) and polymerase chain reaction (PCR) analysis) provide additional information (genomics, proteomics, metabolomics) to refine the understanding of a disease and strengthen the diagnosis.

Why Diagnostic Transmission Electron Microscopy

If the analyzed features are smaller than the resolution limit of the classic light microscope (200 nm), the 1,000 times higher resolving power of transmission electron microscope (TEM) technology (0.2 nm) can visualize small intracellular and extracellular structures in great detail to facilitate a diagnosis that might be uncertain or impossible by Light Microscopy. Examples include cell organelle (nucleus/nucleoli, mitochondria, RER/SER, Golgi complex, lysosomes, melanosomes), various types of inclusions and secretory granules, cytoskeleton components (microtubules, filaments, centrioles), cilia, cell surface specializations (microvilli, cell protrusions, intercellular junctions), extracellular constituents (basal membrane, collagen, amyloid), and a variety of infectious agents.
In many diseases this component displays peculiar abnormalities or lesions.

In conclusion, TEM examination of pathological samples is a method to extend morphologic analysis to the ultrastructural level, providing data not discernible by the other ancillary methods, e.g., on the basis of the antigens expressed by a neoplasm [1]. The new stunning light diffraction limit breaking STED-microscopy (= Stimulated Emission Depletion; resolution range 16-80 nm) developed by Stephan Hell [2] is to date not available for routine diagnostic purposes. TEM was a very popular adjunct in diagnostic procedures in the 1970s and early 1990s, its importance declined in pathology with the emerging use of IHC and molecular methods as well as due to intrinsic EM limitations like insufficient sample processing automation and long turnaround time (TAT). Today a resurgence of TEM as an ancillary diagnostic modality is observed [3], however TEM-diagnostic expertise due to economic and staffing issues is generally available only in larger laboratories or centers with specific interest in ultrastructural pathology [4-6].

**Sample Handling**

In TEM, instead of light, an electron beam passes through the examined section from a sophisticated electromagnetic lens system to give a high-resolution image of the specimen. The limited penetration depth of electrons and their interaction with the specimen (heat and ionization damage) require a special tissue preparation to produce ultrathin sections suitable for TEM examination.

In the literature there is a large number of protocols for embedding different specimens in a variety of dedicated resins according to the addressed issue [7]. Briefly, after sample collection the standard approach is to immerse the specimen immediately in a buffered fixative (Karnovsky formulation = primary aldehyde-based fixative for protein preservation (fig. 1), subsequent osmium tetroxide
postfixation for lipid stabilization and contrast enhancement), dehydrate it in graded ethanol, and embedding in epoxy resin for polymerization (mostly by heat) into hard blocks (including ID-labels). This routine sample processing is nowadays performed in most diagnostic EM labs using computer controlled tissue processors saving reagents, time, and labour (batch processing overnight). The usual routine total sample turnaround time (TAT) is approximately 3 to 5 workdays. In case of urgent clinical cases, microwave-assisted tissue processing (AMW/Leica, KOS/Milestone) can reduce the TAT to less than 6 hours [8].

Once polymerized, tissue blocks are cut with an ultramicrotome equipped with a diamond knife to yield semithin sections for LM screening for specific features (approx. 0.8 µm, rapid staining on glass slides with toluidine blue and basic fuchsin) and to select areas for thin sectioning. The ultrathin resin sections (approx. 80 nm) are collected on copper grids and double-stained on drops of heavy metal salt solutions (uranyl acetate, lead citrate) for contrast enhancement of specimen structures (increased electron scattering). The air-dried sections are usually examined in a TEM with 80 to 100 kV acceleration voltage and equipped with a customized digital camera image acquisition system (recommended resolution 1kx1k or 2kx2k pixels) capable to store the images in a secure databank. Interactive remote TEM operation via Internet allows instant and live "second opinion" consultation of difficult cases worldwide ("ultrastructural telepathology") [9].

**Rapid Diagnosis of Infectious Agents**

One needs to recall, that one of the first samples visualized by Borries and Ruska in the very early days of EM ("Übermikroskopie") were poxvirus samples [10]. Today the negative-staining method is still a very efficient and rapid EM diagnostic procedure (approx. 30 minutes, no sample resin embedding and ultramicrotomy necessary) when applied to potential infectious suspensions: it allows a rapid morphological identification and classification of different agents contained in the specimen, this can be crucial in emerging situations (e.g. SARS, bird flu, anthrax-attack) [5]. We apply this method for e.g. routine TEM examination of blister content of herpetiform skin lesions (herpes virus: yes/no). Another example of routine virus diagnosis, polyoma viruses detected in urine of a patient suffering complications after kidney transplantation, is shown in figure 2 (acute rejection versus virus nephritis can be a challenge to diagnose in renal allograft biopsies by LM).

**Spectrum of TEM Diagnosed Samples**
Based on the example of our centralized EM unit integrated in the diagnostic service of the pathology department in a medical center, we confirm the continuing value of TEM diagnosis in rapid virus detection as mentioned before, as well as in surgical pathology of selected tumours [11], and numerous non-neoplastic indications like renal, muscle, nervous system, skin, cilia defects, storage diseases, liver biopsies of transplanted patients, respiratory diseases, toxic lesions, male infertility (centriolopathy), microsporidia and opportunistic infections, as already referred in detail by others [12-14].

In neoplastic diseases ultrastructural studies are useful in identifying tumor cell structures which are indicative of the line of differentiation or histogenesis (origin) of a neoplasm. The classical examples are different soft tissue tumors presenting immunohistochemically a confusing staining pattern, the evidence of specific cell junctions by TEM, notably desmosomes in the mostly poorly differentiated neoplastic cells, is characteristic for an epithelial tumor (cancer). The discrimination between epithelial mesothelioma (often induced by asbestos exposure) and adenocarcinoma is important for both therapeutic and medico-legal reasons because the special stains and antibodies used for this differential diagnosis are not absolutely specific for either of these two entities. Difficult LM diagnoses can be verified by TEM in a number of other neoplasms, including embryonal myogenic tumors (e.g. rhabdomyosarcomas - evidence of rudimentary sarcomeres), gastrointestinal stromal tumors (GIST), ependymomas, neuroendocrine tumors, dendritic cell sarcoma, granular versus oncocytic renal epithelial tumors, and occasionally TEM contribute to the determination of the primary site of a metastatic tumor [3]. An example of an amelanotic melanoma recognized by the presence of immature melanosomes (premelanosomes, not visible by LM) with a peculiar striated core is demonstrated in figure 3.

From the non-neoplastic conditions mentioned above a small handful will be discussed below. Considering the kidney, TEM is indispensable for effective diagnosis of diseases of the renal glomerulus in concert with LM and immunohistochemical methods. The glomerulus is located in the renal cortex and is the kidney structure where primary urine is formed from blood. Pathologic disturbances of the glomerular components (basal membrane of the capillary loops, endothelium, podocytes, and mesangium) and determination of the character and localization of different deposits can be done by TEM only (fig. 4).

Similarly skeletal muscle, eyelid muscle (ptosis condition), and myocardium shows a broad spectrum of sarcomere abnormalities or presence of inclusions, vacuoles, and deposits (e.g. amyloid) well resolvable by TEM, a typical myopathy lesion related to energy deficiency caused by a mitochondriopathy (mitochondria are the power
From patients, particularly young children, suffering of idiopathic chronic respiratory tract infections biopsy is performed to determine whether the cilia covering the mucosa surface are structurally abnormal and presumably immotile (causing insufficient airways clearing). Normal cilia cross-sections (diameter approx. 280 nm) display a circular arranged axoneme consisting of nine microtubule doublets with an inner and outer dynactin arm projecting from one of each of the doublet tubules and two central tubules (9+2 axoneme). Most biopsies from affected patients show secondary cilia defects including loss of ciliated cell or cilia, compound cilia and megacilia, and abnormal microtubule patterns (loss or supernumerary tubules, tubule dislocations). Patients with primary (mainly hereditary, Kartagener's syndrome = sinusitis + bronchiectasis + situs inversus) cilia dyskinesia (low or absent beat frequencies) generally have reduced or absent outer dynein arms (fig. 5).

Skin Biopsies, CADASIL, NSF

The incidence of diagnostic TEM examinations of skin biopsies diminished recently significantly due to the advent of IHC and molecular methods. However, in several skin diseases it is the method of first choice or verification standard. This include such different disorders like granulomatous lesions (evidence of Birbeck granula in Langerhans cells, fig. 6), amyloidosis, pigmentation disturbances, storage diseases with a typical clinical features, CADASIL (= an inherited vascular disorder affecting predominantly the central nervous system, diagnostic evidence of GOM-deposits in skin arterioles), and the very heterogenous group of mechanobullous dermatoses forming easily skin blisters by mechanical stress. Epidermolysis bullosa is mainly classified into four major groups (simplex, junctional, dystrophic, hemidesmosomal) characterized by the level of blister formation in the skin, which can be excellently resolved by TEM [12].

Since 2000, dermatologists, nephrologists, and radiologists become aware of growing clinical data referring to a heavy disabling disease, now called NSF (=Nephrogenic Sclerosing Fibrosis). This acquired disorder of the skin and systemic tissues emerged in a fraction of patients with renal insufficiency exposed to gadolinium-based (Gd) MR-imaging contrast agents. The pathogenesis of NSF and the mechanism by which Gd acts as a trigger for this condition remained elusive for a long time. A number of TEM examinations of skin biopsies of NSF patients for tissular Gd using the very sensitive Electron Spectroscopic Imaging and Electron Energy Loss Spectroscopic method was performed. The analysis revealed a perivascular and multifocal collagen fiber associated Gd deposition in the deep
dermis. Iron signal was also present in singular Gd-positive deposits as well as in adjacent connective tissue in the skin (fig. 7); this supports the postulated Gd transmetallation hypothesis of NSF pathogenesis [15]. In consequence of these findings worldwide guidelines for Gd-based contrast agents application were updated to protect patients against the new man-made disorder.

Conclusions

Transmission electron microscopy, with the potential of 1000x higher resolving power compared with light microscopy (aside the advent of STED-microscopy), is still used as an ancillary tool, quality control method or gold standard to complement, support, or confirm the result of specific histopathological diagnoses. In this context it is mandatory for each diagnostic EM laboratory to be fully integrated into the diagnostic workflow of the local pathology department and to comply with the accreditation guidelines [6].

Microwave technology and sample processing automation can significantly reduce the sample turnaround time from days to hours providing excellent ultrastructure preservation. Rapid microwave-assisted tissue processing combined with digital image acquisition make the "same-day" EM-diagnosis feasible, which can be crucial in urgent clinical cases.

Ultrastructural telepathology bridges space and time, is a novel tool for instant live second opinion retrieval and to share interesting findings worldwide using modern Internet technology.

Acknowledgments
The author thanks Heiko Siegmund for excellent technical support and Dr. Stephan Schreml / Dermatology, University Hospital Regensburg for manuscript proof reading.

References
A wide range of diagnostic electron microscopy topics would be presented at the ULTRAPATH XVI Conference, held at August 6 -10, 2012, in Regensburg/Germany. Please follow for details the link to the website www.ultrapathXVI.de.

Contact