ELECTRON MICROSCOPY OF ENDOTHELIAL CELLS IN CULTURE: II. SCANNING ELECTRON MICROSCOPY AND OTOTO IMPREGNATION METHOD

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SUMMARY: A method is described whereby endothelial cells are treated with several cycles of osmium and thiocarbohydrazide then critical point dried. Cells grown on Formvar-coated gold grids can be examined directly by transmission electron microscopy, cells grown on glass strips are mounted on stubs for scanning electron microscopy and are scanned without further coating with gold or palladium.

Key words: scanning electron microscopy; endothelial cells; osmium impregnation.

I. INTRODUCTION

The most striking ultrastructural characteristic of endothelium in vivo or in vitro is the extremely flattened morphology of the cells. This means that transverse sections yield an image of a very thin cell with a paucity of organelles (3,6) and surface specializations that occur sporadically, like endothelial projections, tend to be missed. However, scanning electron microscopy (SEM) allows an appreciation of the extent and distribution of surface features and reveals a profusion of surface projections (7,9). Internal organelles such as mitochondria appear as simple circular profiles in transverse sections. Using the OTOTO method, which allows examination of unsectioned thin portions of endothelial cells, mitochondria can be seen to be elongated branching structures (7) with an orientation similar to that of the cytoplasmic filament bundles and the rows of surface caveolae (8).

II. MATERIALS

A. Equipment

Transmission electron microscope (TEM), Philips 301, Philips[1]
Scanning electron microscope, ISI DS 130, International Scientific Instruments[2]
Critical point drier, Polaron E3000, Polaron[3]
Polaron grid specimen carrier, no. 1212, Ted Pella[4]
Polaron specimen boat, no. 1214[4]

B. Glassware and supplies

Grids, gold, no. IGG200 or no. IGG300[4]
Cover glass size 1½, no. M6022-2, American Scientific Products[5]
Stubs for SEM (we use 9/16″ diameter with ¼″ diameter stems airplane rivets with the stems cut off to an overall height of ½″)
Diamond scriber, no. 54463[4]
Falcon plastic tissue culture dishes, no. T4155-1[5]
Small test tubes, no. 1290-3[5]

C. Reagents and supplies

Glutaraldehyde, 25% stock, no. 0376, Polysciences[6]
Cacodylic acid, sodium salt, no. 3880[6]
Sucrose, no. 4072-1[5]
Hanks' buffered salt solution, no. 310-4060, GIBCO[7]
Thiocarbohydrazide (TCH), no. 1211[6]
Osmium, no. 18456[4]
Freon 113, (1,1,2-Trichloro,-1,2,2-Trifluoroethane), ACS, no. W591-9[5]
Formvar, no. 19221[4]
Propylene oxide, no. 18602[4]
Conductive silver paint, no. 18602[4]
Endothelial cells grown on Formvar-coated gold grids (for TEM)
Endothelial cells grown on glass cover slip pieces (for SEM)

III. PROCEDURE

Notes on handling glass strips with cells grown on them for scanning microscopy:

Place the strips of glass in small test tubes in water after step 2 (*below*). Replace fluids in such a manner that the cells are never exposed to air.

Notes on handling Formvar-coated gold grids:

The Formvar-coated gold grids are given a light

carbon film in a vacuum evaporator and sterilized under ultraviolet light before seeding with the endothelial cells. A drop of the cell suspension is placed on each grid and allowed to stand in the covered culture dish for 15 min before flooding with growth medium. This gives the cells a chance to begin their attachment to the coated grid where they will subsequently grow. These grids will generally stay in place in the plastic culture dish through all the handling if care is taken in all steps.

1. Wash medium off the cells with Hanks' balanced saline.
2. Flood on fix: 2.5% glutaraldehyde
   0.05M sodium cacodylate
   6.0% sucrose
   (4% glutaraldehyde may be used in place of 2.5% glutaraldehyde)
3. Wash off fix with distilled water. Use copious quantities but be gentle.
4. Postfix with osmium (1%) in distilled water for 45 min.
5. Wash off osmium with copious quantities of water, be gentle.
6. Treat with 1% TCH for 30 min. (Make up 1% TCH in distilled water and spin down *before use*. Use the supernatant only. Mix immediately before use.
7. Wash off the TCH with copious quantities of distilled water.
8. Repeat step 4 for 30 min.
9. Repeat step 5. One more cycle of TCH and osmium with appropriate washing may be completed (=OTOTO).
10. Dehydrate with an ethanol series, 30, 50, 70, 95, 100% (several changes of 100%). Each step is 10 min with gentle agitation to achieve complete exchange of ethanol.
11. Replace the last 100% ethanol with Freon 113.

    For critical point drying the grids may be placed in a Polaron grid specimen carrier. Many grid spaces will be suitable for examination by TEM at 80 or 100 kV. Again, keep the grids covered with fluids, and carefully perform the final transfer to Freon 113 in the specimen boat under Freon 113. Gold grids are very delicate so special care is needed in all handling. Mitochondria are clearly visualized in the thin peripheral areas of the cells. The Formvar-coated grids with cells and the glass strips treated by OTOTO are critical point dried from liquid CO_2 in the Polaron critical point drier. They are now ready for scanning microscopy (the glass strips) with no further coating. Also the gold grids are ready for direct examination by TEM. Attach the glass pieces to the SEM stubs with conductive silver paint using care that the paint flows onto the edge of the glass pieces but does not flow over the cells.

IV. DISCUSSION

A full appreciation of endothelial cell morphology requires the application of several electron microscopic techniques. Routine examination of thin sections by TEM yields information on intracellular organelles useful for identification, such as Weibel Palade bodies if present, and for monitoring the quality of endothelial cells over a period of time in culture. However, to examine the surface over which, in vivo, the blood flows it is necessary to use SEM or surface replica (5) techniques. Once recognized by SEM (9) endothelial projections can easily be identified in thin sections. The advantage of the procedure described in this paper is that the OTOTO method (1,2) can be used to prepare cells for both SEM (when grown on glass) or for TEM of intact unsectioned endothelial cells (when grown on coated grids). Thin sectioning can lead to a number of artifacts (4) and necessitates serial reconstruction if three-dimensional views are required. However, the OTOTO method allows examination of thin portions of the endothelial cells (peripheral areas not over the nucleus) at 80 to 100 kV and yields information on the spatial organization of cellular organelles.

V. REFERENCES

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[1] Philips Electronic Instruments, Inc., Mahwah, NJ
[2] International Scientific Instruments, Milpitas, CA
[3] Polaron Instruments, Inc., Hatfield, PA
[4] Ted Pella, Inc., Tustin, CA
[5] American Scientific Products, Miami, FL
[6] Polysciences, Inc., Warrington, PA
[7] GIBCO, Grand Island, NY