Characterisation of epithelial cell line from rat cornea

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Abstract

Purpose Characterisation of RtCE-1 cells, newly established rat corneal epithelial cell line.

Methods Morphology of RtCE-1 cells was characterised by light and electron microscopy. Expression of cytokeratins was studied by polyacrylamide electrophoresis and Western blotting. Characterisation of RtCE-1 cells also included karyotype analysis and *in vitro* study of growth kinetics.

Results The line shows morphological similarities to normal corneal epithelium. The cells express cornea-specific cytokeratins. Karyotype analysis revealed that the cells are polyploid with modal number of chromosomes at passage 30 equalling 72 ± 14 . Growth of the line is partially dependent on EGF.

Conclusion RtCE-1 cells may constitute a model for the evaluation of proliferation, function and differentiation of corneal epithelium *in vitro*.

Key words Cell line, Cornea, Epithelium, Rat

Corneal stratified epithelium plays an important role in maintaining homeostasis of the eye and forms a barrier for microbial invasion.¹ Functions of corneal epithelium are facilitated by its high self-renewing capabilities.² Furthermore, corneal epithelium displays a high regenerating potential following trauma.³ The mechanisms of regeneration of corneal epithelium involve an increased rate of cell proliferation, migration and spreading.³⁻⁵ These phenomena were found to be stimulated by some growth factors,^{6,7} and may also be regulated by inflammatory cytokines.^{8,9} There exists a possibility that some of these factors may be useful for treatment of corneal trauma, especially in cases of chemical burns and other extensive wounds.¹⁰ Therefore, studies on the mechanisms of growth and differentiation of corneal epithelium are of significance.

In vitro models for studies of corneal stratified epithelium growth and regeneration utilise mainly primary epithelial cell cultures obtained by enzymatic isolation from human or animal corneas.¹¹ However, these cultures are

not infrequently overgrown by contaminating corneal fibroblasts making specific analysis difficult. Furthermore, studies on cultured normal corneal epithelial cells may also be difficult due to their limited *in vitro* life span.¹² To overcome these problems, SV40-immortalised corneal epithelial cells have been used.¹²⁻¹⁴ However, viral immortalisation usually leads to alteration or even absence of the differentiated phenotype.^{15,16} To our knowledge, extended subculture efforts aiming at generation of spontaneous corneal epithelial cell lines resulted in only one permanent spontaneously transformed rabbit cell line (RCE1).¹⁷ These cells retain the ability to form stratified cultures and express terminal differentiation-associated cytokeratins; nevertheless, their propagation requires the presence of 3T3 feeder cells.

Materials and methods

Culture medium

A modified version of the medium for culture of rabbit corneal cells was used.¹⁷ The standard culture medium consisted of DMEM (Gibco BRL Life Technologies, Paisley, UK) and Ham's F12 Medium (Gibco) 3:1 mixture, supplemented with 10% fetal calf serum (FCS; Gibco), 0.4 µg/ml hydrocortisone (Sigma, St Louis, MO), 10^{-10} M cholera toxin (Sigma), 5 µg/ml transferrin (Sigma), 5 µg/ml insulin (Sigma), 2×10^{-11} M triiodothyronine (Sigma), 2 mM glutamine (Sigma), 1% antibiotic-antimycotic solution (Gibco) and 5 ng/ml epidermal growth factor (EGF; Sigma).

Isolation and culture of corneal epithelial cells

All animal studies conformed to the principles determined by the Association of Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Corneas were excised from 5 inbred male Wistar Albino Glaxo (WAG) rats killed by an overdose of diethyl ether. Each cornea was separated from the globe by a circular incision including the limbus. The corneas were then rinsed in phosphate-buffered saline (PBS; Gibco) and transferred into dispase II (Boehringer, Mannheim, Germany) in PBS

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without Ca^{2+} and Mg^{2+} and incubated at 37 °C for 1 h. Following this the epithelial layer was separated mechanically under a dissecting microscope, cut into small pieces and transferred to 0.05% trypsin, 0.02% EDTA solution (Gibco) for 10 min. The cells were dispersed by gentle pipetting, rinsed with the culture medium and seeded onto a 35 mm plastic culture dish (Costar, Cambridge, MA). After 1 week the cells were trypsinised and transferred into 25 cm² culture bottles (Costar). The medium was changed every 3–4 days. The cells were split when necessary using trypsin/EDTA solution.

The cells were routinely frozen at –7 °C in a solution containing 15% DMEM supplemented with 1.1 M glucose, 10% dimethyl sulphoxide (DMSO; Sigma, St Louis, MO) and 75% FCS at the cooling rate of 1 °C/min and stored in liquid nitrogen.

To produce stratified cultures, the cells were seeded on porous bottom dishes (Transwell, Costar) at a density of 500 000 cells per 6.5 mm diameter well and cultured for 1 week in standard culture medium changed every 2 days.

Evaluation of cell proliferation

For estimation of population doubling time (PDT) the cells were seeded into 25 cm² bottles at a density of $2 \times 10^5/\text{cm}^2$ in the standard culture medium with or without EGF. The cells from duplicate bottles were trypsinised after 12, 24, 48 and 72 h and counted in a Burkert's chamber. The average of four independent cell counts was used to estimate the cell number. PDT was calculated by the least squares method.

Light and electron microscopy

Monolayer cultures grown on 12 cm coverslips were fixed with cold methanol and stained routinely with haematoxylin and eosin. Stratified cultures grown on porous membranes were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h at room temperature. Then they were washed three times in cacodylate buffer, pH 7.4, and fixed in 1% OsO₄ in 0.1 M cacodylate buffer, pH 7.4, at 4 °C for 1 h. After fixation, material was dehydrated in an ethyl alcohol gradient followed by propylene oxide and embedded in low-viscosity epoxy resin.¹⁸ Semithin sections perpendicular to the culture surface were stained with methylene blue. Ultrathin sections were cut on an OmU 3 ultramicrotome (Reichert), stained with uranyl acetate¹⁹ and lead citrate,²⁰ and inspected in a JEM 100 C electron microscope.

Immunofluorescence

The cells grown on glass 12 mm coverslips were fixed in methanol at –20 °C for 15 min. They were exposed to AE1 (specific for most of the acidic keratins) or AE5 (specific for 64 kDa keratin 3) monoclonal antibodies (both from ICN Pharmaceuticals, Irvine, CA), followed by the secondary fluorescein isothiocyanate (FITC)-

labelled goat antimouse IgG (Sigma, St Louis, MO). Stained cells were mounted in Mowiol – DAB CO²¹ (both obtained from Sigma, St Louis, MO) and inspected under a Microphot-FX fluorescence microscope (Nikon, Japan).

Cytokeratin PAGE and Western blotting

Cultured cells (1×10^7) were rinsed twice with cold PBS, scraped from the culture dishes with a rubber policeman, and homogenised in an extraction solution in order to remove water-soluble proteins. Extraction solution contained 5 µg/ml pepstatine A (Sigma, St Louis, MO), 1 mM EDTA, 1 mM EGTA and 1 mM phenylmethylsulphonyl fluoride (Sigma) and 25 mM Tris-HCl (pH 7.4). Keratin-like proteins were obtained by centrifugation at 10 000 g for 30 min. The extraction procedure was repeated and the solution left overnight. After centrifugation the pellets were solubilised in 100 µl of 1% SDS containing 25 mM Tris-HCl and 10 mM dithiothreitol (DTT) by heating at 95 °C for 10 min. Solubilised material was cleared by centrifugation for 5 min at 10 000 g. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide), and transferred onto a nitrocellulose membrane by semi-dry blotting using Biorad's Trans-Blot SD apparatus (Biorad Laboratories, Hercules, CA). Cytokeratins were detected by AE1 or AE5 antibodies followed by an amplified alkaline phosphatase detection system (Biorad). Prestained low-molecular-weight standard (Biorad) was used for estimation of the relative molecular weight.

For visualisation of total PAGE-separated proteins the gel was fixed by soaking in 50% methanol for a least 1 h. Then, the proteins were stained with a solution containing 0.8% of silver nitrate, 0.08% of NaOH and 1.4% of 14.8 M ammonium hydroxide and soaked in developing solution containing 0.025% of citric acid and 0.02% of formaldehyde.²²

Karyotyping

Exponentially growing cells were incubated with Colcemid (0.5 µg/10 ml of medium) at 37 °C for 2 h. The cells were harvested by trypsinisation and treated with 0.075 M KCl at 37 °C for 15 min. After fixation in methanol–acetic acid the cells were pipetted onto slides, air-dried and stained with 10% Giemsa solution. Chromosome number was calculated in 50 metaphases. Chromosome pattern was compared with the normal rat karyotype presented by Levan and Levan.²³

Cell transplantation

The suspension of 5×10^6 cells in 200 µl of PBS was injected subcutaneously at the ear base of 5 male WAG rats. Animals were inspected weekly and the tissues at the site of injection were taken for histological examination after 4 and 8 weeks.

Fig. 1. Appearance of confluent monolayer culture of RtCE-1 cells under phase-contrast microscopy. Original magnification approx. $\times 800$.

Fig. 2. Several layers of RtCE-1 cells grown in a porous bottom dish. The basal layer is continuous and more packed while cells in superficial layers are larger and paler. Light microscopy; original magnification approx. $\times 1000$.

Fig. 3. Electron micrograph showing RtCE-1 cells grown in a porous bottom dish. The arrows indicate cyokeratin filaments. Original magnification approx. $\times 35\,000$.

Fig. 4. Prominent numerous desmosomes (arrows) in adjacent RtCE-1 cells. Electron microscopy; original magnification approx. $\times 70\,000$.

Results

Establishment of the corneal epithelial cell line

Primary cultures of cells obtained from corneal epithelium contained epithelial-like cells which reached confluency in about 5 days. Following the first passage a number of epithelial cells displayed a differentiated phenotype and detached from the culture vessel. The cultures were gradually overgrown by fibroblast-like elongated cells which covered nearly the whole surface of the bottle and masked epithelial cells. One culture containing mostly fibroblastoid cells was left for 1 month with occasional change of medium. Then, a small, slowly growing colony composed of polygonal cells could be observed. After the following 2 weeks these cells covered approximately 70% of the surface. Two-step trypsinisation resulted in elimination of fibroblasts already after the fourth passage. Growth of the epithelial

cells was not affected by the removal of fibroblasts, and to date the cells have reached passage 63. The developed cell line was named RtCE-1.

Evaluation of the morphology of RtCE-1 cells

RtCE-1 cells grown in monolayer culture displayed epithelial-like cuboid morphology (Fig. 1). When seeded at high density onto porous membranes the cells formed multilayer cultures with basal cells displaying basophilic cytoplasm and ovoid slightly flattened nuclei (Fig. 2). This basal layer was covered with a few (usually three or four) layers of cells with pale swollen cytoplasm and nuclei with chromatin concentrated close to the nuclear envelope.

Analysis of multilayer cultures by electron microscopy showed that the basal layer consisted of cells with high electron density and numerous bundles of filaments

Fig. 5. Comparison of proliferation rate of RtCE-1 cells grown with (open bars) and without 5 ng/ml EGF (filled bars).

Fig. 7. Polyacrylamide gel after electrophoresis of RtCE-1 extract, followed by silver staining.

(Fig. 3). These filaments were associated with numerous desmosomes (Fig. 4). Intercellular spaces contained extensions of neighbouring cells, some of which penetrated into the pores of the culture membrane. Cells in superficial layers were larger and of lower electron density than the basal cells. Filaments were more dispersed but the presence of numerous desmosomes marking the limit of a cell was still evidence. Cytoplasm was vacuolated and some cells were degenerated.

Evaluation of the proliferative potential of RtCE-1 cells

The cells cultured in standard culture medium with epithelial growth factor (EGF) displayed a relatively high proliferative potential. Their PDT at passage 47 was 16.1 h. Removal of EGF resulted in a significant increase in PDT to 22.9 h (Fig. 5). During the observation period there were no significant differences in proliferation rate of RtCE-1 cells irrespective of earlier or later cell passage.

Fig. 6. Cytokeratin pattern displayed after immunohistochemical staining of RtCE-1 cells with AE1 antibody. Fluorescence microscopy; original magnification approx. $\times 800$.

Expression of cytokeratins by RtCE-1 cells

Fluorescence microscopic analysis revealed that the cells grown in standard culture medium with EGF stained positively with AE1 antibody. The staining pattern showed a typical filamentous network, particularly dense at the cell periphery (Fig. 6). AE5 antibody specific for 64 kDa (K3) keratin did not reveal significant staining of keratin filaments in the majority of cells. Occasionally cells with weak peripheral fluorescence were observed.²⁴

SDS-PAGE electrophoresis of RtCE-1 water-insoluble cell extracts followed by silver staining detection of proteins showed major bands with molecular weights of 64 kDa, 62 kDa, 58 kDa, 55 kDa, 52 kDa, 50 kDa and 48 kDa (Fig. 7).

Immunoblot detection of K3

AE5 antibody did not show any bands.

Analysis of the RtCE-1 karyotype

A typical karyotype of RtCE cells at passage 30 is shown in Fig. 8. The modal number of chromosomes at passages 30 and 62 was 72 ± 14 and 74 ± 11 , respectively. The arithmetic mean of the chromosome number was 71.62 ($SD \pm 3.69$) and 73.74 ($SD \pm 3.42$). The incidence of passage 30 cells with particular numbers of chromosomes is presented in Table 1. Comparison of normal and RtCE-1 cell line karyotypes is given in Table 2.

Table 1. Number of cells with a particular number of chromosomes

No. of chromosomes	Incidence (no. of cells)
58	1
62	1
66	1
67	3
68	1
69	3
70	7
71	1
72	13
73	2
74	8
75	4
76	4
80	1

Fig. 8 Representative karyotype of RtCE-1 cells. Chromosomes were divided into six groups according to Levan and Levan:²³ group A, chromosomes 1–3; group B, chromosomes 4–10; group C, chromosomes 11–13; group D, chromosomes 14–20.

Tranplantation

There were no signs of tumour formation by RtCE-1 cells either 4 or 8 weeks after injection of 10×10^6 cells in 100 μ l of PBS into the left ear of 5 rats.

Discussion

The present paper describes a new RtCE-1 cell line derived from rat corneal stratified epithelium. The epithelial nature of these cells has been confirmed by both light and electron microscopic examinations. RtCE-1 cells display a typical cobblestone appearance and their cytoplasm contains numerous keratin-like filaments. Furthermore, they are able to stratify and form typical tight cell–cell junctions with numerous desmosomes.²⁵

To confirm the origin of the RtCE-1 line the cells were also analysed for the expression of cornea-specific cytokeratins. Immunofluorescence analysis of RtCE-1 cells revealed a positive reaction with AE1 monoclonal antibody recognising most of the acidic keratins

including 50 kDa class cytokeratins expressed by cultured cells originating from squamous epithelia.^{26–29} Furthermore, SDS-PAGE analysis of RtCE-1 cell water-insoluble extracts showed a cytokeratin pattern similar to that of normal corneal stratified epithelium. This included a pair of 55 kDa (K12) and 64 kDa (K3) cytokeratins which are considered as cornea-specific,^{27,28} thus suggesting the corneal origin of RtCE-1 cells. Schermer *et al.*²⁷ showed that basic 64 kDa K3 keratin detected by AE5 monoclonal antibody is localised in all layers of corneal epithelium, but only in suprabasal layers of the limbal epithelium. However, we were unable to detect significant expression of this keratin in RtCE-1 cells by immunofluorescence or by Western blot analysis using AE5 monoclonal antibody (data not shown). It should be stressed, however, that while AE5 antibody has primary specificity towards K3 of human origin and cross-reacts with monkey and rabbit cytokeratins,²⁸ it has been reported not to react with either fresh or cultured murine corneal epithelium.³⁰ Furthermore, it has been found that expression of K3 keratin may be greatly decreased in exponentially growing cultures.^{27,31}

Epithelial cell cultures, including cultures of corneal epithelium, require the presence of feeder-layer cells¹⁷ or growth factor supplementation.^{6,24,32} Indeed, RtCE-1 cells have been derived using medium containing factors delaying epithelial cell differentiation, i.e. EGF and cholera toxin. In medium supplemented with EGF RtCE-1 cells display a relatively high proliferative potential. Corneal epithelium expresses EGF receptors³³ and EGF supplementation of corneal epithelial cell cultures has been found to result in an increased proliferation and migration rate,^{6,7,9,24} Furthermore, EGF has been

Table 2. Comparison of normal rat karyotype and the karyotype of RtCE-1 cell line

	Normal karyotype	Karyotype of RtCE-1 line ^a
Group A ^b	6	7
Group B ^b	14	26
Group C ^b	6	8
Group D ^b	14	29
Sex chromosomes	2	1 ^c
Marker chromosomes	–	2

^aThe most frequent karyotype

^bChromosome grouping according to Levan and Levan.²³

^cChromosome X was probably included in group B, since it is similar to chromosome 5.

reported to accelerate corneal wound healing.¹⁰ Removal of EGF resulted in a significant decrease in RtCE-1 proliferation; however, the cells still retained the ability to grow. This may suggest that in later passages of RtCE-1 cells EGF may not be absolutely necessary. RtCE-1 cell requirements for other growth factors remain to be established.

Though it was possible to immortalise rabbit¹² and human¹³ corneal keratinocytes, e.g. by virus infection, it is obvious that spontaneously obtained cell lines retain a phenotype closer to that expressed by primary cultures than do cells transfected with a virus. For this reason spontaneously obtained cell lines are more suitable for *in vitro* studies on function of the original tissue.^{34,35}

Since normal corneal epithelial cells display a definite *in vitro* life span and usually differentiate and die following several passages it appears that RtCE-1 cells underwent spontaneous immortalisation. Such immortalisation might be due to spontaneous epigenetic events affecting the function of some growth regulatory genes.³⁸ Genetic events responsible for immortalisation of RtCE-1 cells remain unknown. However, autonomous growth of established immortal cell lines may be, at least partially, explained by polyploidisation which, however, is certainly not sufficient for malignant conversion.³⁵ As other authors report,^{17,34} rodent spontaneous epithelial lines with diploid or nearly diploid chromosome number need to be cultured on feeder layers. Karyotype studies on RtCE-1 support these findings, showing that the modal number of chromosomes in RtCE-1 cells is 72. We did not find differences between the number of chromosomes at early (30) and late (62) passages. Therefore, it appears that the rate of polyploidy of RtCE-1 cells is stable. In some RtCE-1 cells abnormal chromosomes could be seen; however, a possible significance of this finding requires further investigation.

RtCE-1 cells did not display tumorigenic potential when transplanted into syngeneic animals. They also seem to retain features of normal corneal epithelium including the ability to stratify. They may be easily propagated without the need for a feeder layer and may thus constitute a good *in vitro* model for various studies concerning corneal epithelium function, proliferation and differentiation. RtCE-1 cells might also be useful as an alternative or complementary model for various *in vivo* pharmacological tests such as the Draize ocular irritation test,³⁷ as well as for studies on *in vitro* construction of corneal equivalents.^{38,39}

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