Selective growth of rabbit corneal epithelial cells in culture and basement membrane collagen synthesis

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Selective isolation of rabbit corneal epithelial cells was achieved with a medium containing D-valine. Long-term cultures of the epithelial cells synthesize basement membrane–type procollagen doublet chains of 160,000 and 180,000 daltons and a higher-molecular-weight species at 280,000 daltons. Some of these components are disulfide-linked. These peptides gave rise to a single pepsin-resistant collagen chain of approximate molecular size of 115,000 daltons.

Key words: epithelial cells, cornea, cell culture, biosynthesis, basement membranes, collagen

The corneal epithelium is essential to vision and the health of the cornea in various ways. Its expanded surface area which is the result of its projection of microvilli helps to stabilize the tear film, which presents a smooth lenslike surface and prevents desiccation. The corneal epithelium also presents a barrier to both water and bacteria and thereby maintains corneal transparency and prevents infection. The major biosynthetic product of the epithelium is a basement membrane which is primarily or secondarily involved in many corneal dystrophies and slow-healing epithelial syndromes. Many of these disorders are characterized by poor epithelial adhesion.2–4

Investigations of the properties of the epithelial cells can be approached by establishing these cells into culture. At present the only successful long-term culture of surface epithelium has been the growth of the cells on feeder layers of irradiated fibroblasts.5 Epithelial biosynthetic studies are impossible with this technique because the metabolic products of the fibroblast feeder layer will confuse the analysis.

We present a method for long-term cultures of rabbit corneal epithelial cells and partially isolate and characterize their collagenous biosynthetic products.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), minimal essential medium containing D-valine, fetal calf serum, penicillin, streptomycin, and pancreatin were from Grand Island Biological Co., Grand Island, N.Y. [14C]proline (W255 mCi/mmol) was from New England Nuclear, Boston, Mass. N-Ethylmaleimide, phenylmethane sulfonyl fluoride, ethylene diamine tetraacetate (tetrasodium salt), β-aminopropionitrile fumarate (β-APN), and pepsin, were purchased from Sigma Chemical Co., St. Louis, Mo. Diethylaminoethyl (DEAE)-cellulose (DE52) was from Whatman Ltd., Maidstone, U. K. Precoat Bio nephore polyacrylamide gels were obtained from Bio-Rad Laboratories, Richmond, Calif. Collagenase (form III) was purchased from Advance Biofactors Corp., Lynbrook, N.Y.
Corneal epithelial cell culture. Corneas from albino rabbits were excised within the limbus, immediately placed in sterile tissue culture dishes, and covered with DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). An anterior corneal layer consisting of superficial stroma and epithelium was excised with sharp dissection and transferred to separate dishes containing DMEM with collagenase (1 mg/ml). Sterile coverslips were placed over the tissues to keep them in position at the bottom of the tissue culture dishes. After a 6 hr incubation, the collagenase-containing medium was carefully aspirated so as not to disturb the newly loosened cells and replaced with the selective medium.* Subsequent media changes were carried out every 48 hr. Once migration and attachment of epithelial cells onto the dishes were evident (about 48 hr), the corneal tissue was removed and discarded. The epithelial cells were then allowed to spread over the dish. Four to six passages in the selective medium completely removed fibroblast contamination. Confluent monolayer cultures of these cells were serially propagated from 60 mm tissue culture dishes into roller bottles with pancreatin and used in the present study.

Metabolic labeling of corneal epithelial cells. To study the procollagen synthesis, DMEM minus serum but supplemented with 100 μg/ml ascorbic acid and 50 μg/ml β-APN was used. Cultures were preincubated for 16 hr with serum-free medium and then with medium containing 1 μCi/ml [³H]proline for 48 hr. The techniques utilized to purify collagen-procollagen from the radioactively labeled cells and medium have been previously described. However, in the current series of experiments collagenous materials were precipitated with 50% saturated ammonium sul-
fate instead of 30% saturation used previously. With this modification, 90% to 95% of the collagenous material secreted into the culture media was precipitated. This precipitate was redissolved and further purified by ethanol precipitation as described previously.6

**DEAE cellulose chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses.** The ethanol precipitate was dissolved in limiting buffer, dialyzed against starting buffer, and applied to DEAE-cellulose column as previously described.6 The column fractions were pooled separately, dialyzed against deionized water, and lyophilized. These samples were further analyzed by SDS-PAGE.7 Limited pepsin digestion of procollagen or collagen samples was carried out according to the method of Bellamy and Bornstein.6 Duplicate sets of samples were digested with pepsin (100 µg/ml) in 0.5N acetic acid (40 µl) for 6 hr at 4°C. The reactions were terminated by addition of 1N NaOH (15 µl); one set of samples was analyzed by SDS-PAGE after reduction with 50 mM dithiothreitol (DTT), and the duplicate set was electrophoresed without reduction. Procollagen or collagen samples in duplicate were also digested with purified bacterial collagenase according to the method of Peterkosky and Diegelmann.9

**Results**

The use of D-valine as a selective agent for the normal human and rodent epithelial cells in culture was first demonstrated by Gilbert and Migeon in 1975.10 Their study indicated that the epithelial cells synthesize the enzyme D-amino acid oxidase, which converts D-valine in the medium to the essential amino acid L-valine. These cells are therefore able to proliferate. Fibroblasts, however, lack this enzyme and are thus unable to grow. Application of this technique to corneal tissue produced selective growth of corneal epithelial cells from tissue slices containing both epithelial cell and stromal fibroblast populations.

Fig. 1A is a phase-contrast micrograph of...
rabbit corneal epithelial cells migrating out of the corneal epithelial layer 48 hr after the corneal tissue had been placed in the selective medium. Some of these epithelial cells on further incubation rounded up and did not attach to the dish. The presence of collagenase in the medium helped the epithelial cells to disperse from the tissue and migrate onto the culture dish. In other experiments, we found that scrapings from the corneal epithelial layer did not result in the growth of epithelial cells even though there were layers of more than 20 to 30 epithelial cells attached to each other.

Some cells with fibroblastic morphology did grow in the initial outgrowth from the corneal tissue in selective medium along with epithelial cells. However, these fibroblasts did not survive subsequent selective media change and subcultures. It is possible that the fibroblast survival in the early period could be the result of these cells obtaining their required L-valine from the turnover of endogenous proteins.

As the epithelial cells multiplied to monolayer (Fig. 1B), typical polygonal-shaped cells with contact inhibition similar to other established primary cell cultures were observed. These cells, when examined by transmission electron microscopy (Fig. 1C), exhibited desmosomal connections at their points of contact, thus providing additional evidence of pure epithelial cell growth in culture. No evidence of the smaller, more spindle-shaped fibroblastic cells was seen in the long-term cultures used for the study of collagen biosynthesis.

Our pure cultures of rabbit corneal epithelial cells grew very well in DMEM and did not exhibit any additional requirements for either growth factors or trace elements.

Ethanol-purified, collagenous precursor proteins isolated from radioactively labeled media were examined by SDS-PAGE. In the

Fig. 1C. Transmission electron micrograph exhibits desmosomal junctions (arrow) between epithelial cells. (×99,750.)
absence of the reducing agent DTT, the chain structure of the collagenous material was shown to consist of three components. A portion of the material (10% of the total) had a molecular weight of approximately 280,000 daltons, although the major bands are at 180,000 and 160,000 daltons, together accounting for about 90% of the total. All the materials were larger than interstitial collagen α-chains (approximately 95,000 daltons). The collagen α2-chain was not present. This indicates the absence of type I collagen which would be synthesized by contaminating stromal fibroblasts in impure epithelial cell cultures (Fig. 2, A).
Growth of rabbit cornea in culture

The ethanol-precipitated proteins were dissolved in limiting buffer and dialyzed against starting buffer as described in Materials and methods. The dialysate was applied to the column (1.5 by 20 cm) of DEAE-cellulose equilibrated in a starting buffer. The column was developed with a linear gradient of limiting buffer. Radioactive peak 1 (fractions 89 to 98) and peak 2 (fractions 99 to 105) were separately pooled, dialyzed extensively against deionized water, and lyophilized.

In the presence of the reducing agent DTT, the major components were 180,000 and 160,000 daltons (Figs. 2, B and 3, A). Again, no material appeared at the fibrous collagen α-chain region. Bacterial collagenase almost completely destroyed these chains (Fig. 3, B), indicating the collagenous nature of the material. Pepsin treatment, which does not attack the helical portion of the collagen molecule, resulted in destruction of the 180,000 and 160,000 dalton components and generation of single-chain material at approximately 115,000 daltons. About 80% of the applied material appeared in this region. The remainder appeared as low-molecular-weight material near the lower end of the gel (Fig. 3, C). The presence of a collagenase-sensitive molecule which gives rise to a single chain, larger than α1 (type I) with limited pepsin digestion is characteristic of basement membrane collagens. Thus the corneal epithelial cells in culture exclusively synthesized a basement membrane-type collagenous material. This property of basement membrane collagen biosynthesis was maintained after 10 passages in culture.

Further purification of the precursor proteins from ethanol precipitate was achieved on DEAE-cellulose columns (Fig. 4). Three major peaks were observed on chromatography. The material eluted from the column in the starting buffer wash (fractions 20 to 40) when analyzed by SDS-PAGE consisted mainly of a single band at about 115,000 daltons (not shown). The other two peaks (marked 1 and 2) eluted in salt gradients of 5 to 7 mF. Each of the peaks, 1 (fractions 89 to 98) and 2 (fractions 99 to 105), was pooled separately, dialyzed against deionized water, lyophilized, and analyzed individually on SDS-PAGE in the presence and absence of the reducing agent DTT.

Column-purified peaks 1 and 2 were similar when analyzed by SDS-PAGE analyses. Both the peak materials were completely destroyed by collagenase. Peak 1 material (Fig. 5, A) in the presence of DTT resolved into a single polypeptide of 140,000 daltons and in the absence of DTT resolved in three minor components of molecular weights 280,000, 180,000, and 160,000 and a major peak around 140,000. Pepsin digestion brought them into a single polypeptide of approximately 115,000 daltons (Fig. 5, B).
Fig. 5. Electrophoretic analyses of DEAE-cellulose column-purified collagenous protein fractions (peak 1). Lyophilized samples were dissolved in limiting buffer, and aliquots were electrophoresed. A, Without DTT (-----) and after reduction with DTT (—). B, Samples were subjected to limited pepsin digestion and then electrophoresed without DTT (-----) and after reduction with DTT (—). Gels were fractionated, and the radioactivity was determined. The positions of γ-, β-, and α-chains of collagen in the same electrophoresis run are indicated.

DEAE-cellulose column-eluted peak 2 material (Fig. 6, A), when analyzed, revealed two individual peptides 180,000 and 160,000 daltons in the presence of reducing agents. Also, under unreduced conditions, two other polypeptides, one greater than 300,000 and another around 280,000 daltons, were resolved. On pepsin digestion, similar to the material from peak 1, peak 2 proteins resolved into a single protein band corresponding to 115,000 daltons (Fig. 6, B). The presence of lower-molecular-weight material in peak 1 indicates that this peak contained some partly processed chains.

Discussion

We have utilized a selective growth medium containing D-valine which for the first time has established corneal epithelial cells in long-term cultures. We speculate that an undisturbed basal cell layer is important for long-term culture, since our earlier trials of culturing epithelium scraped from the cornea have uniformly failed. In order to obtain an undisturbed basal layer, we included superficial corneal stroma with the epithelium for our initial culture. Fibroblast proliferation was prevented by D-valine because these cells cannot convert D- to L-valine. This seems to allow the slow-growing epithelial cells (which can utilize D-valine for their growth) to predominate.
The establishment of pure corneal epithelial cells in culture provides a system for studying the biosynthetic mechanisms involved in the formation of normal basement membrane.

Several tissues like isolated rat renal glomeruli, chick lens cells, rat parietal yolk sac endoderm, and developing mouse embryos have been investigated for their ability to synthesize basement membrane components. Conflicting reports have arisen from these reports regarding the molecular size of the basement membrane collagen molecules. This confusion mainly appears to be due to differences in the methods of extraction and analysis in different laboratories. Pepsin digestion has been the conventional method applied to solubilize and characterize collagens. With this method, nonhelical crosslinked species are removed by the action of the enzyme, giving rise to pepsin-resistant helical basement membrane chains. The pepsin-resistant basement membrane collagen molecules produced by this method have been reported to range in molecular size between 110,000 to 195,000 daltons, although it is now generally accepted that the major component after pepsin treatment is approximately 110,000 to 140,000.

Our present studies indicate that corneal epithelial cells in long-term culture produce a high-molecular-weight species of basement membrane precursor material composed of major polypeptide chains of 160,000 and 180,000 daltons and higher-molecular-weight species of 280,000 daltons, some of which are disulfide-linked. When subjected to limited digestion conditions with pepsin, these peptides give rise to a single pepsin-resistant polypeptide with a molecular weight of approximately 115,000 daltons. This chain is collagenase-sensitive and is similar in molecular size to other reported basement membrane collagens.

In 1977, Sundar-Raj et al. reported that long-term cultures of rabbit corneal endothelial cells in culture synthesized two precursor chains of basement membrane collagen which migrated in close proximity on SDS-PAGE with approximate molecular weight of 160,000 and 200,000 daltons. These two chains yielded a single pepsin-resistant collagen peptide of 115,000 daltons on limited pepsin digestion. The presence of a doublet on SDS-PAGE gels might very well be a characteristic feature of cells in culture which synthesize basement membrane–type collagenous precursors. The study of Crouch and Bornstein on the synthesis of type IV–like procollagen by human amniotic fluid (AF) cells in culture also shows this doublet on SDS-PAGE gels. The doublet migrated as two closely spaced chains between β-components and type I procollagen chains (approximately 150,000 daltons). However, unlike the corneal epithelial cells reported here and endothelial precursor collagen reported previously, AF procollagen eluted prior to the start of the gradient when examined by DEAE-cellulose chromatography. Pepsin treatment of type I precursor molecules synthesized by AF cells (AF1) produced a collagen chain which migrated slightly more slowly than collagen α1(I) chains.

The basement membrane precursor collagen protein from rabbit corneal endothelial cells in culture eluted as a single peak between 2 and 3 mmho on DEAE-cellulose chromatography. In the present study under similar conditions, the collagenous proteins differed in molecular size (160,000 and 180,000 daltons) and eluted between 5 and 7 mmho.

It is possible that basement membrane collagen precursors synthesized by rabbit corneal epithelial and endothelial cells are related but are separate entities. Perhaps they represent a family of similar molecules as has been reported for collagen type I and type II.

It has been suggested that collagen type A chain, abundant in skin and liver, might originate in epithelial basement membranes, but in the studies reported here this molecular species was not seen. In addition, the absence of type I α1-chains strongly indicates that the corneal epithelial cells in culture synthesize only type IV procollagen. This epithelial cell product has distinguishing properties on DEAE-cellulose columns which suggest that
it is different from AF cell type IV,23 EHS sarcoma17 and rabbit corneal endothelial cell collagen,6 although all species mentioned share the common property of appearing as a doublet on SDS-PAGE under reducing conditions and, when pepsin-digested, give rise to a single pepsin-resistant collagen chain with molecular weight between 110,000 and 140,000 daltons.

We are grateful to Mr. Dan Waruszewski for his expert technical assistance. Our appreciation and thanks are extended to Dr. Takashi Inoue and Mrs. Gloria Limetti for transmission electron microscopy.

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