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BOVINE CORNEAL ENDOTHELIUM IN VITRO

Elaboration and Organization of a Basement Membrane

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SUMMARY

Bovine corneal endothelial cells can be easily grown in culture using conventional techniques. The cultured cells closely resemble the parent, native endothelium. In culture the endothelium synthesizes and deposits, in a polar fashion, a well organized basement membrane that contains molecules which are characteristic of all basement membranes. Membrane deposition continues for at least a year and, during that time, it begins to acquire the unique, ordered substructure characteristic of the native membrane.

The corneal endothelium is a neural crest-derived, simple squamous epithelium that covers the posterior surface of the cornea [1]. This endothelial layer maintains the clarity of the cornea by pumping salts and water out of the connective tissue stroma and into the anterior chamber of the eye [2]. Corneal endothelial cells also elaborate a conspicuously thick basement membrane termed Descemet's membrane, the formation of which begins soon after the invasion of the primary corneal stroma by fibroblasts [3]. Descemet's membrane increases in thickness throughout life, apparently as a result of the ongoing synthesis and deposition of membranous elements by the corneal endothelium [4]. In addition to being unusually thick, the membrane possesses a distinctive internal substructure which, when viewed in tangential section, consists of a series of hexagonally arranged 'nodes' interconnected by fine filaments. This substructure, which is present to a variable extent in amphibians, birds, and mammals, results in a cross-striated or banded appearance when transverse sections of the membrane are viewed [5].

Successful primary outgrowths of corneal endothelium have been observed from explants of a Descemet's membrane–endothelial cell complex that had been manually dissected from the corneal stroma [6–9]. Perlman et al. [10] were the first to describe the synthesis and deposition of a collagen-containing extracellular matrix by cultured (rabbit) corneal endothelial cells. Subsequent studies by Tseng et al. [11] and Gospodarowicz et al. [12, 13] using bovine corneal endothelial cells grown in the presence of an exogenously supplied mitogen, fibroblast growth factor (FGF),

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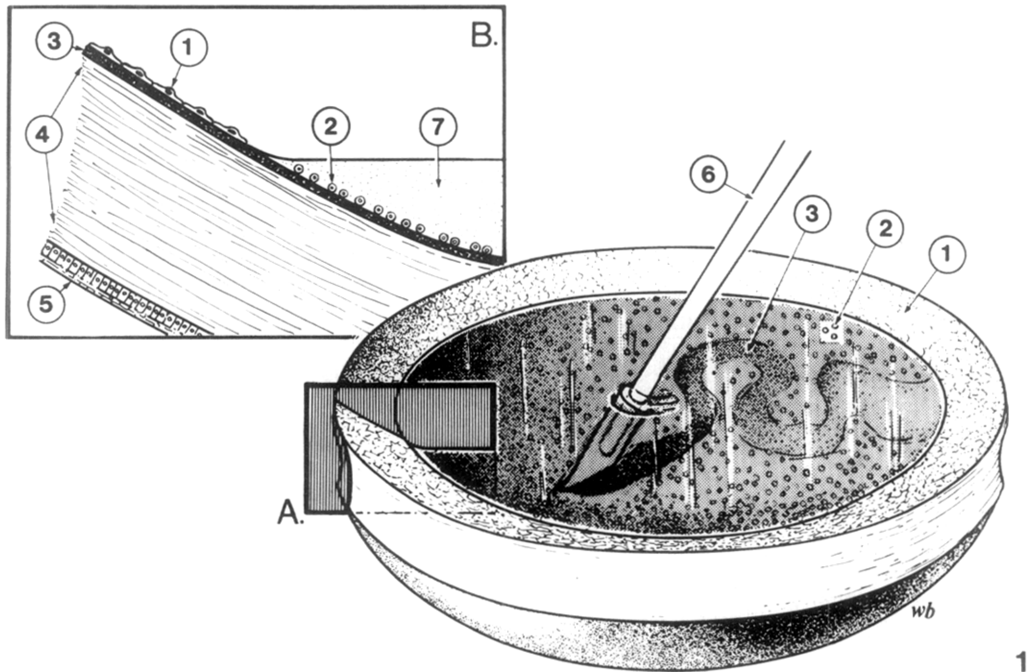

Fig. 1. Schematic illustration of method used to harvest bovine corneal endothelial cells. Endothelial surface is covered with a trypsin–EDTA solution and retracted cells dislodged using a silicone rubber spatula. Care is taken to avoid the cut stromal surface [4] by keeping separating solution level low, thus creating a surrounding ring of uncovered endothelial cells [1]. Area shown in (*B*) represents a section made through the cornea in a plane similar to that indicated in striped rectangle (*A*). *1*, Endothelial cells; *2*, retracted endothelial cells covered with trypsin–EDTA; *3*, Descemet's membrane exposed following removal of cells; *4*, cut stromal surface; *5*, corneal epithelium; *6*, rubber spatula; *7*, trypsin–EDTA solution.

demonstrated that endothelial cells from this species also synthesize collagen types III, I, IV and V, fibronectin and laminin.

In this paper we describe a simple procedure for growing bovine corneal endothelial cells in culture that does not require the addition of exogenous growth factors except those present in serum. Observations on the morphology of the cultured cells, culture-formed basement membrane and some of its constituents are also presented.

MATERIALS AND METHODS

Primary cultures

Adult bovine eyes were obtained from slaughtered cattle within 2–5 h after death. Adhering extraocular tissues were removed from the globes by dissection. The eyes were then vigorously rinsed in room temperature, running tap water and subsequently covered for 15 min with gauze wetted with an aqueous solution of chlortetracycline (50 μg/ml). The cornea was excised with an attached 1–2 mm wide scleral ring and placed, endothelial side up, in a plastic cup having the same shape as the cornea (fabricated from conventional plastic refrigerator egg storage trays). All further culture procedures were conducted on the endothelial surface of the cornea as it sat in the plastic cup which was, in turn, glued to a glass Petri dish. The endothelium was covered with Earle's Balanced Salt Solution buffered to pH 7,3 with 15 mM HEPES, 10 mM TES and 10 mM BES (EBSS) in which the endothelium can be held for 1–2 h. The corneal endothelial cells were dislodged from Descemet's membrane by: (1) rinsing and then subsequently incubating the cells at 34°C for 5–6 min in a solution of Ca^{2+}, Mg^{2+}-free EBSS that contained 5 mM EDTA and 0.18% crude trypsin (1:250). The corneas were removed from the incubator and the endothelial surfaces were gently rubbed with a rubber spatula constructed from a tapered piece of silicone surgical rubber, 2–3 mm wide, affixed to a stainless steel rod (fig. 1).

The spatula edge is curved to conform to the corneal contour and to minimize the chances of penetrating the underlying Descemet's membrane.

In order to reduce the possibility of introducing fibroblasts into culture, fluid contact with the cut stromal surface was avoided. The dislodged cells floating in the trypsin–EDTA solution were aspirated from two eyes with a pipette and added to 5 ml of Eagle's Minimal Essential Medium (MEM) that contained 10% calf serum, 50 μg/ml Garamycin and 160 U Mycostatin. The endothelial cells were gently centrifuged (600 g, 2 min) to form a pellet which was subsequently resuspended in the same medium. The $\sim 0.5 \times 10^6$ cells obtained from two eyes were added in 5 ml of culture medium to 25 cm^2 culture flasks, gassed with 95% air/5% CO_2 and incubated at 34°C for 48 h. The 34°C temperature was selected as it more closely approximates the temperature of the native cornea than the more usual 37°C tissue culture incubator temperature [10].

Our limited experience suggests that the cells grow equally well for at least one month at 37°C. The cultures were subsequently rinsed with medium and cultured from this time on with MEM that contained 10% calf serum and Garamycin (50 μg/ml). Confluence was generally achieved in 5–7 days when the cultures were maintained on a 3-day feeding schedule.

Subcultures

Confluent endothelial cultures ($\sim 2.5 \times 10^6$ cells/flask) were subcultivated using a 3–5 min incubation in EBSS that contained 0.18% trypsin (1:250) and 5 mM EDTA. The free cells were added to an equal volume of MEM containing 10% calf serum. Following centrifugation, the cells were resuspended in sufficient medium to initiate between two and four subcultures per primary culture. Subcultivated corneal endothelial cells both enlarge (hypertrophy) and divide in order to reach confluence in the absence of fibroblast growth factor [40]. Although as many as 128 subcultures may be initiated from a single primary culture, the time required to reach confluence (6–8 weeks) and the resulting cellular hypertrophy render such cultures both time-consuming and morphologically atypical. The results presented in this paper are all from subcultures in the 3rd–5th passage initiated at between 1:2 and 1:4 subcultivation ratios. Biosynthetic experiments were conducted on subcultures initiated from pooled primary cultures in order to obtain a large number of matched cultures that obviated problems of animal age, sex, etc. At confluence, and thereafter, subcultures were fed sodium ascorbate, 50 μg/ml, at every medium change and before any labelling experiment.

Morphological methods

All stages of primary culture and subculture were studied and photographed, where appropriate, using an inverted phase contrast microscope. Native corneal endothelial cells were photographed after manually dissecting Descemet's membrane free from the stroma and subsequently flattening the membrane and attached cells beneath a coverslip wetted with culture medium. For electron microscopy, cultures were rinsed in warm EBSS and then fixed for 2 h at 4°C in a 1:1 mixture of 2.5% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.3, containing 2 mM $CaCl_2$ and 2% aqueous OsO_4. Sucrose (4%, w/v) was present in the final mixture.

The preparations were subsequently stained *en bloc* with 2% uranyl acetate [14], dehydrated in ascending concentrations of ethanol, and infiltrated with a mixture of one part absolute ethanol: one part Epon-Araldite [15], followed by several changes of the pure resin over a period of 48 h, before finally embedding them beneath a 1–2 mm thick layer of resin. Following polymerization, selected areas of the epoxy resin-coated flask bottom were cut out, mounted in a microtome chuck and sectioned using a diamond knife. Thin 50–60 nm sections were collected on carbon-reinforced, Formvar-coated grids and stained with uranyl acetate and lead citrate.

Scanning electron microscopic (SEM) observations were made of the native corneal endothelial cells fixed in situ by perfusing the anterior chamber with 2.5% glutaraldehyde, followed by immersion in the same fixative for 2 h. Dissected corneas were subsequently post-fixed in buffered 1% OsO_4 for 1 h. Cultured corneal endothelial cells used for SEM were grown on glass coverslips and were fixed in the same manner as conventional cultures. The surfaces of native or in vitro formed Descemet's membrane were viewed following removal of the cells by incubating the cultures for 2–4 min at 37°C in an aqueous solution of 0.5% sodium deoxycholate (DOC) [16] and several subsequent rinses of distilled water or EBSS. Fixation of the membranes following removal of the cells with DOC was as described above for conventional cultures. Specimens were transferred from 100% ethanol to a critical-point drying apparatus and dried from CO_2, following which specimens were coated with a layer of gold–palladium prior to viewing.

Immunohistochemical studies

Antisera to both native Descemet's membrane and the in vitro formed membrane were raised by injecting rabbits with homogenized, DOC-cleaned membranes that had been previously reduced and alkylated in the presence of 8 M urea [17]. Two groups of two New Zealand white rabbits were injected in the region of the popliteal lymph nodes with 1 mg of the appropriate antigen that had been resuspended in phosphate-buffered saline (PBS) following exhaustive dialysis and lyophilization. The antigen in PBS was mixed with complete Freund's adjuvant (1:1). Additional intramuscular injections of 0.5 mg of the appropriate antigen in PBS were made at 3–4 week intervals beginning 3 weeks after the initial immunization. The first test bleeding, 7 weeks after the initial injection, yielded antibodies directed against both the native and in vitro membranes.

Antisera directed against type IV collagen [18], laminin [19] and a basement membrane proteoglycan [20] derived from a mouse tumor were kindly provided by Drs J-M Foidart, John Hassell and George Martin

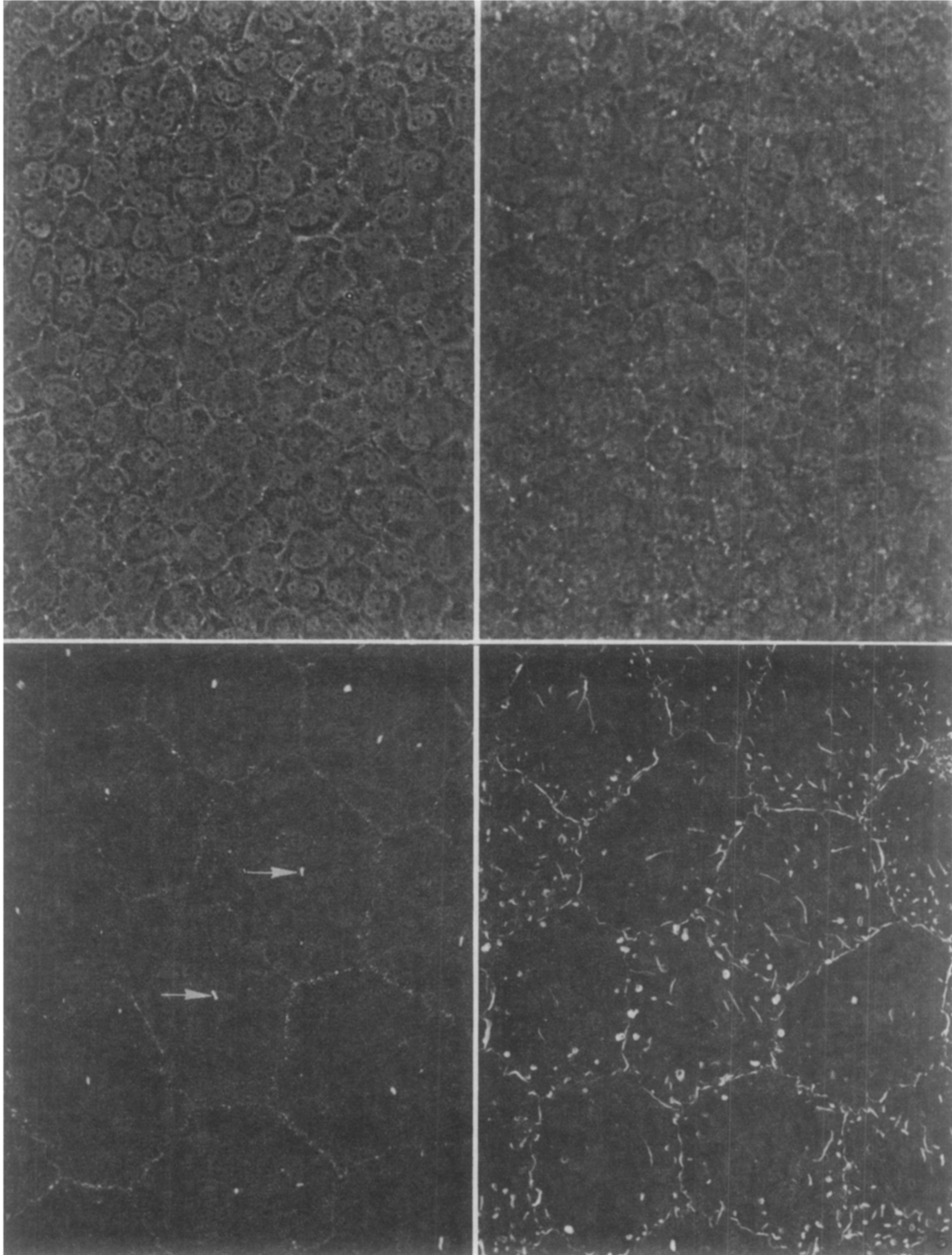

Fig. 2. Phase contrast micrograph of confluent corneal endothelial cell culture. The cells possess excentrically placed nuclei with multiple nucleoli, a juxtanuclear homogeneous-appearing Golgi zone and a peripheral granular cytoplasmic zone composed of rough endoplasmic reticulum, mitochondria and lysosomes. ×275.

Fig. 3. Phase contrast micrograph of native corneal

of the National Institute of Dental Research, Bethesda, MD. The specificity of all antisera was checked by absorption with the molecules of the type used for immunization. Additionally cross-absorptions between laminin and the BM proteoglycan as well as between type IV and V collagens were performed. All antisera employed were specific using these absorption criteria. Immunohistochemical studies using antisera directed against types V and III collagens were kindly performed by Drs Stephen and Renate Gay of the University of Alabama Medical Center, Birmingham, Alab. Antisera against human fibronectin was a gift of Dr Ronald Chung, National Institute of Dental Research, or bovine fibronectin was purchased from Calbiochem-Behring Corp. Coverslips covered by DOC-cleaned basement membranes or unfixed frozen sections of bovine cornea or mouse tongue (for basement membrane specificity) mounted on glass slides were used as the test objects. Indirect immunofluorescence using FITC-coupled IgG was used to demonstrate the presence of specific binding.

Proline labelling experiments

The stability of molecules incorporated into the membrane and the polarity of secretion by corneal endothelial cells were studied by [^{14}C]proline labelling matched subcultures (6×10^6 cells/75 cm^2 flask) maintained 5 weeks beyond initial confluence. Cells were labelled with 0.7 μCi/ml of L-[U-^{14}C]proline (sp. act. 250 mCi/mM) for 12 h in MEM-containing 10% calf serum. Following rinsing and an initial 12-h chase period, the medium was changed every 24 h. Twelve, 36, 84, and 156 h following the labelling period, cells were removed from the membrane using DOC and the cleaned membrane removed using a rubber scraper. The cleaned membrane was stored at −20°C, while the labelling medium, initial chase medium, and the cell lysate were each precipitated overnight in 0.5 N PCA (4°C) and analysed for ^{14}C incorporation into total protein and the distribution of [^{14}C]pro and [^{14}C]OH-pro within the proteins of the various media and cell lysates. Membranes were homogenized and extracted in 1% SDS containing 8 mM dithiothreitol (DTT) for 8 h at 60°C. The SDS-solubilized proteins were separated from the remaining membrane fragments and pooled with two subsequent washes of the membranes. These soluble proteins were dialysed against distilled water containing Dowex 1-8X resin in order to remove the SDS. The *soluble proteins* and the *insoluble residual* membrane were then dried by vacuum evaporation and hydrolysed under nitrogen in constant boiling 6 N HCl. The samples were ultimately dissolved in 0.5 ml of 0.2 N citrate buffer (pH 2.2). Samples from each aliquot were removed for liquid scintillation counting and the remainder was applied to a 23 cm Aminex A-5 column run at 50°C with a flow rate of 80 ml/h. The elution positions of 3-OH-pro and 4-OH-pro and pro had previously been determined by applying standards of these respective compounds (Dr Leonard Franke and the late Dr Elijah Adams kindly provided the 3-OH-pro; [^{3}H-4]OH-pro and [^{3}H]pro were purchased from the Amersham Corp.). Samples were eluted from the column using 0.45 M sodium citrate, pH 2.9. One-ml fractions were collected and dissolved in Formula 963 aqueous counting cocktail (New England Nuclear Corp.) for scintillation counting. Samples of PCA-precipitated cell fractions, labelling and first chase media were dissolved in Protosol (New England Nuclear Corp.) and counted.

Studies were also designed to examine the relative synthesis of non-collagenous and collagenous proteins including the general composition of the latter protein class. At selected times (2, 3, 4, 6 and 16 weeks) cultures were labelled for 24 h in the presence of 80 μg/ml β-aminoproprionitrile with 4 μCi/ml of L-[5-^{3}H]proline (sp. act. 35 Ci/mM). Following DOC lysis of the cell layer the [^{3}H]proline-labelled membranes were scraped from the flask bottom, dialysed exhaustively against distilled water and lyophilized. The lyophilized membranes were hydrolysed in 6 N HCl before being applied to the Aminex A-5 column described above.

In order to obtain a further estimate of the collagen content of the membrane deposited at various times in culture (3, 11, 16 weeks), two separate experiments were conducted that employed matched cultures labelled with [^{3}H]proline as described above (2 T-75 flasks at 3, 11, 16 weeks/experiment). The membranes from each experiment were digested with clostridial collagenase (type III collagenase, Advanced Biofactures Corp.). One hundred μg of lyophilized basement membrane was digested with 350 units (Advanced Biofacture Units) of enzyme for 16 h at 37°C in 0.05 M Tris, pH 7.5, that contained 5 mM $CaCl_2$, 2.5 mM *N*-ethylmaleimide, and 0.05% (v/v) Triton X-100. These conditions were determined experimentally to exceed a 'limit' digestion by a period of 8 h (data not shown). The particular batches of enzyme used were checked for non-specific protease activity by incubating [^{14}C]methyl albumin and unlabelled carrier serum albumin with 350 units of the enzyme followed by precipitation in cold, 5% TCA. The number of counts released was less than 1%, an amount equal to that released when boiled enzyme was used.

The percentage of collagen in the membrane was determined using the methods and calculations of Diegelman & Peterkovsky [21] except that the proline content reported for Descemet's membrane collagen [22] was used rather than the value for type I col-

endothelial cells. In order to make this photomicrograph, Descemet's membrane and the adhering endothelial cells were dissected from the cornea. The close apposition of native cells, as depicted here, is also a feature of the cultured cells. ×275.

Fig. 4. SEM of cultured corneal endothelial cells. The close 'packing' and hexagonal shape of the cells are illustrated, as are centrally located cilia (*arrows*). ×1 100.

Fig. 5. SEM of native bovine corneal endothelial cells. Cells demonstrate usual close ordering and roughly hexagonal shape characteristic of this cell type in most species. Native cells usually possess more microvilli and cilia than do cultured cells. Native cells are also smaller than cultured cells. ×1 850.

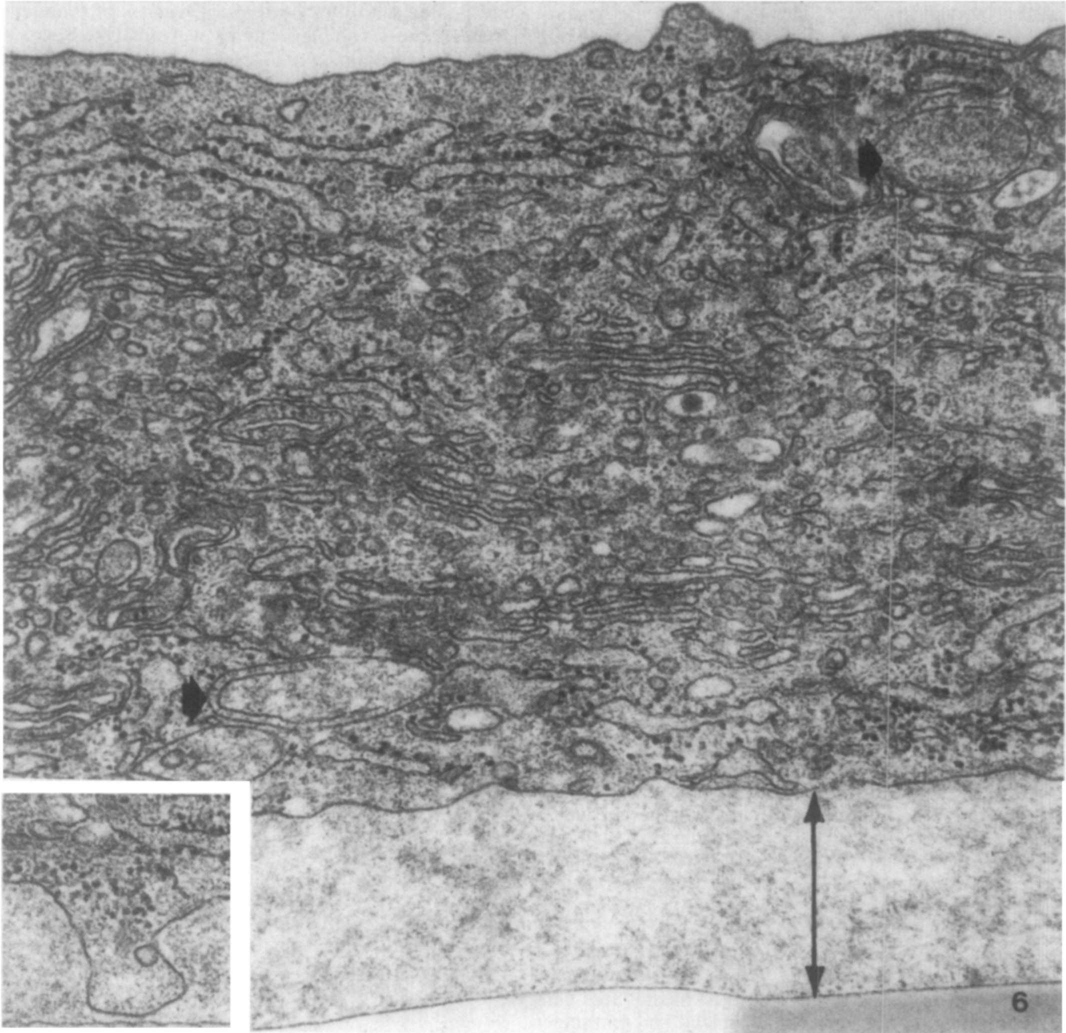

Fig. 6. Cytoplasmic ultrastructure of a corneal endothelial cell, 3 weeks after subcultivation, that possesses numerous profiles of rough endoplasmic reticulum and an extensive Golgi apparatus, only a portion of which is illustrated here. Occasional large vesicles, filled with a moderately electron-opaque material and fragments of membranes, and which are thought to be secondary lysosomes, are present in the Golgi region (*broad arrows*). Illustrated cell has deposited a membrane (*double-headed arrow*) ~0.5 μm thick in 3 weeks. *Inset:* Cultured cells have numerous, small cytoplasmic processes embedded in deposited membranes such as that illustrated in this micrograph. These processes, which are withdrawn as the membrane increases in thickness, are responsible for the pitted surface observed on culture-formed membranes (see fig. 7). ×55 000; *inset,* ×43 000.

lagen. This modification results in a 'collagen factor' of 4.2 rather than 5.4.

RESULTS

Cultured bovine corneal condothelial cells, upon reaching confluence form a highly ordered monolayer that closely resembles the arrangement of the native endothelium (figs 2, 3). When viewed with the scanning electron microscope (SEM), the cultured cells demonstrate the roughly hexagonal shape and close apposition to one another

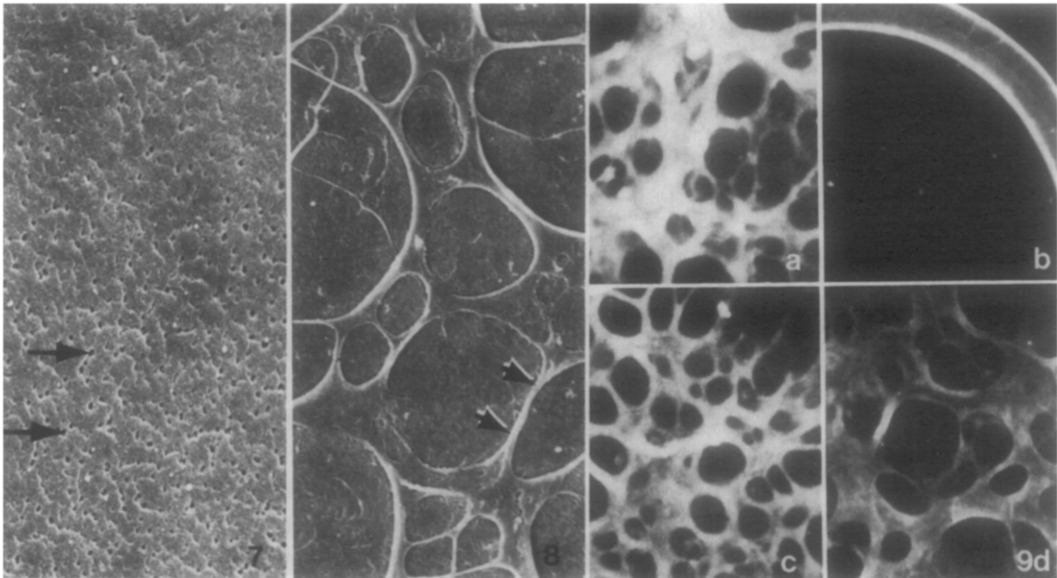

Fig. 7. SEM of a culture-formed membrane following removal of cells with deoxycholate. Arrows mark small pits or depressions on surface caused by cytoplasmic processes of endothelial cell that extend into the membrane (see fig. 7). ×1 000.
Fig. 8. SEM of smooth surfaced ridges (*arrows*) occasionally deposited beneath or around individual cells or groups of cells, especially when cultures are grown on glass. These ridges produce a reticulated pattern convenient for viewing following immunohistochemical procedures (see fig. 9). ×550.

Fig. 9. Immunohistochemical demonstration of type IV collagen (*c*), laminin (*d*) and the cross reactivity of antisera raised against native membranes (*a*) and culture-formed membranes (*b*) when applied to their counterparts. Native Descemet's membrane reacted only on its endothelial and stromal sides with all antisera employed. Apparently reactive sites within interior of membrane are masked. ×350.

that is a distinguishing characteristic of this endothelial type (figs 4, 5) [2]. Ultrastructurally, cultured endothelial cells possess an abundant complement of organelles required for the synthesis of secretory proteins and glycoproteins. There are numerous profiles of rough endoplasmic reticulum, the cisternae of which are filled with a moderately electron-opaque, granular material. An extensive juxtanuclear Golgi complex composed of multiple arrays of flattened lamellae and a wide variety of vesicles is also present (fig. 6). Occasional large (~100 nm) vesicles filled with a granular or flocculent material together with segments of 6 nm wide trilaminar membrane are also present in the cytoplasm. These latter structures are thought to be secondary lysosomes containing sequestered secretory products. Typical secondary lysosomes filled with membranous elements increase in number with prolonged culture.

Soon (24 h) after initiating primary or subcultures, a faintly granular material can be seen interposed between the organizing cell islets and the underlying culture plastic. This subcellular material rapidly accumulates reaching a thickness of 0.5 μm by 21 days in culture (fig. 6). The fine structure of this elaborated matrix is variable but generally contains (1) a random distribution of homogeneous-appearing granules (~30 nm); (2) fine (15–17 nm) irregularly distributed filaments; and (3) a structure-

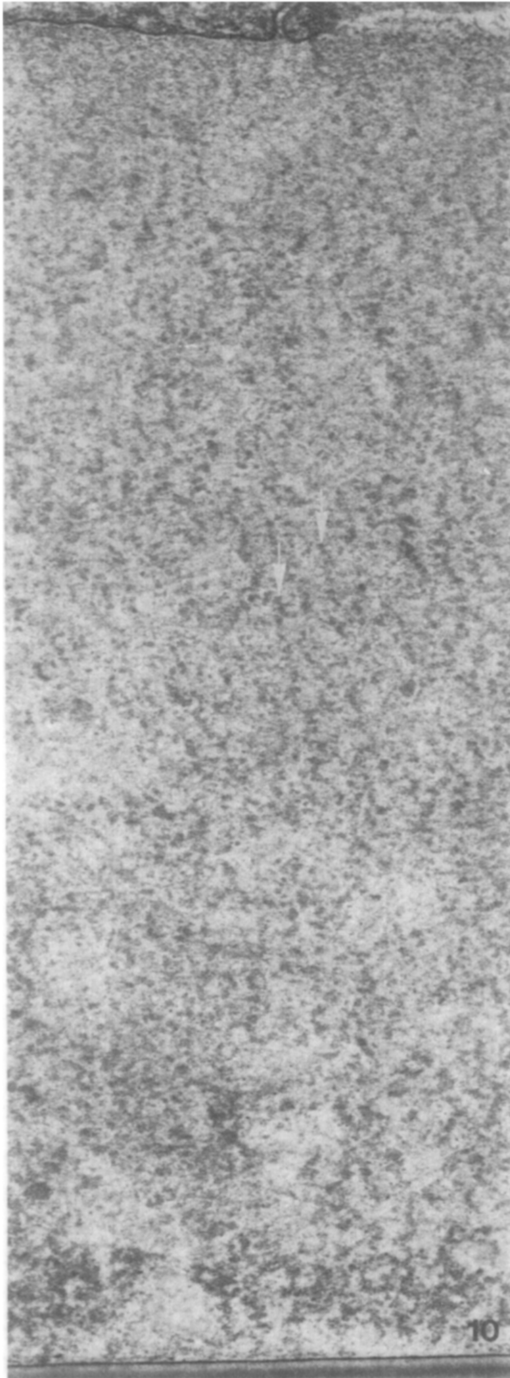

Fig. 10. Transmission electron micrograph (TEM) of a membrane deposited over a period of one year. The membrane is approx. 5 μm thick. Incomplete, irregular bands or striations (*arrows*) are present within the substance of the membrane. In areas where bands are arranged in parallel, the distance between these bands is approx. 90–120 nm. ×33 000.

less, moderately electron-opaque 'fuzz' that occupies the entire depth of the membrane. (The detailed ultrastructure of the membrane in its formative stages is not shown.)

Small cytoplasmic processes of the endothelial cells remain embedded in the matrix (fig. 6). These cytoplasmic processes, which initially contacted the plastic culture surface, are withdrawn as additional membrane is laid down. The spaces occupied by the endothelial cell processes are responsible for the numerous pits observed on the surface of the culture-formed membrane after removing the cells with deoxycholate (fig. 7). Cells cultured on glass coverslips frequently deposited small, smooth textured ridges of membrane either located beneath individual cells or between individual cells or cell clusters composed of three or more cells (fig. 8). This uneven pattern of membrane deposition was less frequently observed when the cells were cultured on plastic surfaces. The resulting reticulated patterns deposited on glass were particularly useful as focusing aids when the properties of the membrane were explored immunohistochemically.

Antisera that were raised against native bovine Descemet's membrane reacted positively when applied to the culture-formed membrane (fig. 9*a*). Antisera raised against the culture-formed membrane reacted with the endothelial and stromal surfaces of native Descemet's *membrane* (fig. 9*b*). This same pattern of endothelial and stromal-sided reactivity was observed in the native membrane with all of the antisera subsequently employed. Even though the antisera were applied to cut sections, the interior of the native membrane reacted

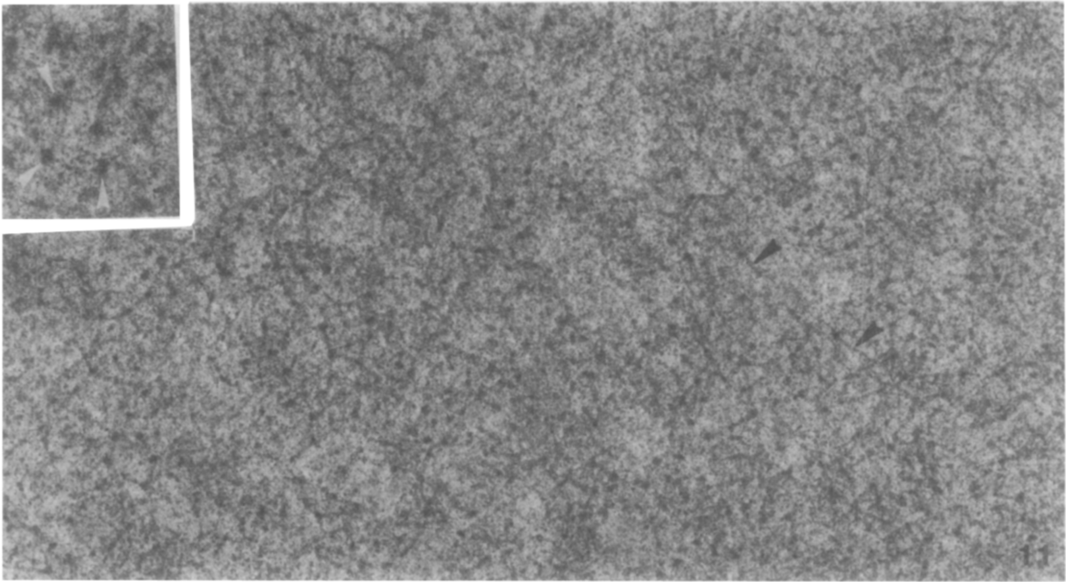

Fig. 11. Tangential section of culture-formed membrane illustrated presence of numerous nodes within the substance of the membrane. In many places the nodes are interconnected by 15–17 nm wide filaments (*black arrows*). *Inset:* Higher power micrograph of membrane nodes (*white arrows*). The 30 nm nodes are separated by a 90–120 nm space and their parallel stacking is apparently responsible for the banding observed in cross-sections through the membrane. ×28 000; *inset*, ×60 000.

weakly, if at all, following application. The culture-formed membrane gave positive immunohistochemical reactions for laminin (fig. 9*c*), basal lamina proteoglycan, types IV (fig. 9*d*), V, and III collagen, as well as for fibronectin.

A progressive thickening of the membrane was observed if the cells were maintained for extended times in culture. Measurements made of membrane thickness at 2, 6 and 12 months gave values of 1.2, 2.5 and 5 μm, respectively, thus indicating an ongoing deposition of membrane for a minimum of one year in culture.

The ultrastructure of membranes that had been deposited for 4 months or longer demonstrated a degree of internal organization. When viewed in cross-section, these membranes demonstrated a distinct, but somewhat irregular banding or cross-striation (fig. 10). Such banding did not, however, extend throughout the depth of the membrane without interruption. The intervals separating the bands measured approx. 90–120 nm. When tangential sections were made of such membranes, numerous regularly spaced (30–34 nm) electron-opaque 'granular nodes' were observed (fig. 11). In many areas, a thin filament (15–17 nm wide) spanned the interval between nodes. Complete hexagonal arrangements of the nodes were, however, rarely observed.

The distribution of [^{14}C]OH-proline and [^{14}C]proline-labelled proteins in the medium, cell layer and membrane is presented in table 1. Roughly 75% of the labelled OH-proline is incorporated into the membrane, while a relatively small amount, 16%, was released into the medium. The bulk of incorporated proline, however, remained

Table 1. *Distribution of [^{14}C]proline and [^{14}C]OH-proline in bovine endothelial cell cultures*

Fraction	Total[a] (%)	Proline (%)	OH-Proline[b] (%)
Membrane	15	11	72
Cell	62	65	12
Medium	23	24	16
Total dpm	416 600	363 200	13 120

[a] Represents total incorporated counts recovered during 12-h labelling period and 156-h chase period.
[b] Sum of 3-OH-pro and 4-OH-pro.

with the cell layer. Those proteins and glycoproteins in the membrane that contained labelled proline and OH-proline became increasingly resistant to extraction by SDS in the presence of a reducing agent. Approx. 80% of the combined labelled proteins and glycoproteins could be extracted with SDS 12 h after the labelling period. This amount was reduced to 46% by 4 days and 40% by 7 days.

The ratio of total OH-proline/proline in the membrane remained relatively constant (0.30±0.02) during 16 weeks. The ratio of 3-OH-proline/4-OH-proline (0.08–0.11) also remained constant over a 16-week period (3-OH-pro dpm : 4-OH-pro dpm/1 000 dpm; 32 : 278, 2 weeks; 24 : 280, 3 weeks; 22 : 275, 4 weeks; 20 : 211, 6 weeks; 22 : 204, 16 weeks). The percentage of collagen in the membrane, based upon collagenase digestions, is shown in table 2. The data indicate that during a 24-h labelling 'window' at 3, 11 and 16 weeks in culture, approx. 16–18% of the in vitro formed membrane is composed of collagen.

DISCUSSION

Bovine corneal endothelial cells can be easily grown in culture using conventional techniques. The resiliency and thickness of Descemet's membrane in the bovine eye interposes an effective barrier between the endothelium and the underlying stromal fibroblasts (keratocytes)—a barrier that is not breached by gentle rubbing of the endothelial surface with a rubber spatula. Therefore, if the separating medium is kept away from the cut stromal surface, contamination of the cultures by stromal fibroblasts rarely occurs.

The resulting cell cultures yield confluent monolayers of tightly packed cells of a uniform size that closely resemble the arrangement of native corneal endothelial cells of most species [2, 9, 23]. We have employed early passage (1–5) subcultures for our studies because they remain morphological-

Table 2. *Collagen content of the basement membrane synthesized by corneal endothelial cells after different times in culture*[a]

Age of culture (weeks)	Collagenase solubilized dpm (% Undigested controls)	Collagenase resistant dpm (% Undigested controls)	% Collagen (± SEM)[b]
3	48	52	16.5±0.8
11	44	56	15.7±0.6
16	46	54	16.8±0.2

[a] 24-hour labelling period with [^{3}H-5]proline; summary of two experiments.
[b] Calculated by the method of Digelmann & Peterkovsky [19] except for a 'collagen factor' of 4.2 (see Materials and Methods).

ly similar to the native cell type. Cells passed 10–20 times become increasingly pleomorphic and resemble subcultivated rabbit corneal endothelial cells [24], the only other reported source of a vigorously growing corneal endothelial cell population in vitro. Addition of the exogenous growth factor, fibroblast growth factor (FGF), greatly facilitates cell division (cells subcultivated at a 1:64 ratio are confluent within 7–10 days through 20 passages—data not shown). However, this exogenous mitogen did not prevent the increasing cellular pleomorphism during multiple passages that we observed with conventional culture techniques. Thus, our experience in cultivating these cells is quite different from that of Gospodarowicz et al. [25], who have had remarkable success in routinely subcultivating this cell type through 200 or so passages in the presence of FGF. As a result, we have avoided the additional expense of adding FGF and have employed conventionally cultured, early passage subcultures.

The native and culture-formed membrane share at least some similar antigenic sites, as demonstrated by the fact that antisera raised against either the native or culture-formed membrane cross-react. Immunohistochemical studies indicate that the membrane deposited by the cultured endothelial cells contains laminin, types IV and V collagen and a basement membrane proteoglycan. The presence of these ubiquitously distributed basement membrane molecules within the culture-formed membrane represents a strong argument that it is an authentic basement membrane. The presence of laminin in the membrane formed by cultured endothelial cells has recently been demonstrated independently by others [13]. Type V collagen has been described as a pericellular collagen in some tissues [26], as well as an integral basement membrane collagen [27]. Fibronectin is made by a large number of cell types, many of which are not associated with basement membranes [28]. However, biosynthetic and immunohistochemical studies by Birdwell et al. [29] have demonstrated that cultured corneal endothelial cells synthesize fibronectin which is subsequently incorporated into the membrane.

The unexpected immunohistochemical demonstration of type III collagen in the culture-formed membrane has also been confirmed independently. Tseng et al. [11] using ion exchange chromatography demonstrated that type III collagen is the major collagen type produced by subconfluent cultured corneal endothelial cells. In our laboratory sequential pepsin and V-8 protease (from *Staphylococcus aureus*) digestions of culture-formed membranes yielded cleavage patterns on SDS–PAGE identical with those derived from purified type III collagen [30]. The 3-OH-pro/4-OH-pro ratios (0.08–0.10) of the total collagens produced by corneal endothelial cells in our system more closely resemble those reported for types IV and V collagen having large amounts of 3-OH-pro [31, 33]. However, Sage et al. [34] have reported that matrix-associated collagens, mixtures of types III and V collagen, formed by cultured bovine aortic endothelial cells have 3-OH-pro/4-OH-pro ratios of 0.07. Our studies indicate that the same mix of collagens, based on 3-OH-pro/4-OH-pro ratios, is produced throughout the 16-week experimental period. A stable collagen phenotype has also been demonstrated in serially passaged rabbit corneal endothelial cells [35].

The polarly deposited membrane formed by conventionally cultured bovine corneal endothelial cells assumes the form of a co-

hesive, homogeneous sheet rather than the tangle of filaments, granular elements and occasional elastic fibrils characteristic of the subcellular matrix formed by cultured vascular endothelial cells [13, 29, 36, 37]. The progressively thickening membrane deposited in culture acquires a degree of internal organization typified by evenly spaced nodes and interconnecting filaments which apparently represents early stages in the formation of the multiple strata of interconnected nodes present in the native membrane. This unique form of internal organization in vivo is thought to help maintain corneal shape by distributing tension throughout the membrane [38]. Growth factor-stimulated cultures [13, 25] or cultures grown on glass form a more irregular membrane.

These conventionally cultured cells are amenable to many experimental manipulations including: (1) biosynthetic and turnover studies of membrane components [39]; (2) population dynamics studies of confluent cultures [40]; (3) transcellular ion transport studies when grown on collagen gels [41]; and (4) the production of basement membranes as growth substrata for other cells in vitro [42].

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