THE DIAGNOSTIC IMPORTANCE OF SEMITHIN AND ULTRATHIN SECTIONS

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Summary

Transmission electron microscopy (TEM) implies an elaborate preparation protocol that includes: glutaraldehyde and osmium fixation, resin embedding, ultrathin sectioning and staining with heavy metal salts. In some cases paraffin-embedded tissue can be used for electron microscopy following a re-embedding protocol. In tumoral specimens various aspects of angiogenesis, including the progression front consisting from sprouts budding from preexisting vessels, have been identified. In glomerular disease the glomerular basement membrane became thickened due to four types of electron dense deposits with different localization. In minimal change disease, considered a disease without glomerular deposits we observed the broadening of pedicles over most of the glomerular basal membrane that presented a normal thickness and appearance. The contribution of electron microscopy for the study of normal and abnormal ultrastructure is important because it provides diagnostic information. Transmission electron microscopy permits the ultrastructural examination in a way that cannot be done using other techniques.

Keywords: transmission electron microscopy, semithin and ultrathin sections

Introduction

Light microscopy of the paraffin embedded sections is the main diagnostic method used in inflammatory and tumoral diseases. Transmission electron microscopy (TEM) is a complementary diagnostic tool, for which the tissues are specifically processed in order to obtain good ultrastructural details (Stirling, & Woods 2002).

In surgical pathology, the diagnostic electron microscopy is usually performed on ultrathin sections obtained after glutaraldehyde-fixation, and resin-embedding.

Sometimes, when the glutaraldehyde fixed biopsy is missing, or when the collected tissue is inadequate the formalin-fixed, paraffin-embedded tissue can be used for electron microscopy following a re-embedding protocol of the wax-embedded tissue used for light microscopy.

Material and methods

In this study we have investigated renal biopsies obtained from patients with tumoral (n=27) and inflammatory disease (n=9).

The formalin fixed biopsies were processed for light microscopy: they were embedded in paraffin and the 5µ thick sections were stained with Hematoxylin–Eosin.
Retrospectively, transmission electron microscopy (TEM) was performed after a protocol (Craciun and Horobin, 1989) that included: cutting of 1 mm³ fragments from the paraffin block that were reprocessed as follows:

- Dewaxing in xylene at 60°C for 2 x 30 min
- Dewaxing in xylene at 22°C for 12 hours
- Rehydration in:
  - Xylene - ethanol (1:1) for 5 min
  - Ethanol 100% for 2 min
  - Ethanol 95% for 3 x 5 min
  - Ethanol 80% for 3 x 5 min
  - Ethanol 70% for 3 x 5 min
- Washing in phosphate buffer 0.1 M for 10 min
- Post-fixing in aqueous OsO₄ 1%, 4°C, for 1 hour
- Washing in distilled water for 2 x 10 min
- Dehydration in:
  - Ethanol 70% for 2 x 15 min
  - Ethanol 80% for 2 x 15 min
  - Ethanol 96% for 2 x 15 min
  - Ethanol 100% for 2 x 15 min
- Infiltration with propylene oxide for 2 x 15 min
- Infiltration with Epon 812 overnight
- Polymerisation of the Epon block at 60°C for 24 – 36 hours

After polymerisation, the Epon blocks were trimmed and sectioned with an ultramicrotome (LKB Ultrotome) using glass knives.

Semithin - 1000 nm thick – sections stained with Richardson's stain, (aqueous solution of Methylene Blue in 1% borax, mixed with Azure II) were examined with the light microscope (Nikon) in order to identify the areas useful for diagnosis. (Richardson and Jarrett, 1960)

The chosen area of the Epon block was cut obtaining 60-100 nm thin sections known as ultrathin sections. These were placed on copper grids, contrasted with uranyl acetate and lead citrate and examined with a Leo 906 transmission electron microscope (Zeiss, Germany).

Results

The TEM is a unique tool that reveals the ultrastructural details in a way that cannot be obtained with other equipment.

In tumoral renal specimens the reprocessing method was used for the investigation of angiogenesis. The HE stained slides permitted the identification of the area with the richest vascularisation, which was sliced from the paraffin block, embedded in Epon and cut with an ultramicrotome obtaining semi- and ultrathin sections.

The semithin sections (1μ) stained with Richardson's stain permitted the identification of mature and new formed blood vessels, in different stages of angiogenesis. The activated endothelial cells of the newly formed blood vessels were supported by a continuous, well developed basal membrane that sometimes displayed reduplications. (fig.1).

![Figure 1. Tumoral vessel with reduplication of the basal membrane, semithin section, Richardson's staining](image1.jpg)

Many vessels, were surrounded by tumoral cells, located close to the vessel wall, some coming in contact with the lumen. (fig.2)

![Figure 2. Tumoral cells next to a vessel, semithin section, Richardson's staining](image2.jpg)
The endothelial cells lining the intratumoral vessels exhibited particular aspects: they had luminal protrusions into the lumen, large nuclei and overlapped other cells.

![Figure 3. Tumoral vessel with luminal protrusions, transmission electron microscopy](image)

The angiogenesis progression front, that consisted from sprouts budding from preexisting vessels was present on some sections.

Special endothelial “tip cells” were present on the forefront of navigating vessels, sharing many similarities with the axonal growth.

![Figure 4. Forefront of a sprouting tumoral vessel, transmission electron microscopy](image)

The sprouts budding from the preexisting vessels were assembling into solid cords which underwent tubulogenesis, in order to form vessels with a central lumen. On ultrathin sections, the pericyte cover was easy to assess.

Aspects of intussusceptive angiogenesis meaning internal division of the preexisting capillaries was present on semi thin and ultrathin sections.

![Figure 5. Tumoral vessels with lumen splitting, semi thin section Richardson's stain](image)

The contribution of electron microscopy to the study of normal and abnormal glomerular morphology is significant: because it provides diagnostic confirmation or additional diagnostic information. All native renal biopsies should be processed for electron microscopy although ultrastructural examination is required chiefly for glomerular diseases.

The renal glomeruli are round structures that consist of a central tuft of fenestrated capillaries separated by supporting cells and the mesangium and surrounded by Bowman's capsule. Four cell
types are present: the endothelial cells, the visceral epithelium represented by the podocytes, the parietal epithelium lining the Bowman's capsule and the mesangial cells.

In normal glomeruli, the glomerular filtration barrier was composed of fenestrated endothelial cells, the glomerular basement membrane and the filtration slit pores between podocyte foot processes. The glomerular basement membrane was continuous and consisted from three layers: a homogenous central dense band called the lamina densa and the two laminae rarae: the lamina rara interna adjacent to the endothelial cells and lamina rara externa adjacent to the podocytes.

In glomerular diseases we have encountered four types of deposits: the subepithelial deposits underneath the podocytes processes, typical for membranous and postinfectious glomerulonephritis, the subendothelial deposits – located underneath the endothelium, characteristically for the membranoproliferative glomerulonephritis and lupus nephritis, the mesangial deposits – present in the mesangial matrix characteristic for the IgA nephropathy, and the intramembranous deposits, located within the glomerular basal membrane characteristic for the dense deposit disease. In thin basement membrane nephropathy, the diagnostic criteria consisted of diffuse, uniformly thin glomerular basement membrane (GBM) compared with age matched controls due mainly to the decreased width of the lamina densa, associated with smooth contours of the GBM and the absence of diffuse lamination of the GMB. The TEM examination of the reprocessed paraffin blocks had limited diagnostic importance in this disease because the formalin fixation affected GBM thickness.

The podocytes located on the outer surface of the glomerular basal membrane join the squamous parietal epithelium covering the Bowman's capsule at the glomerular hilus. The podocytes formed secondary and tertiary processes that branch terminally in thin, bell shaped terminal processes called pedicles that anchor to the glomerular basal membrane. These foot processes interdigitated in a complex manner with similar processes from the same and adjacent cells and surrounded the capillary loops. The spaces between adjacent foot processes called the filtration slits were covered by thin diafragms.

In minimal change disease, considered a disease without glomerular deposits we observed the broadening of pedicles over most of the glomerular basal membrane that presented a normal thickness and appearance.

In the vacuolated cytoplasm of the enlarged podocytes we identified numerous mitochondria, well developed Golgi apparatus, rough endoplasmic reticulum and microvilli facing the urinary surface. Glomerular scars and tubular atrophy were absent.

**Figure 7.** Pedicelle effacement in minimal change disease, ultrathin section-transmission electron microscopy

In the Discussion, Transmission electron microscopy (TEM) implies an elaborate preparation protocol that includes: fixation in glutaraldehyde followed by osmium tetraoxide postfixation, specimen dehydration, infiltration, resin embedding, ultrathin sectioning and staining with heavy metal salts.
We examined by TEM reprocessed renal biopsies performed by percutaneous route in order to reveal the ultrastructural characteristics of the specimen in ways that cannot be examined using other equipments or techniques.

In some cases, when the requirement for TEM were made after tissue collection, we have reprocessed the formalin-fixed, wax-embedded tissue used for light microscopy. (Erlandson, 1994)

Fixation was the most important step that affected the quality of the ultrastructure. Glutaraldehyde is the best fixative for ultrastructural studies although formalin is not the worse. Tissues with an optimal initial fixation in 4% buffered formalin have had a good ultrastructural preservation, but long term formalin fixation had a negative influence on tissue ultrastructure.

The duration of the reprocessing procedure was also important. The first described methods used for the EM preparation of paraffin embedded tissue had lasted up to one week. The majority of this time was spent for paraffin removal from the tissue sample. These time consuming methods have been replaced by simpler methods that can last only half an hour. (van den Bergh, 1984)

Widèhn described a rapid, simple re-embedding method for electron microscopy: the paraffin-embedded tissue was dewaxed in xylene, stained in a 0.01% toluidine blue solution, infiltrated with a propylene oxide/resin mixture, and than embedded in Epon (Widèhn 1988). This quick method that excluded rehydration, postosmification, and dehydration, lasted about 3 h and and produced similar results to previously described, more laborious re-embedding techniques.

In the last decade, for fresh renal biopsies, rapid processing methods have been imagined, implying a direct LRWhite embedding, and a polimerisation at 0°Celsius.

The morphological, immunohistochemical and electrono-microscopical procedures can be applied directly on semithin and ultrathin sections. (Bowdler and Griffiths, 1989)

Other methods that excluded osmium tetroxide post-fixation and dehydration demonstrated similar results. (Tolivia and Navarro, 1994)

In most reprocessed tissues for TEM, the nuclei, desmosomes, sarcomeres, intermediate filaments and electron dense granules were well preserved.

The basal lamina between the glomerular epithelium and the endothelium of the capillaries, appeared in electron microscopy as a dense layer, consisting of a delicate network of fine fibrils called the lamina densa. In addition to the lamina densa there are two pale, electron lucent layers on both sides, called laminae rarae: one of them is adjacent to the foot processes of the podocytes and the other is adjacent to the endothelium.

The basement became thickened in a number of glomerular diseases due to electron dense deposits located on the lamina rara externa, lamina rara interna or the lamina densa.

We also found diffuse glomerular basement thickening due to the presence of highly electron-dense deposits in the lamina densa (intramembranous), discrete electron dense subepithelial deposits widening the lamina rara externa, large electron dense subendothelial deposits widening the lamina rara interna and densa intramembranous deposits, ribbon-like, involving large segments of the glomerular basement membrane.

In thin basement membrane nephropathy, where diagnosis can only be established by electron microscopy, the glomerular basal membrane appeared thinner on reprocessed, formalin fixed tissue, making the reprocessing method useless. (Nasr and Markowitz, 2007)

Polyribosomes, microtubules, mitochondria, Weibel Palade bodies, lipids, and glycogen deposits can exhibit various degrees of degradation. Mitochondria was an exception, because it had a constant poor
preservation of the inner mitochondrial membrane and cristae, in all the used methods. (Yukiko Ogiyama, 2006)

Paraffin embedded tissue reprocessed for TEM will never have the good preservation of rapidly glutaraldehyde fixed tissue. However, ultrathin sections can be obtained from the reprocessed paraffin embedded tissue, in order to provide useful, diagnostic information.

References


