Immortalization of Rabbit Corneal Epithelial Cells by a Recombinant SV40-Adenovirus Vector

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Purpose. Cultured corneal epithelial cell is detrimental because of its short life span and its heterogeneity. We have tried to establish an immortalized epithelial cell line.

Methods. Primary cultured rabbit corneal epithelial cells were infected with a recombinant SV40-adenovirus vector and were cloned three times.

Results. The immortalized cell continued to grow by more than 400 generations through 100 passages. SV40-associated large T antigen was demonstrable on the nuclear membrane of these immortalized cells by immunofluorescence technique. This cell line exhibited a similar cobblestone-like appearance as normal corneal epithelial cells. Transmission electron microscopy showed a line of evidence for stratification, including desmosome formation and microvilli development at the superficial cell layer. As the culture grew, these cells began to express cornea-specific 64 kD cytokeratins. In contrast to cultured normal corneal epithelial cells, this cell line had a good proliferative ability after a long-term storage in liquid nitrogen.

Conclusions. Because this particular cell line shares properties consistent with normal corneal epithelial cells and is easy to handle in vitro, it may serve as a useful tool in corneal epithelial research.

Corneal epithelium comprises of five to six layers of well-differentiated ectodermal cells possessing cornea-specific 64 kD keratins. Cultured rabbit corneal epithelial cells have been widely used in a number of studies. However, they usually stop proliferating after one or two passages and can grow only to seven to eight generations, even with the use of specially designed media. This resulted in a small cell yield, heterogeneity in cell populations, and the necessity of primary culture in each experiment. Therefore, it would be beneficial to culture corneal epithelial cells continuously. Modification of culture media would be required. In fact, it has been reported that rabbit corneal epithelial cells can be maintained up to 15 generations in modified MCDB-153 medium with extremely low calcium concentrations.1

On the other hand, simian virus 40 (SV40), which is one of the papovaviruses endemic to monkey cells, has been intensively used to immortalize several kinds of mammalian cells, such as human epithelial cell of nasal polyp, skeletal myoblast, human and rat embryonic fibroblast, and luminal epithelial cell of the human mammary gland.2-9 Although the molecular basis of immortalization by oncogenes is unclear, a large T antigen derived from SV40 is thought to play an important role in this phenomenon. This strategy also can be applied to overcome the hazards in culturing corneal epithelial cells. Using a recombinant SV40-ad-
enovirus vector, which is a more efficient tool for cell immortalization, we have succeeded in establishing a rabbit corneal epithelial cell line.

METHODS

Primary Culture of Rabbit Corneal Epithelium

All these experiments conform to the ARVO Resolution on the Use of Animals in Research. Primary corneal epithelial cells were obtained as previously described. In brief, a male New Zealand albino rabbit weighing 3 kg was killed with an overdose intravenous injection of pentobarbital. The lamellar corneal button, 8 mm in diameter, was aseptically excised from the eye, cut into 2 × 5 mm blocks, and placed in a 75 cm²-plastic flask (Falcon Labware, Oxnard, CA). Corneal blocks were grown in supplemented hormone epithelial medium (SHEM) consisting of 1 vol of Dulbecco-modified Eagle’s medium and 1 vol of Ham’s nutrient mixture F-12 (GIBCO, Grand Island, NY) supplemented with 15% (vol/vol) heat-inactivated fetal calf serum (GIBCO), 5 µg/ml insulin, 0.1 µg/ml cholera toxin, 10 ng/ml human epidermal growth factor, 0.5% dimethyl-sulfoxide, and 40 µg/ml gentamicin. The cultures were maintained in a humidified atmosphere (95% air and 5% CO₂) at 37°C. The medium was changed every 3 days. To avoid fibroblast contamination, the explanted blocks were removed at 4 days after initiating a primary culture.

Immortalization and Cloning of Rabbit Corneal Epithelial Cells

When reaching 75–80% cell confluency, primary cultured epithelial cells were infected with a recombinant SV40-adenovirus vector according to the method described previously. After two cycles of washing with serum-free SHEM, the epithelial cells were incubated with a recombinant SV40-adenovirus vector for 1 hour at a multiplicity of 1:100. After infection, the cells were cultured in SHEM and serially passaged (1:5). In this recombinant virus, a complete SV40 early region was integrated into the vector in place of an adenovirus early region.

Then, continuously growing, corneal epithelial cells were cloned by the paper filter technique. For this, 200 or 400 cells were seeded in a 60-mm plastic dish (Falcon) and incubated at 37°C. The cells cultured for 2 weeks in other dishes were fixed with 100% methanol at room temperature, air dried, and stained with Giemsas solution. The number of colonies in triplicate dishes was averaged.

Light and Transmission Electron Microscopy

A 60-mm plastic dish (Falcon) was coated with 7 volumes of 0.3% type 1 collagen solution in diluted HCl, pH 3, 2 volumes of 5X solution of minimum essential medium, and 1 volume of reconstitution buffer (2.2 g NaHCO₃ and 4.77 g HEPES buffer solution in 100 ml of 0.05 N NaOH CellMatriX [Nitta Gelatin Biochemical Lab, Osaka, Japan]) at 37°C for 5 minutes in advance. After gelatinization, immortalized corneal epithelial cells were seeded at the density of 1 × 10⁵ cells/ml and subsequently cultured in SHEM. Primary cultured corneal epithelial cells were prepared as described above. One or 2 weeks later, these cells were fixed with 10% paraformaldehyde, embedded in paraffin, and sectioned meridionally for light microscopic observation.

The cells cultured for 2 weeks in other dishes were fixed in 4% glutaraldehyde and postfixed with 1% osmium tetroxide for transmission electron microscopy. Then the specimens were dehydrated with graded alcohols, embedded in Epon, and sectioned for observation.

Immunofluorescence for Keratin and Large T Antigen

An appropriate number of immortalized cells were seeded in a well of a Lab-Teck chamber (Nunc, Naperville, IL) and incubated at 37°C. Primary cultured cells
Corneal Epithelial Cell Line by SV40

SDS-PAGE and Western Blotting of Insoluble Proteins

Insoluble cytoskeletal proteins from immortalized or primary cultured cells were prepared according to the method described previously. After a 1-2-week culture in a 60-mm plastic dish (Falcon), the cells were washed three times with PBS(−), scraped off mechanically, and homogenized in 20 mM Tris-HCl buffer (pH 7.4) for 30 minutes. Then the homogenate was kept at 4°C for 24 hours, sonicated, and centrifuged at 12,000 g for 30 minutes at 4°C. The pellet was solubilized with 2% sodium dodecyl sulfate (SDS) in 10 mM sodium phosphate (pH 7.0) for 10 minutes at 100°C. After protein concentrations were checked by a kit system (Bio-Rad Laboratories, Richmond, CA), 10 μg of each sample was applied to a lane of 10-15% polyacrylamide gradient gel and electrophoresed using Phast-System™ (Pharmacia-LKB Biotechnology, Sweden).

After electrophoresis, proteins were transferred to a nitrocellulose sheet. The sheet was dipped in blocking buffer consisting of 10% bovine serum albumin in Tween-Tris-buffered saline (TTBS), 100 mM Tris (pH 7.5)-0.9% NaCl with 0.1% (vol/vol) tween 20 for overnight, rinsed twice with TTBS, and incubated with anti AE-5 antibody (diluted 1:10 in TTBS) at room temperature for 1 hour. After three cycles of washing with TTBS, it was incubated with biotinylated anti-mouse IgG (Vectastain ABC-Kit, Vector Laboratories Inc., Burlingame, CA) at 37°C for 30 minutes, washed with TTBS three times, and incubated with an avidin/biotinylated anti-mouse IgG alkaline phosphatase complex (Vector Laboratories Inc.). After rinsing three times with TTBS, alkaline phosphatase substrate kit II (Vector Laboratories, Inc.) resolved in 100 mM Tris-HCl (pH 9.5) was applied to the sheet for staining.

Growth Ability in Soft Agar and Tumorigenicity in SCID Mice

The cell growth ability in soft agar was examined according to the method of McPherson. In brief, 100 or 1000 cells/ml of immortalized cell suspension in an aliquot of 0.33% agarose solution was poured onto the 0.66% agarose gel bed prepared in a 35-mm plastic Corning dish and incubated at 37°C for 2 weeks. The number of colonies was counted under a phase-contrast microscope.

In another study, three 10 week-old severe combined immunodeficiency mice received an intradermal injection of immortalized cells (5 × 10⁶ cells) at the back skin.

RESULTS

Growth Characteristics

After infection with an SV40-adenovirus vector, the rabbit corneal epithelial cells uneventfully grew through several passages. After cloning, they continued to grow by more than 400 generations through 100 passages. In culture, they exhibited a cobblestone-like appearance as primary cultured, normal corneal epithelial cells (Fig. 1). By immunofluorescence, large T antigen was constantly expressed in the nucleus of the immortalized cell of any generation (Fig. 2).

The population doubling time of immortalized and cultured corneal epithelial cells (first passage) was 18.8 and 15.6 hours, respectively, indicating that the former cells multiplied a little more slowly than the latter (Fig. 3). The colony-forming efficiency of the immortalized cells was 10.7%, whereas primary cultured cells were incapable of producing a colony.

When thawed from 6-month storage in liquid nitrogen, immortalized cells were notably able to proliferate in culture with the population doubling time (19.6 hours) similar to that of prestorage. In contrast, primary cultured cells showed poor attachment and could not grow well after thawing (Figs. 4 and 5).

FIGURE 1. Immortalized rabbit corneal epithelial cells observed by phase-contrast microscopy. (A) Before confluence. (B) In confluence. They displayed a cobblestone-like appearance.
Cell Morphology and Keratin Expression

On day 7 after initiating the culture, immortalized cells became almost confluent (Fig. 6). At this stage, some immortalized cells began to express positive immunofluorescence to AE-5 (Fig. 7). SDS-PAGE analysis showed that both immortalized and primary cultured cells possessed four major insoluble proteins at 56 kD, 48 kD, 40 kD, and 35 kD levels (Fig. 8).

On day 14, immortalized cells formed two to three layers just as primary cultured cells did (Fig. 6). Transmission electron microscopic analysis of these cells disclosed the development of desmosomes at the intercellular junction and the existence of microvilli at the apical surface of the superficial cell (Fig. 9), suggesting that these transfected cells underwent epithelial differentiation. In addition, all immortalized and primary cultured cells were shown to exhibit a positive immunofluorescence that corresponded to intermediate filaments (Fig. 7). In accordance with this observation, two additional bands were recognized in both immortalized and primary cultured cells at the 64 kD and 55 kD level (Fig. 8a). The 64 kD band was much more prominent and was found to react with cornea-specific cytokeratin antibody (AE-5) by Western immunoblotting method (Fig. 8b).

Tumorigenecity

When seeded in a 35-mm plastic dish, two colonies were formed from 100 transfected epithelial cells (2%) and four colonies from 1000 (0.4%). Thus, the trans-
FIGURE 6. Light microscopy of immortalized and primary cultured rabbit corneal epithelial cells on type 1 collagen gel. (A) Immortalized cells at 7-day culture. (B) Immortalized cells at 14-day culture. (C) Primary cultured cells at 14-day culture. Both cells formed two to three layers at 14 days in culture.

FIGURE 7. Immunofluorescence staining of immortalized and primary cultured rabbit corneal epithelial cells using anti-64 kD keratin antibody (AE-5). (A) Primary cultured cells at 7-day culture. (B) Immortalized cells at 7-day culture. (C) Primary cultured cells at 14-day culture. (D) Immortalized cells at 14-day culture. On day 7, some cells were positive for AE-5. On day 14, primary cultured cells and immortalized cells showed positive staining of well-defined intermediate filaments in all cells.

FIGURE 8. (a) One-dimensional SDS-PAGE profile of cytokeratins from immortalized and primary cultured rabbit corneal epithelial cells. Lanes A, D: molecular markers; phosphorylase B (106 kD), bovine serum albumin (80 kD), ovalbumin (49.5 kD), carbonic anhydrase (32.5 kD), soybean trypsin inhibitor (27.5 kD), lysozyme (18.5 kD). Lane B: immortalized cells at 7-day culture. Lane C: primary cultured cells at 7-day culture. Lane E: immortalized cells at 14-day culture. Lane F: primary cultured cells at 14-day culture. Immortalized cells expressed four bands at 56 kD, 48 kD, 40 kD, and 36 kD levels at the 7-day culture and, additionally, one major (64 kD) and one minor band (55 kD) at the 14-day culture. The cytokeratins were similarly expressed in primary cultured cells. (b) Western blotting of cytokeratins from immortalized and primary cultured rabbit corneal epithelial cells. Lane A: molecular markers; Lane B: immortalized cells; Lane C: primary cultured cells. Note the strong immunoreactivity of 64 kD protein with AE-5.
FIGURE 9. Transmission electron microscopy of immortalized rabbit corneal epithelial cells on type 1 collagen gel. Note that the superficial cells express microvillar structures (A), and desmosomes can be recognized between the cells (B). Bar = 1 μm.

infected epithelial cells were able to grow in soft agar, although rather inefficiently. A solid tumor, 10 mm × 5 mm × 10 mm, was slowly formed in one of the three severe combined immunodeficiency mice (33%) that had received an intradermal injection of the transfected epithelial cells. This tumor was not invasive to the surrounding tissues and was found to consist of homogeneous epithelial cells with round, dark-stained nuclei and intense immunofluorescence to SV40-associated large T antigen.

DISCUSSION

As reported, we have successfully immortalized rabbit corneal epithelial cells using a recombinant SV40-adenovirus vector. They continued to grow by more than 400 generations in vitro over 100 passages.

SV40 virus has been extensively used for immortalization of cells. However, the recombinant SV40-adenovirus vector used in this study has provided us with more advantages than SV40 itself. First, cell immortalization occurs more efficiently through the specific attachment of the vector to epithelial cells via an adenovirus receptor and subsequent intracellular introduction of a large T antigen. Secondly, viral replication does not occur in the immortalized cells because this vector does not include the origin of replication. A large T antigen shown to be expressed in every immortalized corneal epithelial cell may act as a suppressor on such negatively regulatory genes as p53 or retinoblastoma oncogene. To our knowledge, this is the first virus-immortalized cell line derived from corneal epithelial cells.

Undoubtedly, whether or not these immortalized epithelial cells retained properties consistent with corneal epithelium is the critical question. Although layered immortalized cells seemed merely to pile up rather than stratify on light microscopy, good evidence for cell-stratification came from transmission electron microscopic observations that they expressed an apical surface property such as microvilli and an intercellular connection such as desmosomes.

In support of this finding, SDS-PAGE gel electrophoresis clearly showed that these immortalized epithelial cells displayed the same keratin pattern as normal corneal epithelial cells. Especially as the culture grew, they began to express cornea-specific 64 kD keratin in addition to four major hyperproliferative-type keratins. This accords well with the previous report that normal basal corneal epithelial cells synthesize 48/56 kD keratins when growing exponentially in vitro and that they begin to express 55/64 kD keratins when becoming confluent and heavily stratified. Thus, it can be said that these transfected epithelial cells shared morphologic and biochemical properties with differentiated corneal epithelial cells. Further investigations on other cell functions such as integrin expression or collagenase production would certainly determine the significance of this particular cell line.

One may argue that these transfected epithelial cells acquired some properties indicating malignant transformation, such as anchorage-independent growth and tumorigenicity in an immunocompromised host. However, the tumor was formed at a lower incidence and the efficiency of growth in soft agar was not high. Of course, these immortalized epithelial cells cannot be used clinically; nevertheless, they will bring about benefits in basic research of primary cultured corneal epithelial cells. Probably the best application would be collection of mRNA or DNA because large amounts of homogeneous epithelial cells can be easily yielded. In addition, they are easy to handle in culture because of their rapid growth in inexpensive culture media and good cell preservability in liquid nitrogen. This rabbit corneal epithelial cell line may be a useful tool in corneal epithelial studies.
Corneal Epithelial Cell Line by SV40

Key Words
corneal epithelial cell, rabbit, large T antigen, immortalization, cell culture

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References