Corneal endothelium in vitro:
Characterization by ultrastructure and histochemistry

George M. Lowry

Rose chamber primary explant cultures of rabbit corneal endothelium were studied in comparison with freshly excised endothelium by means of phase and electron microscopy and dehydrogenase histochemistry. Rapid outgrowth, high mitotic rate, and monolayer covering of the growth surface with polygonal cells were characteristics of the explant cultures. Electron microscopy of endothelial outgrowth into a gelatin foam substrate revealed well-developed desmosomes and intracellular fibrillar systems in the cultured cells. These structures were not present in freshly excised endothelium and are considered to be adaptations to growth in a three-dimensional substrate. Cultured cells contained increased amounts of glycogen and decreased rough-surface endoplasmic reticulum and mitochondria in comparison with freshly fixed tissue. Histochemical reactions specific for lactic dehydrogenase demonstrated high activity in cultured cells and in areas of fresh endothelium subjected to slight mechanical trauma. No significant lactic dehydrogenase activity was evident in freshly excised non-traumatized endothelium. Rapid morphologic and physiologic adaptation to certain radical environmental changes has been demonstrated by corneal endothelial cells in these experiments.

The corneal endothelium is a monolayer of mesodermally derived squamous cells lining the posterior corneal surface and continuous with the endothelium of the trabecular meshwork. Primary endothelial dystrophies and the rapid response of the endothelium to penetrating corneal injuries have long been recognized. Knowledge of the physiology of normal corneal endothelium, however, has been difficult to obtain because the small endothelial cell mass present on the cornea is relatively unapproachable by the standard methods of cell physiology. The work of Leber published in 1873 is probably the earliest report indicating the importance of normal corneal endothelium to preservation of vision. Recent experimental studies involving donor corneal tissue stored for use in full-thickness corneal grafting have demonstrated the necessity of maintaining a viable corneal endothelium if corneal transparency is to be preserved following implantation in the host eye. Further investigation has shown that endothelial cells are apparently more sensitive to environmental conditions imposed by usual donor tissue storage techniques than are the other cellular elements of the cornea.

Certain features of the corneal endothelium environment in vivo are uniquely analogous to conditions imposed upon cells maintained in vitro. These features

From the Department of Ophthalmology, Washington University School of Medicine, St. Louis, Mo.
This study was supported in part by a research grant, B-621, from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Md.
include monolayer growth on an acellular substrate and direct contact with a relatively large volume of acellular, nutrient-containing medium. The possibility of adapting corneal endothelium to growth in tissue culture was demonstrated in 1929 by Matsuo. Stocker and associates have recently described techniques for establishing pure cultures of each of the three major cell components of the cornea, i.e., epithelium, stromal fibroblasts, endothelium. The relative ease of establishing tissue cultures of corneal endothelium stands in marked contrast to the apparent sensitivity of these cells to storage conditions which maintain viability of the other corneal cellular elements.

Phase microscopy, electron microscopy, and certain histochemical reactions were used in the present study to define the characteristic pattern of endothelial adaptation to an in vitro system and to determine differences in structure and function between endothelial cells in vivo and in vitro. Evidence that corneal endothelium assumes a higher degree of morphologic differentiation in vitro than in vivo is presented.

Materials and methods

Culture methods. Young male albino rabbits averaging 500 grams were killed instantly by a blow to the neck, and the eyes were immediately removed. Corneas were excised aseptically and placed in Petri dishes containing culture medium. By microdissection (see reference 5), it was possible to separate from the corneal cup a layer consisting only of Descemet's membrane with its endothelial lining. With minimal trauma to the endothelial surface, the composite membrane was cut into explants averaging 2 by 3 mm. Cultures were established and maintained in Rose chambers beneath dialysis membrane. Eagle's basal medium supplemented with fetal bovine serum to 10 per cent final concentration was used for all cultures. Penicillin (10 units per liter) and streptomycin (100 μg per liter) were added to the medium. Cells to be studied by light microscopy or histochemistry were grown directly on glass. Tissue to be processed for electron microscopy was explanted onto thin strips of Gelfoam.

surgical sponge, a nonantigenic preparation of expanded gelatin. This procedure permits cellular outgrowth into a substrate which can subsequently be processed and sectioned for the electron microscope along with the cell population.

After a period of seven to ten days in vitro, medium having been changed once during that time, cultures were fixed and processed as required. Control material consisting of fresh tissue obtained by the above dissection methods was immediately either fixed for electron microscopy or stained for light microscopy.

Electron microscopy. Tissue and cultures to be used for electron microscopy were fixed at 4°C for 60 minutes in 1 per cent osmium tetroxide or for 90 minutes in 3 per cent glutaraldehyde followed by 60 minutes post fixation in 1 per cent osmium tetroxide. Both fixatives were buffered to pH 7.4 with phosphate buffer. After rapid dehydration, tissues were embedded in Araldite.

Light gold and silver sections cut with glass knives on a Porter-Blum ultramicrotome were mounted on uncoated copper grids and stained with lead hydroxide. Some sections were counterstained in saturated alcoholic uranyl acetate. Confirmation of the presence of glycogen indicated in lead stained sections was obtained by floating representative grids for 2 minutes on a filtered 2 per cent solution of crude bacterial amylase in isotonic phosphate buffer at pH 7.0, followed by washing and lead hydroxide staining. Micrographs were taken on an RCA EMU-3G electron microscope at 50 kilovolts.

Histochemistry. Dehydrogenase activity specific for lactate, succinate, and glucose-6-phosphate was assayed by means of tetrazolium dye reduction reactions. Cultures or freshly dissected tissue were incubated for 45 minutes at 37°C in a solution consisting of 5 c.c. of 0.1M substrate, 4 c.c. of Sorensen's phosphate buffer (pH 7.0), 5 c.c. of p-nitro blue tetrazolium f (1.0 mg. per cubic centimeter), and 5 c.c. of DPNf (TPN for glucose-6-phosphate) (1.0 mg. in 5 c.c. of distilled water). The pH of the final mixture was adjusted to 7.0 with 1.0M NaOH. Phase microscopy and the May-Grunwald-Giemsa stain were used routinely to demonstrate in vitro morphology.

Results

Tissue culture. Microdissection with direct explantation has yielded a series of over two hundred successful Rose chamber cultures with rapid outgrowth from each of the three to five explants per chamber. Mitotic figures of all stages in stained

*Microbiological Associates, Bethesda, Md.
1Through the courtesy of the Upjohn Company, Kalamazoo, Mich.
2Through the courtesy of the Upjohn Company, Kalama-zoo, Mich.
preparations averaged thirty per thousand when cultures were terminated after seven to ten days in vitro. Cells subcultured from Rose chambers have been maintained in flask cultures for five to six passages before being discarded. In flask culture, individual cells retained a polygonal form and paved the growth surface in typical monolayer distribution. Outgrowth from the primary explant appeared within 24 hours. Cells of the early outgrowth were polygonal with an undulating cytoplasmic membrane directed away from the explant (Fig. 1). Cell division and migration produced a halo of outgrowth surrounding the explant and averaging 40 to 50 cells in breadth at the time cultures were terminated. A gradient in cell surface area was evident radially across the outgrowth with large, irregular cells at the periphery and smaller polygonal cells predominant in the remainder of the culture (Fig. 2 and 3).

In areas of especially dense cellularity a spindle form was occasionally seen.

Ovoid nuclei with one to five nucleoli were characteristic in living cells observed by phase microscopy. Occasional binucleate cells indicated that amitotic division probably occurs in vitro as well as in vivo. Outgrowth from tissue explanted onto the gelatin sponge preparation extended through the interstices of the substrate and spread onto the glass surface of the chamber in a typical monolayer of polygonal cells indistinguishable from the outgrowth of cultures explanted directly onto glass. Rate of outgrowth as assessed by daily observation of the extension of the outgrowth halo was similar for both substrates. The form assumed by cells within the Gelfoam sponge appeared to be dependent upon configuration of the sponge and upon attachments to the substrate or to other cells. Thus cellular forms rang-

Fig. 1. Twenty-four hour outgrowth from primary explant of corneal endothelium/Descemet's membrane preparation. Polygonal cells with broad undulating cell membranes predominate. (Original magnification ×120.)
Outgrowth after ten days in vitro. Polygonal epithelioid form has been maintained. Fig. 2. Central area of outgrowth. Explant is densely stained mass at lower right. Fig. 3. Peripheral outgrowth. Note presence of large cells at the periphery and occasional binucleate cells. (May-Grunwald-Giemsa. Original magnification ×125.)

From flat polygonal to irregularly spherical were present within the sponge substrate (Fig. 4). An area of undulating cytoplasmic membrane associated with intracellular vacuoles varying in their refractive index was characteristic of cells in the early outgrowth and of some cells at the periphery of the later outgrowth halo. By time-lapse phase contrast photomicrography of various cell types in vitro, Pomcrat\(^{11}\) has associated this membrane/vacuole pattern with pinocytosis.

**Electron microscopy.** Control tissue fixed immediately after dissection appeared quite similar to descriptions of the ultrastructure of in situ corneal endothelium\(^{12, 13}\) (see Fig. 5 and 6). The cells were flattened and closely adherent to Descemet's membrane. Cytoplasmic membranes formed complex intercellular junctions by interdigitation, but cell surfaces in contact with Descemet's or with aqueous humor were smooth. Occasional terminal bars were present at intercellular regions on the cell surface normally contacting aqueous. These structures were evident only in sections of tissue fixed in osmium tetroxide alone and were not a constant feature. The cytoplasm contained extensive sacculations of rough-surface endoplasmic reticulum and many ovoid mitochondria with concentric or transverse cristae. Nuclei contained a dense karyoplasm and were flattened parallel to Descemet's membrane. Invaginations of cytoplasm formed frequent clefts and depressions in the nuclear membrane. No intracellular fibrils were observed in the control tissue. Desmosomes were not observed either between adjacent cells or at the endothelium/Descemet's interface. Glycogen granules were not a prominent

---

**Fig. 2** (left) and **3** (right). Outgrowth after ten days in vitro. Polygonal epithelioid form has been maintained. **Fig. 2.** Central area of outgrowth. Explant is densely stained mass at lower right. **Fig. 3.** Peripheral outgrowth. Note presence of large cells at the periphery and occasional binucleate cells. (May-Grunwald-Giemsa. Original magnification ×125.)

**Fig. 4.** Outgrowth into surgical sponge substrate (see text) from primary explant. The explant occupies the left half of the field. Cells of the outgrowth (arrows) assume varying forms. (Original magnification ×125.)
Fig. 5. Electron micrographs of control tissue fixed immediately after dissection from cornea. Full thickness section of dissected layer similar to that used for culture explants. Descemet’s membrane has no adherent stromal material attached. (Original magnification ×8,000.)

Fig. 6. Higher power micrograph of endothelial layer. Extensive rough surface endoplasmic reticulum (er) and many ovoid mitochondria (m) are present. Nuclear chromatin is dense. An intercellular junction (if) is seen but no junctional complexes are evident. (Glutaraldehyde–osmium tetroxide fixation. Lead hydroxide stain. Original magnification ×8,000.)
feature in the rapidly fixed, lead-stained sections of control tissue."

Endothelium maintained in vitro underwent several striking modifications visible in electron micrographs. No longer confined to the flat surface of Descemet's membrane or to the smooth glass surface of a culture vessel, the endothelial cells assumed various forms apparently in response to the shape of the gelatin sponge substrate and to newly developed intercellular connections (Fig. 7 and 8). The simplest type of junctional complex consisted of parallel osmiophilic thickenings in segments of adjacent cell membranes. The intercellular space between the thickened membranes was approximately 250 Å across and frequently contained two parallel bands of osmiophilic material (Fig. 9 and 10). A localized area of dense cytoplasm surrounded most junctional regions. Cytoplasmic fibrils about 40 Å in diameter converged on this dense area in many observed complexes. Fibrils associated with

Fig. 7. Low power electron micrograph of outgrowth from primary corneal endothelium explants, ten days in vitro, illustrating intertwined villous projections and many junctional complexes joining adjacent cells. Intracellular fibril bundles are prominent especially in central cell. (Glutaraldehyde-osmium tetroxide fixation, Lead hydroxide stain. Original magnification ×4,000.)

Fig. 8. Junctional zone between two flattened cells (see Fig. 7). Two junctional complexes are visible (arrows). (Original magnification ×8,000.)
Ultrastructure and histochemistry of corneal endothelium

Fig. 9. Junctional complexes in vitro closely resembling the in vivo desmosome. Sections from ten-day outgrowth of corneal endothelium explanted onto gelatin sponge. Prominent junctional complex at right center. Slight adjacent cytoplasmic density is present. (Glutaraldehyde-osmium tetroxide fixation. Lead hydroxide stain. (Original magnification x19,000.)

A junctional complex could often be traced into a fibril bundle elsewhere in the cytoplasm. In one example (Fig. 10), two adjacent complexes oriented roughly perpendicular to each other not only were seen to be associated with fibrils from bundles deep within the cytoplasm but also appeared to be connected by short fibrils running at the cell periphery between the areas of cytoplasmic density. Villous cellular extensions frequently bore junctional complexes at their distal tips if in contact with another cell (Fig. 11). The deeper cytoplasmic fibrils referred to above were characteristically in bundles of parallel strands oriented approximately parallel to the nearest segment of cell membrane. The number of fibril bundles observed per cell varied widely but seemed more numerous in cells no longer attached to Descemet's membrane.

Cytoplasm of the cultured cells contained large numbers of coarse granules which stained with lead. The appearance

Fig. 10. Adjacent junctional complexes oriented approximately perpendicular to each other (see Fig. 9). Long fibrils from within the cytoplasm extend to each complex. In addition, short fibrils appear to connect the junctional regions directly. (Original magnification x25,000.)
of these granules was consistent with descriptions of intracellular glycogen aggregations.\textsuperscript{14} Amylase digestion markedly reduced the number of these granules. Ovoid mitochondria with concentric or transverse cristae were present but were not as numerous as in normal in situ endothelium. Rough-surface endoplasmic reticulum with closely spaced ribosomes lining the sacculations was prominent in the cultured cells but to a lesser extent than in uncultured tissue. A Golgi region was present in many cells, frequently in association with vacuoles of varying electron density (Fig. 12).

With the exception of dense nucleolar areas, nuclei of the cultured cells contained karyoplasm less dense than that observed in control nuclei. Deep clefts and invaginations in the nuclear membrane produced occasional bizarre nuclear forms. Complex interdigitation of narrow cytoplasmic processes was seen in many areas. This pattern was reminiscent of the tortuous contours formed by adjacent cells in the endothelium of the normal eye.\textsuperscript{15} Sections which contained areas of transitional growth from Descemet's membrane into the substrate demonstrated the tendency of endothelial cells to migrate from Descemet's in contact with other cells (Fig. 13). A general tendency to maintain intercellular contact was evident.

**Histochemistry.** Tetrazolium reduction techniques utilizing p-nitro blue tetrazolium demonstrated the presence of lactic, succinic, and glucose-6-phosphate dehydrogenases in cultured endothelium. The same techniques applied to freshly excised tissue revealed succinic and glucose-6-phosphate dehydrogenases but no significant reduction of the tetrazolium salt with lactic acid as the substrate. Exceptions to this were areas subjected to slight mechanical trauma during the dissection. Such areas retained normal cellular morphology by
Fig. 12. Corneal endothelial cell 10 days in vitro. The section demonstrates a well developed Golgi region (g) associated with a membrane-limited vacuole (v). (Glutaraldehyde-osmium tetroxide fixation. Lead hydroxide—uranyl acetate stain. Original magnification ×13,000.)

Table I

<table>
<thead>
<tr>
<th></th>
<th>Lactic dehydrogenase</th>
<th>Succinic dehydrogenase</th>
<th>Glucose-6-phosphate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>±</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Control</td>
<td>±</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Cultured tissue</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Experimental</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

light microscopy, but were characterized by intensely positive lactic dehydrogenase reactions. In each of the enzyme reactions activity appeared to be limited to cytoplasmic granules. Those reactions are summarized in Table I.

Discussion

Methods originally described by Stocker and colleagues for obtaining pure cultures of corneal endothelium have been applied in this laboratory with consistently good results. In any experimental work dealing with tissue cultures ostensibly of single
cell type, satisfactory evidence that the cells in question are actually of a pure histologic type must be presented. Because of the uniform population of polygonal cells in these cultures and because electron microscopic examination of tissue from a series of eight animals revealed no adventitious stromal cells on the anterior surface of Descemet's membrane, we have concluded that the cultures obtained by these methods are composed exclusively of corneal endothelium. Aggregations of cells immediately beneath Descemet's membrane which tended to cling to micro-dissected Descemet's/endothelium preparations have been described by von Sallman, working with rabbits of approximately the same age as those used in this work. We have not observed similar aggregations in the course of these experiments.

Chi and associates have described the pattern of endothelial renewal in vivo following mechanical denudation of Descemet's membrane. Their description of broad irregular cells at the advancing border of the regenerating endothelium and their observation that mitosis in the healing area is concentrated within the advancing sheet rather than at its leading edge are both strikingly similar to patterns seen in explant cultures. Von Sallman emphasizes a parallel idea in his observations that the mitotic index of in vivo corneal endothelium decreases with age, approaching a zero rate in the full-grown animal, and that surface area of endothelial cells increases with age either by a process of flattening or by increments in cell volume. His conclusion is that cellular flattening and volume increase become more important than mitotic rate in maintaining the endothelial covering as an animal ages. Thus two mechanisms which maintain an intact endothelial layer, i.e., cellular proliferation and increase in cell surface area, appear to function in vitro as well as in vivo. Mitotic indices in cultured endothelium (averaging 30 per thousand) and in areas of healing in situ endothelium are much higher than those calculated by von Sallman for normal endothelium in young rabbits (10.4 per 10⁶ in two-week-old animals). A logical conclusion is that physical interruption of the in situ monolayer is necessary to stimulate full response of the mitotic mechanism. Cutting the Descemet's/endothelium preparation into explants apparently simulates that condition.

Tetrazolium dye reduction reactions employing incubation systems containing specific enzyme substrates and necessary cofactors to demonstrate qualitatively or semiquantitatively the presence of certain dehydrogenases have proved useful in several studies of ocular tissue metabolism (see, e.g., references). Bamji has described lactic, succinic, and glucose-6-phosphate dehydrogenase activity in fresh, quick-frozen rabbit corneas. Studies of endothelial dehydrogenase activity in stored donor eyes has resulted in the use of tetrazolium reactions for lactic dehydrogenase as a rough measure of endothelial viability. For example, Pena-Carrillo and Polack have demonstrated minimal endothelial lactic dehydrogenase activity in fresh non-traumatized tissue but gradually increasing activity with time in storage at 4°C. The latter authors have also described a rapid rise in activity following freezing or slight mechanical trauma to the endothelium. Kaufman and colleagues have demonstrated increased lactic dehydrogenase activity in an endothelial halo surrounding an area of endothelium killed by full-thickness corneal freezing. They attribute this increased activity to the higher rate of energy metabolism necessitated by the healing process.

The studies presented here have confirmed the observation that lactic dehydrogenase activity rises in areas of slight mechanical trauma. In addition, this work has demonstrated that colonies of rapidly proliferating endothelial cells in vivo develop a similar but homogeneous increase in lactate dehydrogenase activity. Increased glycogen deposition in cultured endothelium as compared with the in vivo cells tends to support the concept of increased...
activity in vitro along pathways of energy metabolism involving lactic dehydrogenase. Whether the apparent increase in activity might also be secondary to altered membrane permeability cannot be determined by the methods used here. It should be pointed out, however, that highly positive tetrazolium reactions for lactic dehydrogenase will in some circumstances indicate an actively metabolizing corneal endothelium rather than a non-viable one.

The work of Jakus\textsuperscript{12, 21} and others\textsuperscript{13, 22, 28} on the ultrastructure of in situ corneal endothelium in various species offers a comprehensive background to further discussion of endothelial ultrastructure. Jakus\textsuperscript{21} has noted that junctional complexes are seen infrequently between corneal endothelial cells but that terminal bars are present in some species. Kaye and Pappas\textsuperscript{13} have observed continuous terminal bars between cells at the posterior endothelial surface of adult rabbit eyes. These authors further described hemidesmosomes on cell membranes in contact with Descemet's membrane. Iwamoto and Smelser\textsuperscript{22} have presented excellent electron micrographs of freshly fixed human corneal endothelium which illustrate desinate terminal bars at the posterior endothelial surface. In addition, these authors report the presence of scattered areas of thickened adjacent cell membranes, some of which contain an apparent intercellular dense band. These structures are felt to be poorly developed desmosomes. Current knowledge therefore suggests that terminal bars are present in the corneal endothelium of rabbit and man but that typical desmosomes, as described by Farquhar and Palade,\textsuperscript{24} are not found in this tissue in situ.

The present study has shown that corneal endothelium not only survives and proliferates in the artificial tissue culture environment but achieves a state of morphologic differentiation which is perhaps more advanced than that found normal conditions. Structures such as desmosomes and epithelial fibrils, which are not prominent components of in situ corneal endothelium, are characteristic of highly differentiated epithelia in other anatomic sites.\textsuperscript{24} Leure- duPree\textsuperscript{26} has used the appearance of junctional complexes and associated cytoplasmic fibrils as an index of maturity in ultrastructural studies of the developing lens. The significance of these structures in endothelium maintained in vitro is unknown at present; it is felt, however, that they are related to physical requirements of growth in a three-dimensional substrate. The seemingly facile adaptation of these cells to a radical environmental change, together with the apparent ability to develop structural components more advanced in a morphologic sense than those present in vivo, suggests that corneal endothelium maintains a high degree of differentiation in the tissue culture environment. Current investigation is directed toward application of these findings to problems of normal endothelial physiology, endothelial response to pharmacologic agents, and donor tissue storage.

The author wishes to acknowledge the generous advice and support of Dr. Bryce Munger, formerly of the Department of Anatomy, Washington University Medical School, and Dr. Bernard Becker, Department of Ophthalmology, Washington University Medical School. He wishes further to acknowledge the use of the facilities of the Department of Anatomy, Washington University Medical School, made available through the courtesy of Dr. Allen C. Enders.

REFERENCES

5. Stocker, F. W., Eiring, A., Georgiade, R., and Georgiade, N.: A tissue culture technique for growing corneal epithelial, stromal, and...


