FINE STRUCTURE AND COLLAGEN SYNTHESIS ACTIVITY OF MONOLAYER CULTURES OF RABBIT CORNEAL ENDOTHELIUM

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The corneal endothelium is a single layer of polygonal cells covering the posterior surface of the cornea. The apical free surface of these cells faces the anterior chamber and the basal surface rests on an unusually thick basement lamina, Descemet's membrane. This membrane is collagenous (1, 6, 11, 12) and is believed to be secreted by the endothelial cells (6, 13, 18). These cells form the primary barrier across which metabolic substrates enter and waste products leave the cornea (19, 23), and are believed to be responsible for the active transport processes by which the adult cornea maintains its normal hydration state (8, 14, 15).

Recently, a technique for mass culture of pure rabbit endothelial cells has been described (16). The present study was undertaken to ascertain by electron microscopy if these are pure endothelial cultures and to describe their fine structure. Further studies were undertaken to correlate biochemical evidence of collagen synthesis and fine structure in sister cultures.

MATERIALS AND METHODS

Corneas were excised from adult male albino rabbits sacrificed with 5 ml intravenous pentobarbital. Under sterile conditions in a complete culture medium (20), Descemet's membrane and endothelium were dissected from the underlying stroma of the corneas of 96 rabbits (21). Descemet's membrane-endothelium layers from six pairs of eyes were explanted into each of 16 30-ml Falcon tissue culture flasks (Falcon Plastics, Div. of B.-D. Laboratories, Inc., Los Angeles, Calif.) containing 3 ml of culture medium per flask. Cultures were incubated at 35°C in a mixture of 5% CO_2-95% air. At the same time Descemet's membrane-endothelium layers from three pairs of eyes were fixed immediately after dissection in 2% glutaraldehyde in Sorensen's phosphate buffer, post-fixed with 2% OsO_4 in Sorensen's phosphate buffer, and embedded in Epon for light and electron microscope evaluation of the possible contamination of the dissected layer with stromal fibroblasts.

When initial outgrowth (Fig. 1) from the explant was about 25 cell diameters, approximately 2–3 wk, the culture flasks were rinsed with 0.2% EDTA in sterile buffered saline and suspended by incubation for 1 h with 0.4 ml of 0.5% trypsin plus 0.4% EDTA in buffered saline. The suspended cells from each initial culture were transferred to a new flask with 3 ml of complete medium and the remnants of the original Descemet's membrane were discarded. 7–10 days after trypsinization, a confluent monolayer of endothelium (Fig. 2) was obtained which could be used for experimental or analytical purposes or subculture.

Confluent cultures intended for chemical analysis of

[^{3}H]proline incorporation were supplemented for 24 h with 75 μg/ml of sodium ascorbate before exposure to [^{3}H]proline. Cultures for electron microscopy and/or autoradiography were continually supplemented with ascorbate from selection to fixation (24 h–3 mo).

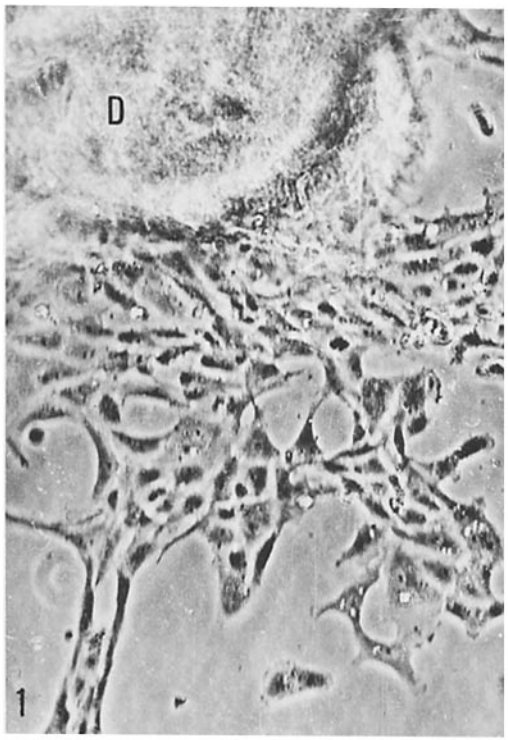
Some cultures were exposed for approximately 24 h to 5 mCi of L-[2,3H]proline (New England Nuclear, Boston, Mass., NET-323 45.7 CI/mmol) in 0.1 ml phosphate-buffered saline added to the medium. Of these, three monolayers were processed individually for chemical analysis of [^{3}H]proline uptake and synthesis of hydroxyproline by the method of Green, Goldberg, and Todaro (5), or by the method of Switzer and Summer (22), while sister cultures were fixed and processed for autoradiography and electron microscopy.

One 3-mo old culture with a hyaline layer evident beneath the degenerating cells was trypsinized, rinsed with distilled water, and subjected to three freeze-thaw cycles to disrupt the remaining cells. 24 h later, the distilled water was poured off and the membrane-like material scraped from the flask with a spatula. This material was resuspended in distilled water to which was added 3 ml of ether to dissolve any residual polystyrene. The suspension was then centrifuged, the supernate discarded, and the precipitate analyzed for hydroxyproline according to the method of Switzer and Summer (22).

Cultures were fixed either with 1% OsO_4 in Sorensen's phosphate buffer, pH 7.4, or with 2% glutaraldehyde in the same buffer, followed by rinsing and storage in buffer plus 8% sucrose, and postfixation in 2% OsO_4 in the same buffer. Cultures were dehydrated in ethanol and infiltrated and embedded in Epon 812 without passing through acetone or propylene oxide (4). For autoradiography, 1-μm Epon sections previously stained with toluidine blue in borate buffer were dipped in Kodak NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, N.Y.), exposed for 1 and 2 wk, and processed according to our routine procedures (10).

RESULTS

Light and electron microscope examination of the dissected Descemet's membrane-endothelium preparations from three rabbits showed no contamination by stromal fibroblasts. The plane of shearing is at the interface of Descemet's mem-



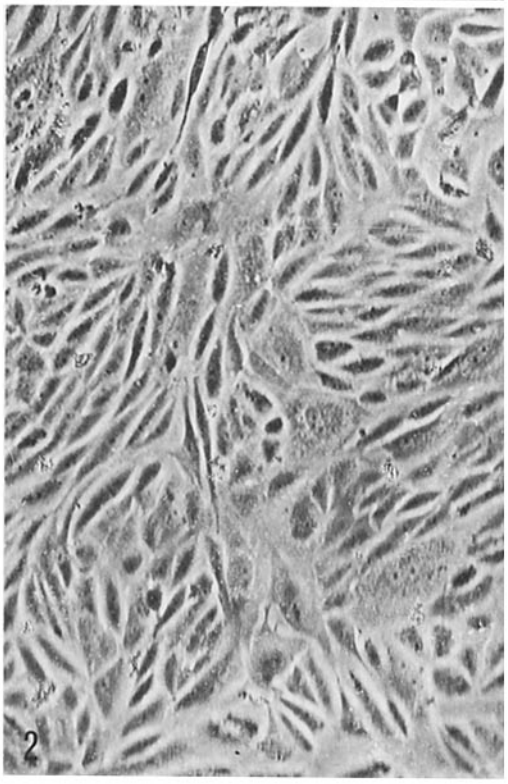
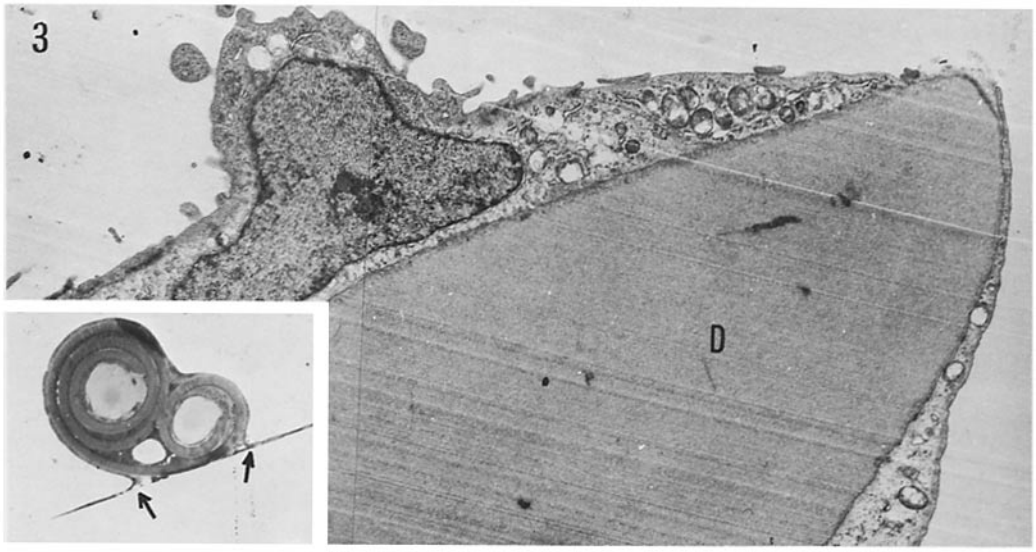


FIGURE 1 Initial outgrowth of rabbit corneal endothelium from Descemet's membrane-endothelium preparation placed in culture. *D*, Descemet's membrane. × 350.

FIGURE 2 Confluent monolayer culture of corneal endothelium approximately 3 wk after trypsinization and replating. × 350.



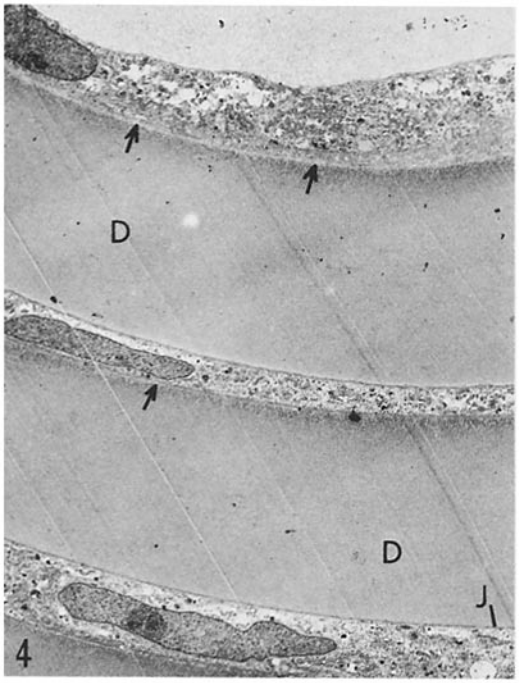
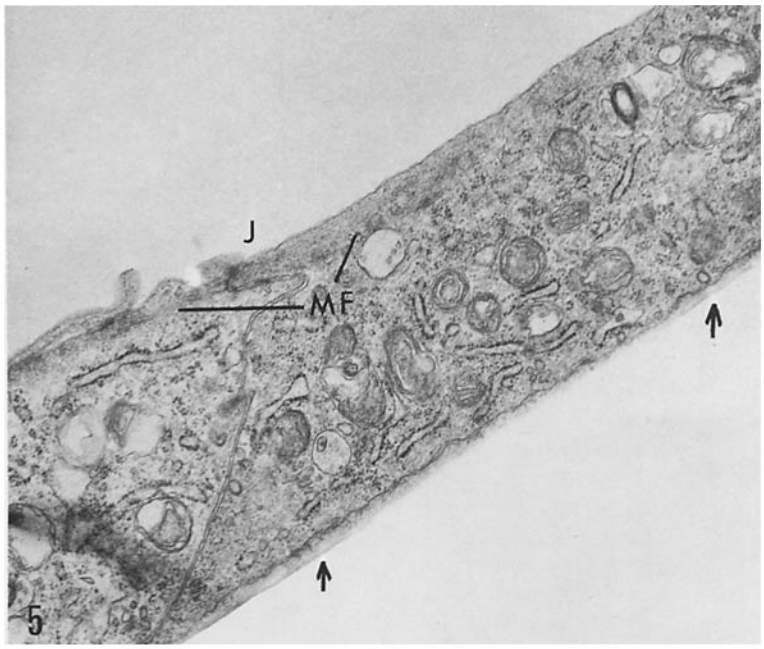


FIGURE 3 Initial outgrowth culture of corneal endothelium: Descemet's membrane forms a "jelly roll" in the culture medium (inset) and endothelium grows out to cover all surfaces of Descemet's membrane and then turns to grow out along the polystyrene surface of the culture flask (arrows). The composite electron micrograph shows one corneal endothelial cell migrating to cover the cut surface of Descemet's membrane (*D*) and a highly attenuated process of another extending over the cut surface. × 6,100. Inset, × 96.

FIGURE 4 Low-power electron micrograph of section through the "jelly roll" of the initial explant (see inset on Fig. 3). The endothelial cells maintain their normal polarity despite being in contact with Descemet's membrane (*D*) at both apical and basal surfaces. Newly secreted basement lamina material having a lower density than the mature Descemet's membrane is seen underlying the endothelial basal plasma membrane (arrows). *J*, junctional zone. × 2,000.



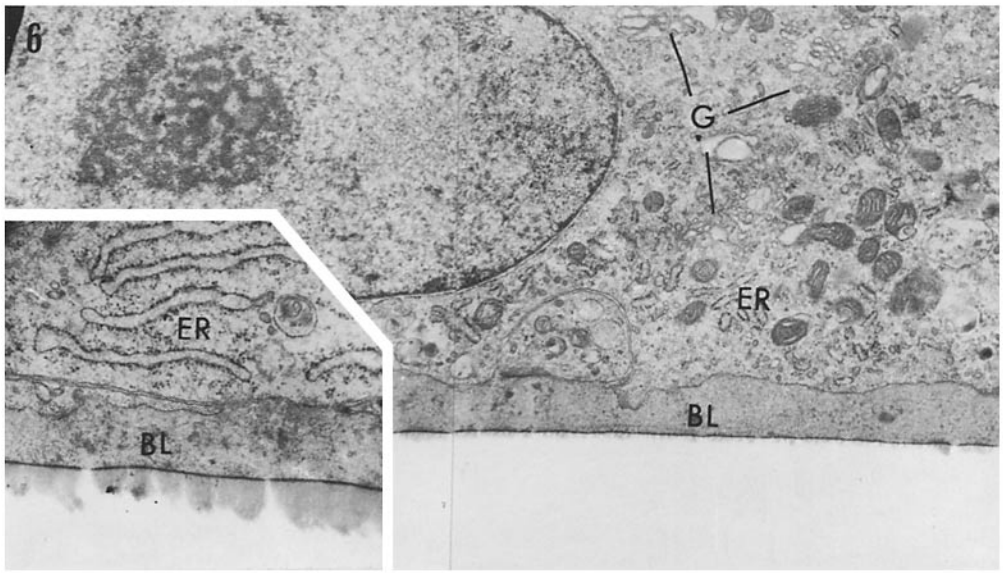


FIGURE 5 Electron micrograph of endothelial outgrowth along polystyrene surface in initial culture. The cells exhibit normal polarity with a junctional zone (*J*) at the apical end of the intercellular space and a microfilament network (*MF*) underlying the apical plasma membrane. Newly synthesized basement lamina material can be seen between the basal plasma membrane and the plane of the interface with the culture flask (arrows). Granular endoplasmic reticulum appears concentrated in the basal cytoplasm. × 11,500.

FIGURE 6 Composite electron micrograph showing a portion of a corneal endothelial cell in confluent monolayer culture. There is an increased number of cisternae of granular endoplasmic reticulum (*ER*) concentrated near the basal surface (see inset) and an increased number of Golgi complexes (*G*). Newly synthesized basement lamina (*BL*) is seen between the basal plasma membrane and the wispy remnants of the polystyrene of the culture flask. × 12,200. Inset × 17,000.

brane with the posterior surface of the most posterior collagen lamella. Previous morphologic studies (7, 9) have shown that the most posterior layer of stromal fibroblasts is at the anterior face of this collagen lamella.

Light and electron microscope examination of initial outgrowth cultures shows that the endothelial cells spread, divide, and migrate to cover all surfaces of Descemet's membrane (Fig. 3), and grow out in a monolayer where they come into contact with the polystyrene surface of the culture flask (Fig. 3).

The endothelial cells retain their normal polarity and intercellular junctions even when they are entrapped in the inner coils of the "jelly-roll" (Fig. 4) and are in contact with Descemet's membrane at both their apical and basal surfaces. The cells in culture show an increase in the number of cisternae of granular endoplasmic reticulum and of Golgi complexes (Fig. 5), particularly in the basal half of the cytoplasm.

Newly secreted material with a somewhat lower electron density than mature Descemet's membrane accumulates between the basal plasma membrane and the original Descemet's membrane (Figs. 4 and 5), and between the basal plasma membrane and the polystyrene of the culture flask in the original explant and initial outgrowth, respectively.

Similar secreted material accumulates between the corneal endothelial basal plasma membrane and the surface of the culture flask in the confluent monolayer cultures (Fig. 6). The thickness of this layer increases with length of time in confluent culture, reaching 0.5 μm in 3–4 wk after confluency. One culture maintained for as long as 3 mo had a subendothelial layer thick enough to be scraped from the flask with a spatula after lysis of the cells, and this was also subjected to chemical analysis. This layer was previously shown to be PAS positive (17).

Autoradiographs of both initial outgrowth cultures and confluent monolayer cultures exposed to [^{3}H]proline for 24 h before fixation showed heaviest localization of silver grains in a line immediately subtending the basal surface of the endothelial cells at the interface with the culture flask.

Chemical analysis of cultures grown in the presence of [^{3}H]proline and supplemented with ascorbate (75 μg/ml) shows an average incorporation of labeled imino acid into protein and hydroxylation of 4.8% of the protein-incorporated proline to hydroxyproline. This indicates an average ratio of collagen synthesis to total protein synthesis of 2.27% (Table I).

TABLE I

Rate of Proline Hydroxylation and Collagen Synthesis by Confluent Monolayer Cultures of Rabbit Corneal Endothelium

	HYPRO/ PRO	HYPRO/ PROnc	ΔC/ΔP
	%	%	%
Endothelial cells	5.7	5.8	2.7[4]
	3.9	4.0	1.8[4]
	4.8	4.9	2.3[22]
Control killed cells	0	0	—

Analysis was made by the method of Green, Goldberg, and Todaro (4), or by that of Switzer and Summer (22). Calculation of the ratios was done according to the equations of Green, Goldberg, and Todaro (4) with correction factors and constants derived by Kefalides (11) for basement membrane collagen as follows: proline/hydroxyproline 0.41–0.60 (used 0.5); and 4.1/8.8 = basement membrane collagen correction factor; PROnc cpm = PRO cpm, 0.5 (HY PRO cpm).
PRO, proline; PROnc, noncollagen proline; HY PRO, hydroxyproline.

The hyaline membrane in the 3-mo old culture which was analyzed for hydroxyproline by the method of Switzer and Summer (22) has an average hydroxyproline content of 7.35% by weight, whereas adult rabbit Descemet's membrane has an average hydroxyproline content of 7.7% as analyzed by the same method.

DISCUSSION

The present study demonstrates that rabbit corneal endothelium retains and reestablishes its normal morphology in initial outgrowth and confluent monolayer cultures, respectively. Incorporation and hydroxylation of labeled proline indicate a collagen synthesis rate equal to that of some fibroblastic cell strains (ratios of 1.5% to 10%) and greater than that of most established lines of fibroblastic origin (0.15% to 1.5%) (2, 3). Electron microscope studies of monolayer cultures show a secreted basement lamina which increases in thickness with time in culture and which labels with tritiated proline. Further, this basement material has a hydroxyproline content similar to that of normal adult rabbit Descemet's membrane (1, 12). This basement lamina produced in vitro morpho-

logically and biochemically resembles Descemet's membrane, which rabbit corneal endothelial cells secrete in vivo.

Not only do corneal endothelial cultures provide a system in which collagen synthesis may be studied, but they offer a unique model for a "pure epithelial" system in which many of the physiological and pharmacological studies previously conducted only on whole corneas in vivo and in vitro may be reexamined on the cell layer believed to be responsible for most of the transport and absorptive functions of the cornea (8, 14, 15).

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