Expression of the 55-kD/64-kD Corneal Keratins in Ocular Surface Epithelium

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According to the concept of keratin pairing defined by tissue coexpression, a 55-kD/64-kD keratin pair is a marker of “corneal-type” differentiation. Intermediate filament (IF)-enriched preparations from guinea pig and bovine corneal epithelium were analyzed, and a rabbit antiserum was generated against a 55-kD polypeptide enriched in these preparations. This antiserum generated a typical IF-like pattern in cultured bovine corneal epithelial cells. Immunofluorescence microscopic analysis of frozen sections of guinea pig and bovine tissue revealed that the 55-kD antiserum labeled corneal and limbal epithelium. In addition, the antiserum stained a subpopulation of peripheral limbal cells that were distributed in both basal and suprabasal layers of the epithelium. The monoclonal antibody AE5 was used to investigate the distribution of the 64-kD polypeptide in guinea pig and bovine tissue. Immunoblotting analysis revealed that AE5 antibodies recognized a 64-kD polypeptide in guinea pig cornea, but recognized a 66-kD polypeptide in bovine cornea. Immunofluorescence microscopic analysis of guinea pig tissue revealed that AE5 antibodies labeled suprabasal layers of corneal and limbal epithelium. In contrast, in the bovine eye, AE5 antibodies generated staining in all layers of corneal epithelium, in suprabasal layers of limbal epithelium, and in groups of cells in the peripheral limbal epithelium. We discuss the possibility that the ocular epithelial cells recognized by either the 55-kD or the 64-kD antibodies in the peripheral limbus may play a role in the reepithelialization of the cornea after wounding. Invest Ophthalmol Vis Sci 31:448-456, 1990

Materials and Methods

**Explant Culture**

Microfilaments (5-6 nm in diameter), microtubules (20-25 nm), and intermediate filaments (IFs) (7-11 nm) are the major constituents of the cell cytoskeleton. Although the subunit polypeptides of microfilaments and microtubules are conserved in epithelial cells, IFs are composed of a complex family of about 30 polypeptides called keratins, ranging in molecular weight from 40 to 70 kD.1-5

It has been shown that the keratin subunit composition of epithelial cell IFs differs between species and between different tissues within the same species.3-5,6 One noteworthy example of differences in IF composition is found in the ocular surface epithelium, which is comprised of three morphologically and biochemically distinct cell types, ie, cornea, limbus, and conjunctiva.7-9 Because the corneal and conjunctival epithelium are separated by only several millimeters of limbal epithelium, ocular surface epithelium provides an excellent system in which to study the differences in IF composition between closely juxtaposed tissues.

Sun and co-workers1 have suggested rules for the pairing of keratin subunits based on frequent tissue coexpression. In this regard, they proposed that in the cornea, the 64-kD keratin (no. 34) pairs with a 55-kD keratin (no. 12) to form IF.1 This keratin pair has been described as a marker for “corneal-type” differentiation.

Although a monoclonal antibody (AE5) specific for the 64-kD corneal keratin has been described,2 no antibody has been produced which is specific for the 55-kD corneal keratin. Therefore, we generated a rabbit antiserum directed against the guinea pig corneal 55-kD polypeptide. Using the 55-kD antiserum and AE5 antibodies, we compared the distribution of the 55-kD and 64-kD polypeptides in guinea pig and bovine ocular surface epithelium.

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house, and then rinsed with povidone iodine (Chas-
ton Medical, Dayville, CT), 70% ethanol, and sterile
phosphate buffered saline (PBS: 8 mM Na2HPO4, 1.5
mM KH2PO4, 171 mM NaCl, 3 mM KCl, pH 7.4.
All chemicals were obtained from Sigma, St. Louis,
MO, unless otherwise noted). Explants were removed
from the periphery of the cornea with a 3-mm derma-
tology punch (Baker Cummins, Miami, FL) com-
monly used for skin biopsies and placed epithelial
side up on polylysine-coated coverslips.10 For cor-
neal-limbal cocultures, the punch was centered over
the limbus so that corneal and limbal epithelial cell
types were removed in one explant. The explants
were incubated for 30 min at 37°C to allow the
stroma to adhere to the coverslip prior to the addition
of culture medium. Explants were maintained at
37°C in minimum essential medium (MEM) supple-
mented with 10% fetal calf serum, 50 U/ml of peni-
cillin and 50 μg/ml of streptomycin (Gibco, Grand
Island, NY).

IF Preparations

IF-enriched preparations were isolated from guinea
pig and bovine corneal epithelium.11 Epithelial cells
were scraped from the corneal surface of globes with a
no. 11 scalpel blade (American Scientific, McGraw
Park, IL) and solubilized in 8 M urea, 5 mM sodium
phosphate, pH 7.2, 1 mM phenylmethane sulfonyl
fluoride (PMSF), 1 mg/ml p-toluenesulfonyl-L-argi-
nine methyl ester (TAME), and 0.2% β-mercaptoeth-
anol, with stirring, at room temperature for 2 hr.
Insoluble material was pelleted by centrifugation at
130,000 g for 45 min with a Beckman (Palo Alto, CA)
TLA 100.3 rotor in a Beckman TL 100 ultracentri-
fuge. The supernatant, which contained disassembled
IF subunits, was then dialyzed against 5 mM phos-
phate buffer, pH 6.6, 1 mM PMSF, and 0.2% β-mer-
captoethanol overnight at 4°C to induce IF reassem-
bly. Pelleted IF preparations resulting from high-
speed centrifugation were solubilized in gel sample
buffer (8 M urea, 0.63 M Tris-HCl, pH 6.8, 1% SDS,
10% glycerol, and 1% β-mercaptoethanol) and stored
at −80°C for later use.

Whole-Cell Extracts

Epithelial cells were scraped from corneal surfaces
of guinea pig and bovine eyes (see above) and homog-
enized in a Potter-Elvehjem homogenizer (Wheaton,
Millville, NJ) in the gel sample buffer described
above. Samples were stored at −80°C for later use.

Antibodies

A rabbit antiserum directed against the guinea pig
corneal 55-kD polypeptide was prepared as follows.12

Bands containing the 55-kD polypeptide were excised
from Coomassie-blue-stained sodium dodecyl sulfate
(SDS)-polyacrylamide gels (see below), homogenized
in PBS, and emulsified in Freund’s complete adju-
vant (Cappel, West Chester, PA). A New Zealand
white rabbit was immunized in multiple sites along
the back. Three weeks later, the rabbit was boosted
with protein emulsified in Freund’s incomplete adju-
vant. The rabbit was bled at weekly intervals begin-
ing 1 week after the boost. Blood was collected from
the ear vein and allowed to clot at 37°C for 1 hr and
at 4°C overnight. The serum was clarified by centrif-
gulation at 1200 g for 15 min with a Beckman AH-4
horizontal rotor in a Beckman AccuSpin FR table-
top centrifuge, and stored at −20°C. Antibody speci-
ficity was determined by Western immunoblotting
and immunofluorescence analysis. All animals used
in this investigation were maintained in accordance
with the ARVO Resolution on the Use of Animals in
Research.

AE5, a monoclonal antibody preparation specific
for the 64-kD corneal keratin,2 was kindly provided
by Dr. T.-T. Sun (New York University School of
Medicine, New York, NY). For immunoblot analysis
of IF-enriched preparations, hybridoma cells secret-
ing the anti-IF monoclonal antibody described by
Pruss et al13 were obtained from the American Type
Culture Collection (Rockville, MD). A monoclonal
antibody recognizing desmoplakins I and II was pur-
chased from Boehringer Mannheim (Indianapo-
lis, IN).

SDS–Polyacrylamide Gel Electrophoresis (PAGE)
and Western Immunoblotting

IF-enriched preparations and whole cell extracts
were analyzed with 7.5% acrylamide slab gels with
3.5% acrylamide stacking gels (BioRad, Richmond,
CA).14 Poly peptides were visualized by staining the
gels in 0.075% Coomassie blue, 45% methanol, and
10% glacial acetic acid, and then destaining in 10%
glacial acetic acid. IF-enriched preparations and
whole cell extracts separated by SDS-PAGE were
transferred to nitrocellulose (Schleicher and Schuell,
Keene, NH).15

Total transferred protein was visualized by staining
nitrocellulose blots in 0.1% amido black, 5% metha-
nol, and 10% acetic acid, and then destaining in 5%
methanol and 10% acetic acid. Comparable blots
were blocked in a solution of 5% nonfat dry milk in
PBS for at least 1 hr. Culture supernatants of AE5
and anti-IF antibody were used for immunoblots,
with the 55-kD antiserum used at a 1:500 dilution.16

Two-dimensional gel electrophoresis was also per-
formed on the bovine corneal IF-enriched prepara-
After electrophoresis, one of two procedures was performed: either the two-dimensional gels were stained with Coomassie blue and subsequently stained with a BioRad (Richmond, CA) silver stain kit, or separated polypeptides were transferred to nitrocellulose, as detailed above. Total transferred protein was visualized by staining with Ponceau S and destaining in 5% acetic acid. After photography, the same blot was blocked in nonfat dry milk and processed using the 55-kD antiserum as described above.

**Immunofluorescence**

Sections of tissue containing corneal, limbal, and conjunctival epithelium were dissected from five bovine and guinea pig eyes, frozen in liquid N₂, and embedded in OCT compound (Miles, Elkhart, IN). From each of the tissue pieces, approximately ten nonserial 6–8-μm sections were taken on a Tissue-Tek cryostat and processed for double-label indirect immunofluorescence. For these studies, the 55-kD antiserum was added to AE5 antibody culture supernatant, resulting in a final dilution of 1:50 for the 55-kD antiserum.

Cell culture coverslips were rinsed in PBS and then fixed in -20°C acetone for 2 min. After air-drying, the cultures were processed for immunofluorescence. For single-label immunofluorescence, AE5 antibody culture supernatant was used, and 55-kD antibodies were used at a 1:20 dilution. For double-label immunofluorescence, the 55-kD antiserum and the desmoplakin monoclonal antibody were mixed together to final dilutions of 1:20.

**Results**

**Biochemical and Immunologic Characterization of Corneal IF-Enriched Preparations**

IF-enriched preparations from guinea pig corneal epithelial cells were isolated by the method described by Steinert et al, taking care to exclude limbal epithelium from the preparation. Guinea pig corneal IF-enriched preparations (Fig. 1, lane 2) contained four major polypeptides (64-kD, 60-kD, 58-kD, and 55-kD) and several minor polypeptides.

In order to confirm the characterization of the IF of certain corneal polypeptides, we performed an immunoblotting analysis using a monoclonal antibody described by Pruss et al, which is broadly reactive for all classes of IFs, including epithelial keratins. A number of polypeptides in the molecular weight range of 40–70-kD, including the 55-kD and the 64-kD polypeptides, are labeled by the anti-IF antibody in IF-enriched preparations and whole cell extracts (Fig. 1, lanes 5, 10).

In order to characterize further the 55-kD component of corneal IF-enriched preparations, we prepared a rabbit antiserum directed against this polypeptide. Immunoblot analysis revealed that the antiserum was specific for the 55-kD polypeptide in guinea pig corneal IF-enriched preparations (Fig. 1, lane 3), and in whole cell extracts (Fig. 1, lane 7) prepared from guinea pig corneal epithelium (Fig. 1, lane 8).

The 55-kD antiserum is not species-specific, but was found to cross-react with bovine ocular surface epithelial tissue. IF-enriched preparations were isolated from bovine corneal epithelium in a manner similar to that described in Materials and Methods for guinea pig tissue. Bovine corneal IF-enriched preparations (Fig. 2, lane 2) contained three major bands (66-kD, 55-kD, and 47-kD) and two minor bands (60-kD and 50-kD). The 55-kD and 66-kD polypeptides were recognized by the antibody described by Pruss et al (Fig. 2, lanes 5, 10), indicating that they were IF proteins. Immunoblotting analysis of the bovine preparations revealed that the 55-kD antiserum recognized a 55-kD corneal polypeptide in IF-enriched preparations (Fig. 2, lane 3). In whole-cell extracts prepared from bovine corneal epithelium...
Fig. 2. IF-enriched preparations (lanes 2–5) and whole cell extracts (lanes 7–10) of bovine corneal epithelium were subjected to SDS-PAGE and transferred to nitrocellulose. Lanes 1 and 6 show molecular weight standards. Lanes 2 and 7 are amido-black stains to show total transferred protein. Lanes 3 and 8 are immunoblots using the 55-kD antiserum. Lanes 4 and 9 show immunoblots using AE5 antibodies, and lanes 5 and 10 are immunoblots using the anti-IF antibody.13 The 55-kD antiserum labeled a polypeptide in IF-enriched preparations (lane 3) and whole-cell extracts (lane 8). AE5 antibodies labeled primarily a 66-kD polypeptide (lanes 4, 9). Several lower-molecular-weight polypeptides are recognized also by AE5 in the whole-cell extract preparation. These may correspond to proteolytic breakdown products of the 66-kD polypeptide. The 55-kD and 66-kD polypeptides were stained by the anti-IF antibody13 (lanes 5, 10).

(Fig. 2, lane 7), the antiserum again recognized a 55-kD polypeptide (Fig. 2, lane 8).

Two-dimensional electrophoretic analysis revealed that the 55-kD polypeptide appeared as a spot, with a small tail (Fig. 3). The 55-kD polypeptide possessed an isoelectric point of about 4.9 (Fig. 3A).22 This spot was recognized by the 55-kD antiserum on the two-dimensional immunoblot (Fig. 3B).

AE5 monoclonal antibodies recognized a 64-kD polypeptide in guinea pig corneal IF-enriched preparations (Fig. 1, lane 4) and whole-cell extracts (Fig. 1, lane 9). In addition, weak reactivity was observed with a 66-kD polypeptide in the guinea pig corneal IF-enriched preparations. In bovine corneal IF-enriched preparations (Fig. 2, lane 4) and whole-cell extracts (Fig. 2, lane 9), AE5 antibodies recognized primarily a 66-kD polypeptide. Such differences in molecular weights of polypeptides recognized by AE5 antibodies in different species have been reported previously.20,23

Immunofluorescence Analysis of Ocular Surface Epithelium

In order to compare the distribution of the 55-kD and 64-kD IF polypeptides, frozen sections of guinea pig and bovine ocular surface epithelial tissue were processed for double-label immunofluorescence using the 55-kD antiserum (Fig. 4A) and AE5 monoclonal antibodies (Fig. 4B).

In guinea pig tissue, AE5 antibodies generated staining primarily in the suprabasal layers of the corneal and limbal epithelium (Fig. 4B). This labeling by AE5 antibodies stopped abruptly (Fig. 4B, large arrow). These observations agree with those described in rabbit ocular surface epithelium by Schermer et al.2 The 55-kD antiserum labeled all layers of the corneal epithelium (Fig. 4A). The limbal epithelium was also labeled, with the exception of some small areas of basal limbal cells (Fig. 4A, arrow). It is noteworthy that the 55-kD antiserum labeled a subset of cells that extended beyond the area labeled by the AE5 antibodies.

All layers of the corneal epithelium of frozen sections of bovine eye prepared for double-label indirect immunofluorescence using the 55-kD antiserum...
Fig. 4. Cryostat sections of guinea pig ocular tissue were prepared for double-label immunofluorescence using the 55-kD antiserum (A) and the AE5 antibody preparation (B). In these micrographs, the cornea is to the left (S, corneal stroma), and the small arrows mark the beginning of the limbus. The limbus is identified by the increase in the number of cell layers. In the peripheral limbus, there was an abrupt end to AE5 antibody labeling (large arrows). Note that all of the conjunctival epithelium was AE5-negative. The arrowheads in (A) show groups of peripheral limbal cells that are 55-kD-positive. Bar = 5 μm.

(Fig. 5A) and AE5 antibodies (Fig. 5B) were stained by both antibody preparations. In the bovine eye, we defined the area at which Bowman’s layer ends and where the number of epithelial cell layers increases as the beginning of the limbus.24 Also, the basal cells of this area are characteristically heavily pigmented in the bovine eye. In the limbus, the 55-kD antiserum and AE5 antibodies labeled the suprabasal layers. In contrast to guinea pig tissue, AE5 antibody labeling did not stop abruptly, but labeled groups of cells in the peripheral limbus before stopping (Fig. 5B, arrow). Whereas some of these cell groups were labeled by both the 55-kD antiserum and the AE5 antibodies, certain other groups were labeled only by AE5 antibodies (Fig. 6, arrow), and were not recognized by the 55-kD antiserum.

The 55-kD antiserum did not recognize other types of stratified epithelia such as tongue stratified squamous epithelia (data not shown).

Distribution of the 55-kD Polypeptide in Cultured Cells

The distribution of the 55-kD polypeptide was studied in cultured bovine ocular surface epithelial cells, using a tissue explant system described by Ebato et al.10 The explants were prepared by removing the epithelium, along with a portion of the stroma, from...
the eye using a dermatology punch. The epithelial cells migrated off the stroma and proliferated. Since the punch could be placed on any part of the eye, we were able to generate cultures consisting of only corneal epithelial cells by placing the punch on the cornea proper. We also were able to coculture corneal and limbal cells by positioning the punch so that these two types of epithelium were removed.

In cultured cells derived from the corneal epithelium, both the 55-kD antiserum and the AE5 antibodies generated a typical IF network pattern in every cell. Co-cultures of corneal and limbal epithelial cells also were processed for double-label immunofluorescence using the 55-kD antiserum. However, not all of the cells in these cultures were labeled by the 55-kD antiserum. This fits with our fluorescence studies in that not all of the limbal cells stained with the 55-kD antiserum. However, the epithelial nature of those nonlabeled cells was confirmed by analysis with monoclonal antibodies directed against desmoplakin. Desmoplakin is a major component of the desmosome located at the sites of IF attachment to the cytoplasmic plaque.\(^\text{25,26}\) Figure 7A shows a single cell that was not labeled, surrounded by many cells that were labeled by the 55-kD antiserum. Figure 7B shows a group of 55-kD positive cells next to a group of 55-kD-negative cells. Even in 55-kD negative cells, desmosomes were formed with 55-kD positive cells as determined by labeling with desmoplakin antibodies (Fig. 7C, D; arrows).

**Discussion**

We analyzed IF-enriched preparations of guinea pig and bovine corneal epithelium by SDS-PAGE, and characterized a 55-kD polypeptide that is a major component of the preparations in both species (Figs. 1, 2). We produced a rabbit antiserum directed against the guinea pig 55-kD polypeptide. This antibody cross-reacted with the 55-kD polypeptide in bovine tissue and generated a typical IF pattern in cultured bovine corneal epithelial cells processed for immunofluorescence. On immunoblots, the 55-kD polypeptide was labeled by a monoclonal antibody known to recognize all classes of IF, including epithelial cell keratins.\(^\text{13}\) These results suggest that the 55-kD polypeptide is a keratin structural protein.

Detailed one- and two-dimensional gel analyses of keratins in human, rabbit, and bovine tissue showed that the 55-kD (K12, as suggested by Moll et al\(^\text{1}\)) and 64-kD keratins (K5) have a limited tissue distribu-
Fig. 6. A cryostat section of bovine peripheral limbal epithelium was prepared for double-label immunofluorescence using the 55-kD antiserum (A) and the AE5 antibody preparation (B). The arrows point to a group of cells that were labeled by the AE5 antibodies, not by the 55-kD antiserum. S, stroma. Bar = 5 μm.

... that can be regarded as a marker for corneal-type differentiation. Indeed, the corneal specificity of the 64-kD keratin in rabbit ocular surface epithelium has been demonstrated by immunofluorescence microscopy using the monoclonal antibody AE5. However, no antibody preparations have previously been generated against the 55-kD keratin, and so its distribution in ocular surface epithelial tissue has not been established firmly.

In the current study, we analyzed the distribution of the 55-kD and 64-kD keratins in bovine ocular tissue by immunofluorescence microscopy. In bovine tissue, both AE5 antibodies and the 55-kD antiserum labeled all layers of the corneal epithelium. In addition, both the AE5 antibodies and the 55-kD antiserum labeled suprabasal layers of the heavily pigmented limbal epithelium and a subset of cells which extended into the peripheral limbus.

The distribution of keratins in the guinea pig ocular surface epithelium has not been well established. The results of our immunofluorescence analysis of the distribution of the 55- and 64-kD keratins deviated from both the bovine and the rabbit data, suggesting a species difference. In guinea pig tissue, AE5 antibody labeling stopped abruptly in the limbal epithelium. The 55-kD antiserum, however, labeled a subset of peripheral limbal cells which extended a short distance beyond the end of the AE5 antibody labeling.

Schermer et al showed that in cultured corneal epithelial cells, the synthesis of the 64-kD keratin precedes that of the 55-kD keratin by 1–2 days. Roop et al showed also that in cultured mouse epidermal cells, the appearance of the K1 subunit of another keratin pair, K1/K10 (56.5-kD/65–67-kD), can precede the appearance of the K10 subunit. Therefore, the expression of members within a keratin pair is not always linked. Our results revealed that the above phenomenon is not restricted to cultured epithelial cells, but also occurs in situ in tissues. Specifically, although both the AE5 antibodies and the 55-kD antiserum appeared to colocalize in corneal epithelium, this is not the case in peripheral limbal epithelium. In guinea pig peripheral limbal epithelium, no cells were labeled with AE5 antibodies, but groups of cells were labeled with the 55-kD antiserum (Fig. 4). In bovine peripheral limbal epithelium, the reverse was true, ie, groups of cells were 55-kD-negative but AE5-antibody-positive (Fig. 5, 6). Certainly, we cannot rule out the possibility that the absence of immunoreactivity with either the 55-kD antiserum or AE5 antibodies was due to a reduced concentration of keratins in these cells or to epitope masking.

In guinea pig cornea, AE5 antibodies recognized a 64-kD polypeptide. In bovine corneal epithelium,
Fig. 7. Cocultures of bovine corneal and limbal epithelium were processed for double-label immunofluorescence using the 55-kD antiserum (A, B) and a monoclonal antibody directed against desmoplakin (C, D). (A, C) A 55-kD negative cell surrounded by many 55-kD positive cells. (B, D) A group of 55-kD-negative cells next to a group of 55-kD positive cells. The small black arrows in (B) indicate pigment granules in a 55-kD positive cell. The large white arrows in both (A) and (B) point to an area of contact between a 55-kD-negative and a 55-kD-positive cell. The arrowheads in (A) and (B) indicate contact between two 55-kD positive cells. Both of these contact sites show labeling by desmoplakin antibody. Bar = 10 μm.

However, AE5 antibodies recognized a 66-kD polypeptide, confirming the results of Cooper and Sun,\textsuperscript{20} ie, in corneas of different species, AE5 recognizes polypeptides of variable molecular weight. It now will be relevant to determine whether the 66-kD conjunctival polypeptide mentioned above merely shares a common epitope with the 64-kD corneal polypeptide or whether the 64-kD and 66-kD polypeptides are the same but are modified, eg, by phosphorylation, in different tissues.

One noteworthy result of the double-label immunofluorescence analyses of cultured cells using the 55-kD antiserum and desmoplakin antibodies suggests that desmosomes form between morphologically distinct cell types, ie, between cells that express different IF structural polypeptides (Fig. 7). Indeed, it appears that an individual desmosome may have different IF polypeptides associated with each of its cytoplasmic plaques; ie, desmosomes may be asymmetric at least as far as the components of the associated IF structure are concerned.

Stratified epithelia, such as the epithelium of the ocular surface, are excellent models in which to study the cell and the molecular biology of the processes leading to the differential expression of IF and the associated polypeptides that result as a consequence of cell stratification.\textsuperscript{29,30} The ocular surface is of particular interest, since two connected stratified epithelial tissues appear morphologically and biochemically distinct. One of the initial purposes of our study of the ocular surface epithelium was the production of antibody probes specific for corneal epithelium in order to study such differential keratin expression. We produced an antiserum directed against a 55-kD
corneal keratin which is proposed to be corneal specific. Immunofluorescence data revealed that this polypeptide was present in a subpopulation of peripheral limbal epithelial cells. Therefore, there are certain peripheral limbal cells that express major corneal polypeptides and so may be more “cornealike” than the surrounding cells. One may speculate that the more cornealike cells may be a source for epithelial cell renewal of the cornea after wounding. Using in vitro and in vivo model systems, we are currently in the process of investigating this possibility.

Key words: cornea, cytoskeleton, epithelium, intermediate filaments, keratins

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References


