MYOEPIHELIAL CELL STAINING BY LEVANOL FAST CYANINE 5RN APPLIED TO EPOXY-EMBEDDED SEMITHIN SECTIONS

ADEL MOHAMED EL-BARDAIE* AND HIROMASA NIKAI

*Department of Oral Pathology, Hiroshima University School
of Dentistry, Minami-ku, Hiroshima 734*

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The present communication reports a modified technique for staining myoepithelial cells in normal and neoplastic human salivary gland tissues in 2–3 μm thick sections from epoxy blocks conventionally prepared for transmission electron microscopy. The method employs tannic acid-phosphomolybdic acid-levanol fast cyanine 5RN procedure by Puchtler et al. after intense oxidation of plastic-free sections using 5% potassium permanganate for 20 min followed by bleaching in 1% oxalic acid for 20 min. As for the resin-removal procedure, treatment with saturated solution of potassium hydroxide in absolute ethanol for 5–10 min yields an optimum result. Direct correlations of light and electron microscopic observations indicate that the cells positively stained with levanol fast cyanine 5RN correspond well to the myoepithelial and neoplastic modified myoepithelial cells containing bundles of microfilaments. This approach is useful in matching the corresponding areas at the light and electron microscopic levels in a single block of tissue for evaluation of myoepithelial participation in various salivary gland tumors.

Many investigators have offered methods of staining semithin sections from the epoxy resin-embedded tissues with a variety of differential staining techniques for light microscopy in order to allow direct comparison of their specific staining properties and the ultrastructures (3, 4, 13). However, only a few reports have been published on such an application of some staining methods to salivary gland tissues (4, 5) and no further work on the identification of myoepithelial cells (MCs) has been performed since the application of staining methods to epoxy-embedded tissues was adopted.

Although the identification of neoplastic cells with myoepithelial features within a tumor parenchyma can be considered to be an argument in favor of the role of MCs in tumor histogenesis, understanding of their distribution is always hampered by the lack of real specificity of most of the classical light microscopic staining procedures.

On the basis of previous research into dye structure and affinity for muscle

* Present address: Department of Oral Histology and Pathology, Faculty of Dental Medicine, Al-Azhar University, Cairo, Egypt.

fibers, Puchtler *et al.* (9, 10) have made extensive studies on the tannic acid-phosphomolybdic acid-various milling dyes procedure and its affinities for striated and smooth muscle cells, myoendothelial cells of blood vessels and MCs of salivary and eccrine sweat glands in Carnoy or methacarn-fixed, paraffin-embedded tissues. Review of the literature concerning the utilization of this technique in neoplastic tissues revealed only a few studies illustrating MCs and myofibroblasts in mammary tumors (11, 12).

The present communication permits the use of the tannic acid-phosphomolybdic acid-levanol fast cyanine 5RN (TPL) method to identify normal and modified MCs, by staining microfilaments in their cytoplasm, in plastic-free semithin sections from the epoxy blocks initially prepared for transmission electron microscopy.

MATERIALS AND METHODS

To check the effect of glutaraldehyde fixation on TPL staining, preliminary observation was made on 2.5% glutaraldehyde-fixed, paraffin-embedded small specimens selected from the tissues including skeletal muscles, salivary glands and pleomorphic adenomas. The stainability was compared with that of the materials fixed with Carnoy's No. 2 solution and satisfactory staining of positive structures was confirmed.

Experiments were carried out on 24 human biopsy specimens from the following types of tissues: normal and chronically inflamed submandibular gland, pleomorphic adenoma, adenoid cystic carcinoma and epithelial-myoepithelial carcinoma of intercalated duct origin. These tumor types were chosen as test objects because

FIG. 1. Carnoy-fixed, paraffin-embedded tissue from chronic sclerosing sialadenitis. Myoepithelial cells in proliferating ductules are intensely stained by Levanol Fast Cyanine 5RN. TPL $\times 400$

FIG. 2. TPL staining of normal submandibular gland following removal of the epoxy resin. Myoepithelial cells surrounding acini are clearly recognizable. $\times 400$

myoepithelial participation in their formation has been well documented.

The fresh specimens were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 2–3 hr and washed overnight in the same buffer. They were postfixed in cold cacodylate-buffered 1% osmium tetroxide for 1 hr, dehydrated in graded ethanols and embedded in Epon 812-Araldite 502 mixture (6). Sections, 2–3 μ m thick, were cut on a LKB Ultratome III with glass knives, floated onto clean glass slides with a drop of distilled water, flattened by warming gently and dried on a hot plate at 80°C. Ultrathin sections serial to these semithin sections were examined with a HITACHI H-500H electron microscope at 75 kv.

For removal of the epoxy resin, slides with semithin sections were placed in a saturated solution of potassium hydroxide (KOH) in absolute ethanol (2) for 5–10 min and subsequently washed in four changes of absolute ethanol in order to remove KOH completely. Sections were washed well in distilled water for 5 min prior to oxidation using 0.5% potassium permanganate (KMnO₄) for 20 min. Sections were finally bleached in 1% oxalic acid and washed in three changes of distilled water. Other oxidants such as peracetic acid and hydrogen peroxide were also tested.

Staining with levanol fast cyanine 5RN (Color Index: 26360) was carried out according to the procedure described by Puchtler *et al.* (9). For purposes of control, sections were stained without prior oxidation in KMnO₄. Staining without

FIG. 3. Modified myoepithelial cell in pleomorphic adenoma. Note the presence of developed bundle of microfilaments (mf). $\times 8,800$ Inset: Corresponding deresined section. The majority of tumor cells exhibit positive reaction. TPL $\times 400$

removing the resin was also tried. No special modification was introduced in the staining technique other than pretreating the tissues with KMnO_4 and prolonging the action of tannic acid up to 30 min for maximum color contrast.

RESULTS

With the modified TPL procedure described above, the intact epoxy sections showed non-specific, pale blue coloring of resin, while the sections stained after resin removal displayed specific stainability. Although the color contrast was less intense than that in Carnoy-fixed, paraffin-embedded sections (Fig. 1), MCs in normal salivary glands (Fig. 2) and both the classic and modified MCs in salivary gland tumors (Figs. 3–5 insets) were easily identifiable. Deep blue cytoplasm of these cells contrasted well with red nuclei and yellow cytoplasm of other cells. Smooth muscle cells in the blood vessel walls, skeletal muscle fibers and red blood cells, if present, were also colored dark blue and could be used as good positive control. The best staining properties of different samples were obtained at various time intervals of KOH treatment ranging from 5 to 10 min. Except for a notable degree of cellular shrinkage, the structural detail of the tissues appeared to be undamaged by the solvent action. The patchy staining of the connective tissue by the dye often seen in glutaraldehyde-fixed, paraffin-embedded sections was significantly abolished in deresined sections.

FIG. 4. Myoepithelial-like cell in adenoid cystic carcinoma. Note developed microfilaments (mf) with focal densities and replicated basal laminae (bl). $\times 10,000$ Inset: Corresponding deresined section showing positivity of peripheral cells. TPL $\times 200$

FIG. 5. Epithelial-myoeplithelial carcinoma of intercalated duct origin. Outer myoeplithelial-like cell contains microfilaments (mf) with focal densities. $\times 6,000$

Inset: Deresined section from the same epoxy block. Cells of the outer layer in neoplastic ducts are positively stained (arrows). Apical cytoplasm of the inner luminal cells are also faintly stained (arrow heads). TPL $\times 400$

Staining without prior oxidation of the sections abolished binding of the dye with MCs. Pretreatment with KMnO_4 yielded successful staining, whereas no such effect was obtained with other oxidants tested in this study.

Stainability of MCs and modified MCs with TPL was revealed by electron microscopic observation of the adjacent ultrathin sections to correspond with the presence of developed bundles of microfilaments with peripheral plaques and occasional focal densities (Figs. 3–5). Faint staining of the apexes of the duct-lining epithelial cells was in accordance with the presence of microfilaments in the terminal webs.

DISCUSSION

The TPL technique has been used for demonstration of myofibrils in striated and smooth muscle cells, myoendothelial cells and MCs in paraffin-embedded tissues. Different fixatives were tested for this staining method. Puchtler *et al.* (10) recommended Carnoy or methacarn fixation for successful staining. According to van Bogaert *et al.* (14), the best results were achieved with Baker's formol-calcium. These authors (15) also recommended the modification of some steps

of the TPL procedure such as oxidation, nuclear staining and differentiation, to enhance the selectivity of MC staining.

The application of TPL staining has never been tested in plastic-embedded tissues. Our rather good results in glutaraldehyde-fixed, paraffin-embedded small tissues prompted us to apply this method to epoxy-embedded tissues. Staining of underresined sections was found to produce no reactivity, indicating inability of the dye to penetrate the epoxy resin, and selective staining was obtained only after the resin removal. The sections from recently polymerized blocks required shorter resin-removal time than those from the blocks stored for several years. Therefore, the time of resin-removal procedure to achieve optimum plastic-free condition must be controlled by trial and error for each sample.

In previous reports (1, 7), successful staining of osmicated tissue sections for light microscopy was achieved after release of the osmium blockade using oxidant agents. Our experience with TPL staining without the oxidation step was also not successful. Munger (7) found that the best results could be obtained by pretreatment of the sections with peracetic acid. Aparico and Marsden (1) also reported successful staining using hydrogen peroxide. In our hands, however, these oxidants were not satisfactory, whereas specific stainability of the tissues was obtained following KMnO_4 pretreatment.

The deresin-modified TPL method established in the present study allowed screening of that particularly interesting class of cells, neoplastic MCs, in epoxy-embedded tissues from various salivary gland tumors and could facilitate the examination of sections from a single block of these tumor tissues for direct correlation of light and electron microscopic observations (8).

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