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Development of a spontaneous permanent cell line of rabbit corneal epithelial cells that undergoes sequential stages of differentiation in cell culture

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SUMMARY

Established epithelial cell lines that retain their differentiation potential and growth regulatory characteristics can provide valuable tools for studying gene regulation, extracellular matrix synthesis or growth factor response. They are also useful for drug development and toxicity testing. Experiments were therefore carried out to optimize culture conditions for the long-term, serial transfer of corneal epithelial cells in the presence of 3T3 feeder layers; and to establish a permanent cell line. In such experiments, rabbit corneal epithelial cells were seeded at low inoculation densities, and transferred every 5 days. After 80 population doublings, an epithelial cell line, RCE1, emerged. The cell line is heteroploid, with an average population doubling time of 15.5 hours (vs 18 hours for primary cultures). When RCE1 cells reached confluence, they stratified to form a three- to five-layered epithelium and expressed the differentiation-related keratin pair K3/K12 as shown by immunoblot and immunostaining. Biosynthetic labeling of proliferating, confluent and stratified cultures further showed that RCE1 cells expressed keratin pairs K5/K14, K6/K16 and K3/K12, thus mimicking faithfully the stage-dependent differentiation of primary cultures of rabbit corneal keratinocytes. The results demonstrated that RCE1 cells provide a useful model for studying corneal cell growth and differentiation.

Key words: corneal epithelium, cell culture, keratin expression, established cell line

INTRODUCTION

Analysis of epithelial cell growth and differentiation has been frequently hindered by the limited life-span of primary cell cultures, and by the requirement for repeated isolation of tissues used as source of cells for experimental work. These difficulties can be circumvented by the establishment of cell lines that are capable of differentiating under defined culture conditions. Thus, many authors have attempted to develop epithelial cell lines. There are two major strategies. First, one can 'immortalize' cells with oncogenes introduced by transfection (Howeling et al., 1980), viral infection (Chang et al., 1982; Reddel et al., 1988, Scholte et al., 1989), or using retroviral vectors (Cone et al., 1988; Emami et al., 1989; Agarwal and Eckert, 1990). Most authors have obtained epithelial cell lines through infection with SV40 (Steinberg and Defendi, 1983; Hronis et al., 1984); the expression of transfected SV40-LT antigen encoding sequences (Agarwal and Eckert, 1990; Bartek et al., 1991; Stoner et al., 1991; Schiller et al., 1992) or oncogenes from epitheliotropic viruses such as HPV16 (Halbert et al., 1991; Willey et al., 1991), HPV18 (Willey et al., 1991) or adenovirus (Cone et al., 1988; Kuppuswamy and Chinnadurai, 1988). However, although immortalization frequency is increased, results from this approach are quite variable and dependent on cell type (Kuppuswamy and Chinnadurai, 1988), and in most cases the expression of differentiated phenotype is altered or absent (Hronis et al., 1984; Rosen et al., 1988; Scholte et al., 1989; Agarwal et al., 1991). Secondly, one can generate spontaneous cell lines by extended subculture, particularly in rodent tissues, e.g. those obtained from rat small intestine (Quaroni et al., 1979; Nègrel et al., 1983) or 3T3 fibroblasts (Todaro and Green, 1963). Although spontaneous establishment of human epithelial cell lines has been obtained by serial passage (Baden et al., 1987) or enhancing proliferation and delaying terminal differentiation (Boukamp et al., 1988), in general, spontaneous human cells lines have been difficult to obtain. Nevertheless, the cell lines obtained by extended cell culture tend to show close similarities to populations in primary cell culture (Quaroni et al., 1979; Nègrel et al., 1983; Boukamp et al., 1988). Thus, a spontaneous differentiating cell line should be advantageous compared with those immortal cells obtained by oncogene manipulation.

Corneal epithelial cells from human and rabbit have been succesfully cultured (Sun and Green, 1977; Doran et al., 1980; Jumblatt and Neufeld, 1983; Chan and Haschke, 1983), and availability of viable corneal epithelial sheets for research and drug testing has been reduced due to the limited proliferative abilities of such cultured cells. This problem could be solved by choosing an adequate source of cells for tissue culture, such as limbal epithelium (Lindberg et al., 1993), by the improvement of corneal keratinocyte culture, or by the establishment of a differentiating corneal epithelial cell line. Experiments were therefore designed to achieve an increased number of in

vitro passages for rabbit corneal epithelial cells and to develop a spontaneous cell line. After 80 population doublings, the RCE1 cell line was obtained. These spontaneously established cells retain many of the differentiation properties of corneal epithelium. Therefore, they provide an important tool with which to study corneal epithelial growth and differentiation.

MATERIALS AND METHODS

Materials

Insulin, hydrocortisone, human transferrin, cholera toxin, L-triiodothyronine and mitomycin C were obtained from Sigma Chemical Co. (St Louis, MO). The [^{35}S]methionine and Amplify™ were purchased from Amersham (Amersham Place, UK). Fetal bovine serum and calf bovine serum were purchased from HyClone Laboratories (Logan, UT). The AE5 monoclonal antibody and R167 antiserum were generous gifts from Dr Tung-Tien Sun (New York University School of Medicine). The biotinylated horse anti-mouse IgG, goat anti-rabbit IgG and FITC-streptavidin were provided by Vector Laboratories (Burlingame, CA); HRP-anti-mouse IgG and HRP-anti-rabbit IgG were from ICN Biomedicals Inc. (Costa Mesa, CA); Tissue-tek OCT compound was from Miles Inc. (Elkhart, IN), Epon 812 was from Polysciences Inc. (Warrington, PA.) and Gelvatol was obtained from Monsanto (St Louis, MO). All other reagents used were analytical grade.

Cell culture

Excised central corneas from freshly killed 1.0 kg albino rabbits, were used to prepare the primary cultures of corneal epithelial cells. Briefly, after removal of the iris-ciliary body, conjunctiva, lens and corneal endothelium, tissue was rinsed thrice with PBS, and transferred to a mixture (1:1, v/v) of trypsin 0.25% and EDTA 0.02%. Then, corneas were incubated at 37°C for 40 minutes and cells were gently scraped off. Disggregated corneal keratinocytes were seeded at 2.7×10^3 cells/cm^2 together with mitomycin C-treated 3T3 cells (Rheinwald and Green, 1975a; Sun and Green, 1977; Rheinwald, 1980), using Dulbecco-Vögt's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FBS) and 0.4 μg/ml hydrocortisone (basal medium) (Sun and Green, 1977). Three days after inoculation, primary cultures were changed to a (3:1, v/v) DMEM and F12-Ham's nutrient mixture supplemented with 5% FBS, 5% calf serum, 5 μg/ml insulin, 5 μg/ml transferrin, 0.4 μg/ml hydrocortisone and 2×10^{-9} M triiodothyronine (M-I). Where indicated, 1×10^{-10} M cholera toxin was added (M-II). The latter culture medium was used in most experiments. Medium was changed every other day. For subculturing, subconfluent cultures were incubated with a (1:1, v/v) mixture of 0.15% trypsin and 0.02% EDTA for 20 minutes at 37°C. Cells were always inoculated in the presence of 3T3 feeder cells as above. For colony forming efficiency quantitation, cells were inoculated at 1000 cells/60 mm culture dish. After 6 days, cultures were fixed and stained with Rhodamine B (Rheinwald and Green, 1975a,b).

Biosynthetic labeling of keratins

RCE1 cultures were incubated at 37°C for 1 hour in serum-free DMEM containing [^{35}S]methionine (50 μCi/ml) and a low concentration of cold methionine (3.0 μg/ml). After removal of 3T3 cells with EDTA, RCE1 cells were extracted with 25.0 mM Tris-HCl (pH 7.5) containing 1.0% Triton X-100, 1.0 mM EDTA, 1.0 mM EGTA, 1.0 mM PMSF, 87 μg/ml aprotinin, 0.5 μg/ml leupeptin to remove the water-soluble proteins. The remaining cytoskeletal preparation was solubilized by heating at 95°C for 5 minutes in 1.0% SDS and 25.0 mM Tris-HCl (pH 7.4) (Eichner et al., 1984).

Immunoblot analysis of keratins

Keratins from post-confluent cultures were separated by 12.5% SDS-PAGE, transferred to a sheet of nitrocellulose paper, reversibly stained with 0.5% (w/v) Ponceau S (Salinovich and Montelaro, 1986), and then stained with monoclonal antibodies against K3 (AE5) or rabbit antiserum raised against K12 (R167) as primary antibodies.

Immunofluorescence staining

RCE1 cells were grown on 18.0 mm × 18.0 mm glass coverslips, fixed and permeabilized with ice-cold methanol/acetone (1:1, v/v) for 5 minutes and stained with antibodies AE5 or R167 as described (O'Guin et al., 1985). When staining was performed on frozen sections, two-day post-confluent epithelia were detached from plates, draped over a piece of Whatman no.1 paper as described (Watt, 1984), and fixed with Zamboni's solution. After washing with PBS, epithelia were frozen and 5 μm sections were obtained. Primary antibodies were detected with FITC-streptavidin and biotinylated horse anti-mouse IgG or goat anti-rabbit IgG as secondary antibodies.

Electron microscopy

After fixing, confluent cultures were gently scraped off from the culture dish with a rubber policeman and pelleted. Pellets were embedded in Epon 812. In other experiments, cultures were directly embedded in the Petri dish. The thin sections were observed with a Carl-Zeiss M9 electron microscope.

Chromosome number quantitation

Chromosome number was determined in metaphase spreads from exponentially growing cultures. Cell cultures were incubated with 0.05 μg/ml colchicine for 5 hours. After incubation, fibroblasts were removed and epithelial cells were processed as described (Slack et al., 1976).

RESULTS

Serial transfer of rabbit corneal epithelial cells

A general strategy to maximize the number of passages for rabbit corneal epithelial cells could consist in cell culture in the presence of additives that can be described as stimulators of epithelial cell growth. Hence, I carried out several experiments to optimize their culture conditions. Rabbit corneal keratinocytes were inoculated with mitomycin C-treated 3T3 feeder cells (see Materials and Methods). After 3 days, while control cultures were fed again with basal medium (Sun and Green, 1977), some cultures were changed to M-I medium or M-I plus 1×10^{-10} M cholera toxin (M-II medium). After 5-6 days, subconfluent cultures were trypsinized and cells were seeded at the same inoculation densities. Cell passages were continued until cell populations died. Simultaneously, colony forming efficiency (CFE) was evaluated after each transfer.

Cells grown with M-I or M-II medium showed a greater longevity than those from control keratinocyte cultures. When

Table 1. Cell population duplications obtained in corneal epithelial cell cultures supplemented with different media

Culture conditions	Population doublings	Passages
Control (basal)	22.8	4.0
M-I	55.3	10.0
M-II	77.0	14.0

Rabbit corneal epithelial cells were maintained with the indicated culture media (see Materials and Methods), and subcultured as many times as possible. When cells were passaged, total cell number/dish was determined and cell population doublings were calculated.

corneal epithelial cells were grown in M-I medium, the number of cell population doublings was more than 2-fold higher than that from cells maintained with basal medium (Table 1). Also, when keratinocytes were fed with M-I plus 10^{-10} M cholera toxin (M-II), the highest number of passages was obtained; population doublings were about 77, i.e. 3.5-fold the values found in control cultures (Table 1). These results were confirmed when CFE was evaluated after each passage (Fig. 1). Control cultures showed a sharp decrease in CFE, and after the 4th passage cells did not form any colonies and did not grow even when they were inoculated at higher densities (not shown). When cells were grown in M-I or M-II, the CFE values were high for longer periods. At least 5.0% of the inoculated cells were able to grow into colonies up to the 8th or 10th transfer (Fig. 1). These cell populations were lost in the 10th and 14th passages, respectively.

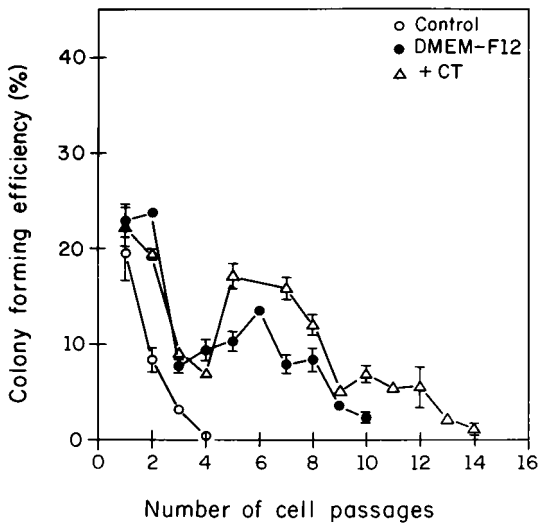

Fig. 1. Change in colony forming efficiency during serial transfer of rabbit corneal keratinocytes. Subconfluent cultures were trypsinized and cells were subcultured as many times as possible. To evaluate plating efficiency, cells from each transfer were inoculated as described (see Materials and Methods); after 6 days, cultures were fixed, stained, and colony number was determined. (○) Basal medium (control); (●) M-I medium (DMEM-F12); and (△) M-II medium (M-I plus 1×10^{-10} M CT).

Establishment of the RCE1 cell line

Using the M-II mixture, which appears to be superior for the long-term serial transfer of corneal epithelial cells, experiments were initiated to establish a spontaneous cell line of rabbit corneal epithelial cells. Keratinocytes were obtained from central corneas and seeded at 2.7×10^3 cells/cm^2. Five days after inoculation, subconfluent keratinocyte cultures were subcultured and the result of successive passages was assayed. On each passage, cells were grown for 5 days, counted and seeded, always at the same cell density. The results are shown in Fig. 2. After 6-8 transfers, cells showed a decrease in their growth rate, and CFE had the lowest values between the 12th and 14th passages (Fig. 2B). During this period, non-proliferative cells with an altered morphology appeared and were most frequently observed in the oldest cultures; however, cell population did not decrease in size, and growth rate began to increase at the 15th transfer (Fig. 2A, Table 2). By the 20th subculture, the CFE was augmented (Fig. 2B) and doubling time decreased (Table 2). It was considered that the RCE1 cell line was established when 80 population doublings had elapsed, and experiments were carried out in order to characterize it and to examine its differentiation abilities.

Growth of RCE1 cells, and chromosome complement

The average doubling time for rabbit corneal epithelial cells is about 15 hours, although this value is dependent on the animal and on the handling of tissue samples used as a source of cells; in some cases, primary cultures showed slower (22 hours) population doubling times. The growth rate of the RCE1 cells was

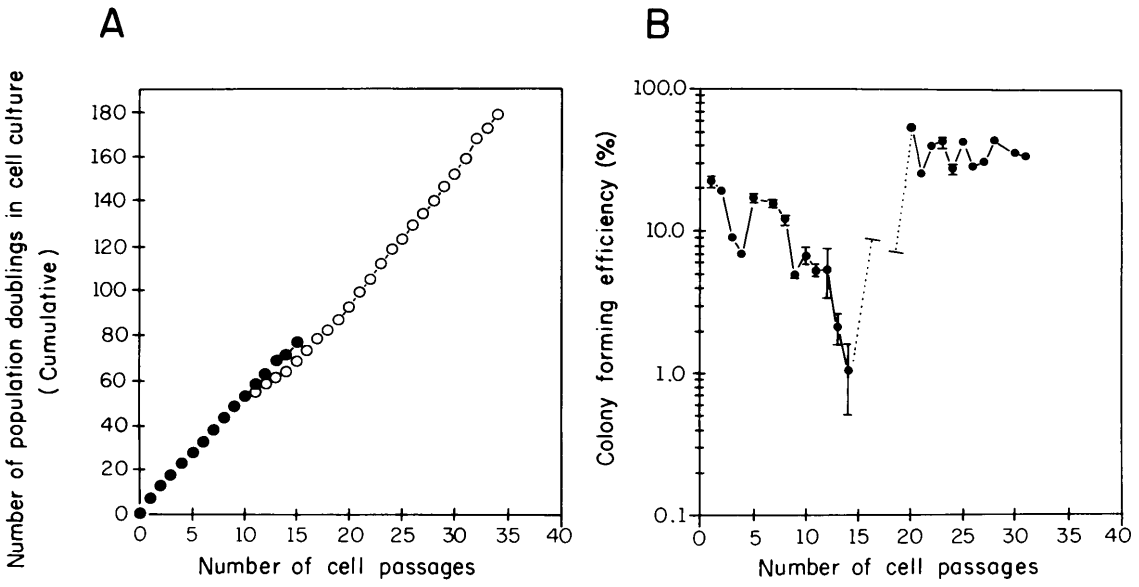

Fig. 2. (A) Establishment of the RCE1 corneal keratinocyte line. Rabbit corneal epithelial cells were inoculated as described (see Materials and Methods). When corneal epithelial cells were subcultured 6-7 days after inoculation (●), cell population was lost after 14 transfers. However, cells maintained with a 5-day transfer schedule (○), survived to crisis and developed into an established cell line. (B) Colony forming efficiency was evaluated as a measure of the proliferative abilities of the corneal epithelial cells. Note the decrease in CFE, which reached its lowest value at 75 population doublings. Afterwards, proliferative abilities increased and after 80 population doublings cells had a steady CFE, ranging between 30 and 40%.

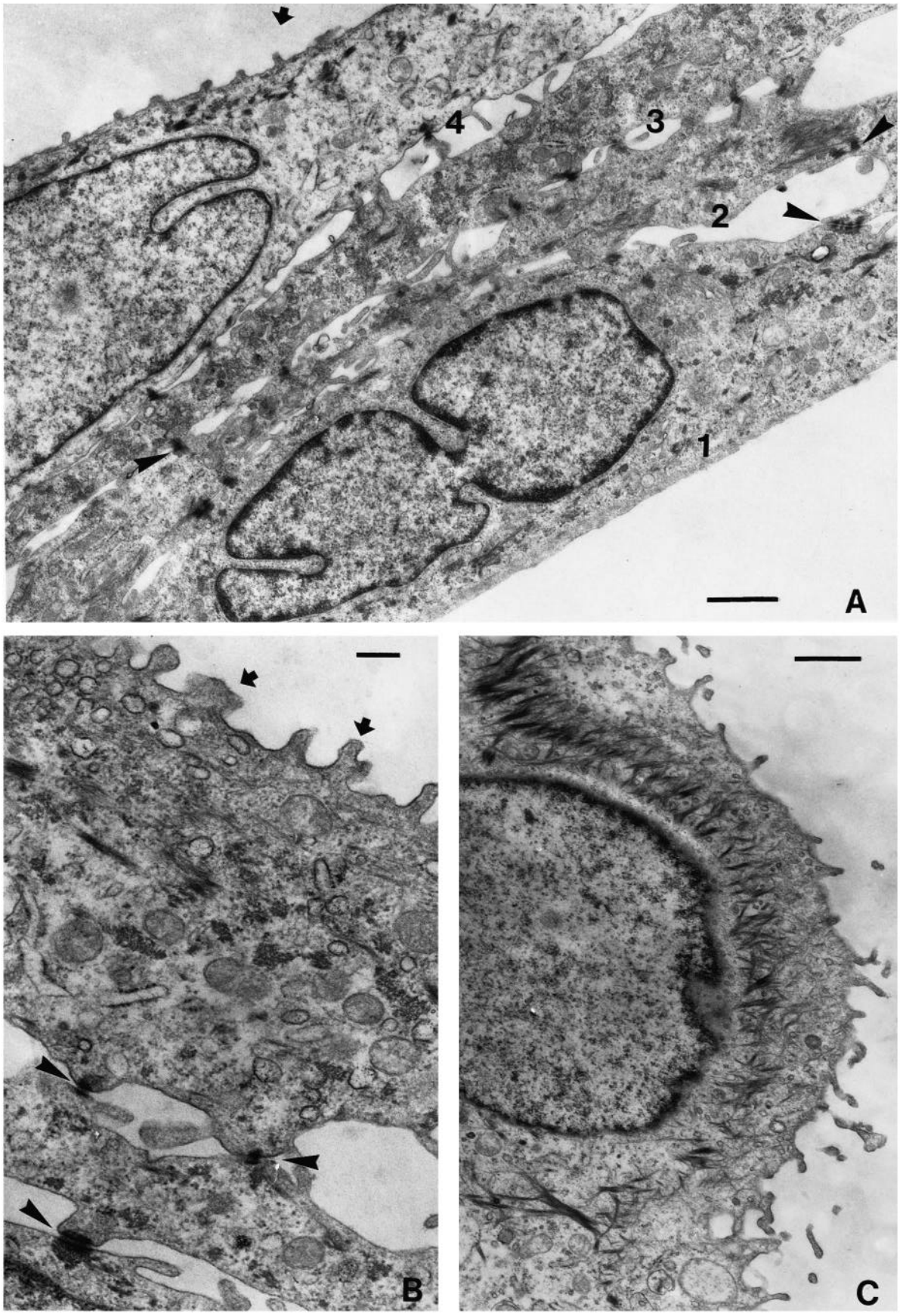
4
3
2
1
A
B
C

Table 2. Change in growth rate during rabbit corneal epithelial cell line establishment

Number of cell passages	Population doubling time (h)
1	18.0
12	59.0
14	67.0
20	19.0

Rabbit corneal epithelial cells were grown with M-II medium and serially subcultivated as described in Materials and Methods. Population doubling times were determined at every other passage, at the exponential growth phase of cell cultures inoculated at 2.7×10^3 cells/cm^2 .

compared with that obtained from primary cell cultures. During the exponential phase, RCE1 cells showed an average population doubling time of 15.5±2.2 hours. In RCE1 confluent, stratified cultures, cell densities were about 2.18×10^5 cells/cm^2. These values were similar to those observed in stratified primary cultures, which had about 2.0×10^5 to 3×10^5 cells/cm^2. On the other hand, when RCE1 cells were inoculated onto soft agar, even in the presence of 3T3 feeder cells, they did not grow, suggesting that they do not show a transformed phenotype.

The established corneal keratinocyte cell line RCE1, analyzed after 225 doublings, was found to have a heteroploid chromosome complement. The modal chromosome number was 37, with a range about ±19.

The RCE1 cells form stratified epithelia and express the terminal differentiation-linked keratin pair K3/K12

Light microscopy studies of RCE1 cultures, and the similar cell densities found in confluent primary corneal epithelial and RCE1 cell cultures, suggest that the cell line was undergoing cell differentiation. Further confirmation about the expression of the corneal phenotype by RCE1 cells was obtained from keratin analysis and examination by electron microscopy.

Five day post-confluent cultures were cut at right angles to the culture dish, or at a tangent to the epithelial surface. Thin sections of those epithelia constituted by RCE1 cells were composed of 3-5 flattened cell layers. Fig. 3 shows the stratified organization of a 4-layer epithelium; as can be seen, all cell layers showed abundant desmosomes interconnecting adjacent cells (Fig. 3A,B). Basal cells contained very few components of the granular or smooth endoplasmic reticulum, and mitochondria did not show a highly organized internal membrane. Keratin filaments were observed in all cell layers (Fig. 3A,B,C), but they were generally more prominent within suprabasal cells, where they were aggregated (Fig. 3C). Surface cells also exhibited short cytoplasmic projections on their surface (Fig. 3A,B).

On the other hand, immunofluorescence studies with the AE5 monoclonal antibody, which is specific for the basic 64 kDa rabbit and human corneal keratin 3 (Schermer et al., 1986), or with the R167 antiserum, which is specific for the acidic 55 kDa rabbit corneal keratin 12 (T.-T. Sun, personal communication), showed that RCE1 cells expressed the corneal-type keratin pair. AE5 staining was observed in a subpopulation of cells (Fig. 4C). In more advanced cultures (2-5 days after confluence) both AE5 and R167-positive cells were mainly located in the suprabasal layers of the stratified cultures (Fig. 4E-H) as demonstrated by frozen sections stained with AE5 antibody (Fig. 4H). Consistent with the results, the immunoblot analysis of keratins extracted from 5 day post-confluent RCE1 cells demonstrated high levels of keratins K3 and K12 (Fig. 5).

Finally, the changes in keratin synthesis during successive days in culture in RCE1 cells were examined. RCE1 cells were inoculated at 6.2×10^2 cells/cm^2, cultures were pulse labeled with [^{35}S]methionine, and their newly synthesized keratins analyzed by SDS-PAGE followed by fluorography (Fig. 6). During exponential growth (lanes 1-5), RCE1 cells expressed the keratin pair K5/K14, characteristic of basal cells from stratified epithelia, as well as the K6 keratin related to hyperproliferative states (Fig. 6, lanes 1-5, arrowheads). Low levels of K3 expression were observed from day 4 after inoculation; when cells reached confluence (7th day), the expression of the differentiated keratin pair K3/K12 was increased (Fig. 6, lanes 6-11). In similar experiments, keratin bands were sliced from SDS-PAGE gels, and keratin synthesis was evaluated by quantitation of radioactivity incorporated into individual keratins, which was plotted in relation to cell growth. The results indicate that K6 is synthesized only during exponential growth of RCE1 cells (Fig. 7). On the other hand, the expression of the differentiation-related keratins K3/K12 was strongly augmented after RCE1 cell cultures reached confluence, suggesting a close correlation with the stratification of the cultured epithelia (Fig. 7).

Fig. 3. (A) Transverse section of an epithelial sheet of RCE1 cells; ×13,300. Arrow points to apical cell surface. Note the cytoplasmic projections, similar to those microvilli found in corneal epithelium. Each layer in the epithelium has been numbered according to its position in relation to the culture surface: (1) basal cell layer, (2-4) suprabasal cells. Bar, 1.0 μm. (B) Another section of the epithelial sheet, showing microvilli on the epithelial surface; ×32,000; bar, 0.25 μm. (C) Sagital section of a cell located in the epithelial upper layer showing the abundant keratin filaments that are characteristic of all the suprabasal cells of RCE1 epithelium; ×12,250; bar, 1.0 μm. Arrowheads indicate desmosomes; arrows point to microvilli on epithelial surface.

DISCUSSION

Spontaneous cell lines have been difficult to obtain, since the results depend on cell type, animal species or even animal strain. In some animals, mainly rodents, cultured cells readily give rise to immortal cell lines as a result of epigenetic events (Todaro and Green, 1963; Denhardt et al., 1991). In rabbit, the chances for spontaneous establishment could be similar to those found for rodents, as suggested by the existence of the SIRC cell line obtained from rabbit cornea (Leerhoy, 1965; ATCC, 1988). Hence, I sought to obtain a spontaneous rabbit corneal epithelial cell line, following a general approach that has been used for mouse fibroblasts (Todaro and Green, 1963), and which could be useful by being extended to other mammalian cell types.

Culture conditions were optimized to maximize the number of cell passages and, therefore, to increase the chances for spontaneous establishment. As shown, the use of some additives that stimulate epithelial cell growth and delay

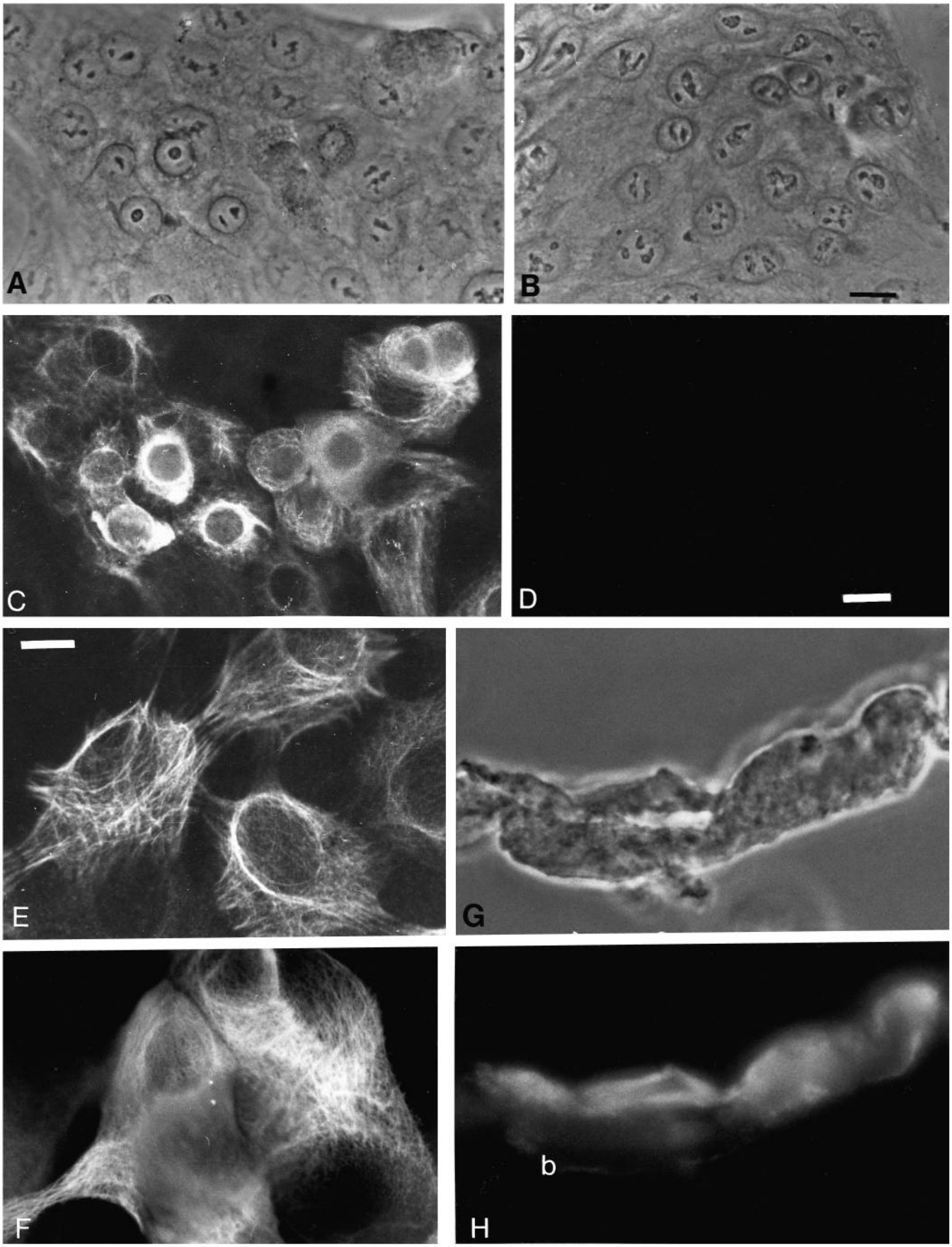

Fig. 4

Fig. 4. Immunofluorescent staining of RCE1 cell cultures. (A,B and G) Phase-contrast images corresponding to those fields showed in C,D and H, respectively. Note the staining of a subpopulation of RCE1 cells within the colony (4 days after inoculation) shown in (A) with the AE5 antibody (C). Those control cultures incubated with non-specific purified mouse IgG (B) did not show any significant staining (D). In more advanced cultures (5 days after confluence), cells were strongly stained with the R167 antiserum raised against K12 (E), or the AE5 antibody (F). A two-layered epithelium (two days after confluence; G,H) stained with the AE5 antibody; as shown, the immunostaining was observed mainly in suprabasal layers, although some basal cells were also stained (see right side of the figure); b indicates the basal side of the epithelium. Bars: (A-D), 9.0 μm; (E-H), 5.0 μm.

terminal differentiation increased the number of cell passages; the best results were obtained when corneal keratinocytes were grown in the presence of cholera toxin (Green, 1978). Afterwards, serial transfer of corneal cells was attempted using low inoculation densities and a 5 day transfer schedule. Since cells were always transferred before reaching confluence, terminal differentiation was avoided. After 15 passages cells went through a growth crisis but the population was not lost and the RCE1 cell line was established.

As demonstrated, RCE1 cells meet all the basic criteria that are considered useful for cell differentiation studies. First, the cells showed growth properties similar to those found in primary cultures of corneal keratinocytes (Schermer et al., 1989). Secondly, after reaching confluence, RCE1 cells were able to organize a three- to five-layered stratified epithelium with many of the morphological features shown by corneal epithelium (see Sheldon, 1956; Pedler, 1962; Kaye and Pappas, 1962; Hogan et al., 1971; Nichols et al., 1983). Finally, the immunostaining and western blot studies demonstrated specific expression of the differentiation-related keratins K3/K12 (Schermer et al., 1986, 1989), mainly in those cells leaving the basal layer. These findings suggest a high similarity between RCE1 cells and primary cultures of corneal epithelial cells.

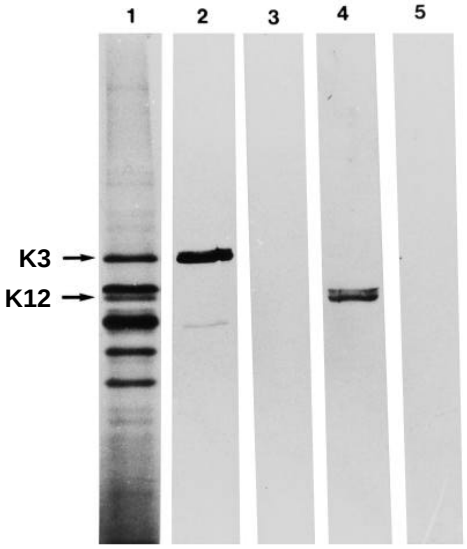

Fig. 5. One-dimensional immunoblot analysis of RCE1 keratins. Keratins extracted from stratified RCE1 cell cultures (5 day post-confluence) were incubated with: the AE5 antibody (lane 2), the R167 antiserum (lane 4) or the corresponding non-specific antibodies (lanes 3 and 5). Lane 1 shows the Coomassie Blue staining of the keratin extract. Note the strong reaction of the AE5 antibody and the R167 antiserum with the K3 and K12 keratins, respectively (arrows).

Additional evidence was provided by keratin pulse-labeling experiments. The basal cell-associated K5/K14 keratin pair was expressed as expected for a stratified epithelium (see Nelson and Sun, 1983; Cooper et al., 1985; Schermer et al., 1989); and although K16 was not resolved in the electrophoretic analysis, K6 was detected during exponential growth (see Fig. 6, lanes 2-5; Fig. 7) by following the expression pattern observed in primary cultures of corneal keratinocytes (see Schermer et al., 1989). Thus, the evidence clearly show the reciprocal synthesis of the hyperproliferation markers (K6/K16) and the differentiated keratins (K3/K12) described by Schermer et al. (1989). Moreover, K12

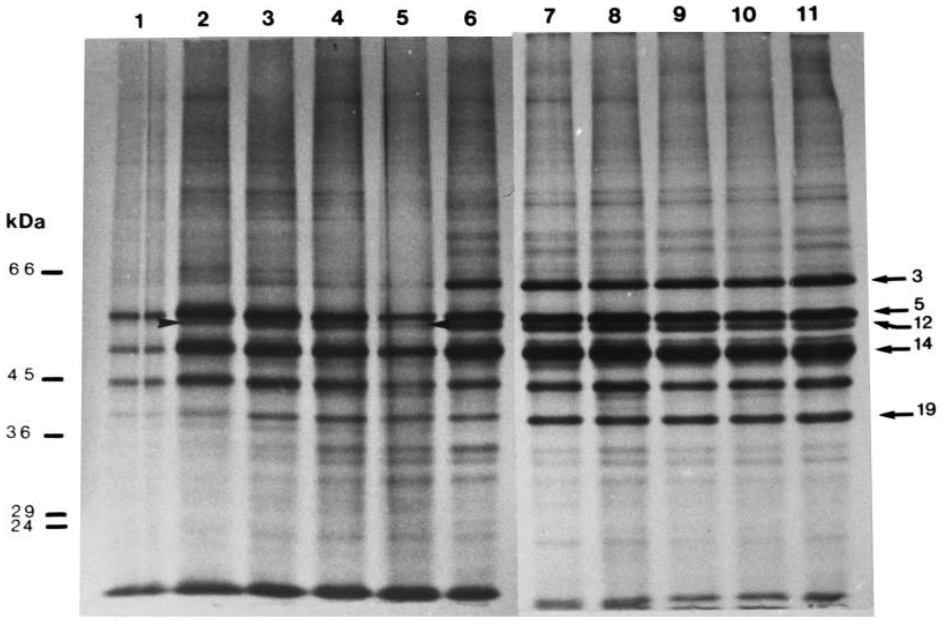

Fig. 6. Changes in keratin expression during culture of RCE1 cells. Beginning on the 3rd day of culture, duplicate RCE-1 cultures were pulse-labeled with [^{35}S]methionine on successive days (see Materials and Methods). Keratins were extracted; radioactivity incorporated in TCA-insoluble material was determined, and the extracts were analyzed by 12.5% SDS-PAGE; 200,000 cpm were loaded into each lane. Lanes 1-11 show keratins synthesized by cells grown for 3-13 days, respectively. Numbers on the left show the molecular mass marker mobilities; numbers on the right show the keratins identified. Arrowheads in lanes 2 and 5 indicate K6.

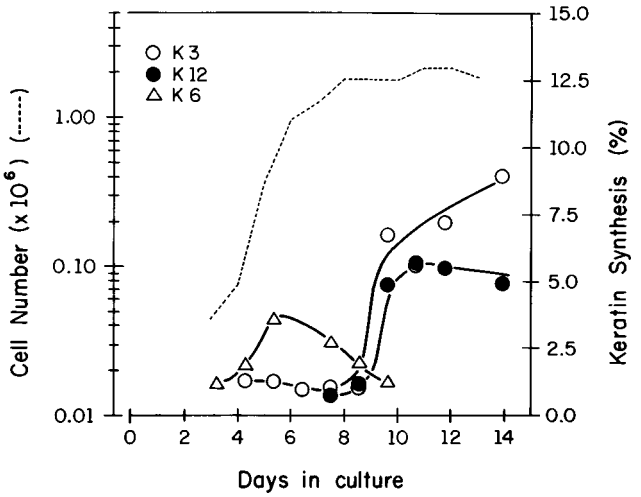

Fig. 7. Synthesis of K3/K12 and K6 during culture of RCE1 cells. In experiments similar to those described for Fig. 7, cells were pulse-labeled as indicated. Water-insoluble material was obtained, and 200,000 cpm of each sample were loaded onto 12.5% SDS-PAGE gels. After electrophoresis, keratin bands were sliced and radioactivity incorporated into individual keratins was quantitated. Rate of synthesis was calculated from total radioactivity incorporated into total water-insoluble protein extracted from cell cultures (see Materials and Methods). Broken line denotes cell number per 35 mm dish.

expression was preceded by K3 synthesis in a similar way to that described in those reports that demonstrated that K3 and K12 expression is not strictly synchronized (Schermer et al., 1989; Chaloin-Dufau et al., 1990; Kurpakus et al., 1990), and that the basic member of a keratin pair usually precedes that of the acidic partner (Schermer et al., 1986). Altogether, the results strongly support the conclusion that RCE1 cells mimick faithfully the differentiation process of cultured rabbit corneal keratinocytes.

Recently, rabbit (Araki et al., 1993) and human corneal keratinocytes (Kahn et al., 1993) have been immortalized. These cell lines share biochemical and morphological features with corneal epithelial cells but, in comparison with RCE1 cells, they show lower K3/K12 keratin levels (see Araki et al., 1993; Kahn et al., 1993) and, in some cases, growth properties related to the transformed phenotype (see Araki et al., 1993). It is clear that RCE1 cells express a phenotype more closely related to their tissue of origin than those cell lines obtained through oncogene manipulation. Thus, as suggested by others (Quaroni et al., 1979; Nègrel et al., 1983; Boukamp et al., 1988) and in this report, the spontaneous cell lines must be superior as experimental systems. The use of RCE1 cells should be advantageous in trying to understand cell differentiation; upstream sequences such as that of the K3 keratin (Wu et al., 1993) or antisense cDNAs encoding the K3/K12 or K6/K16 keratins could be used to gain some insight into tissue-specific regulation of keratins.

RCE1 cells are the first spontaneous cell line obtained from corneal epithelium. Previously, SIRC fibroblasts have been used to study corneal metabolism and physiology (Grabner et al., 1982, 1983; Shams et al., 1986), but few data about their origin and differentiation abilities are available. Therefore, the results obtained from SIRC cells should not be extended to corneal keratinocytes. In conclusion, the RCE1 cells should be an excellent model with which to study cell differentiation and keratin gene regulation. Also, they may be valuable in developing in vitro drug assay systems as an alternative to the eye irritation test (Draize and Kelley, 1952; Draize et al., 1944).

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