Cultured epithelial cells of cornea, conjunctiva and skin: absence of marked intrinsic divergence of their differentiated states

Tung-Tien Sun & Howard Green

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Keratinocytes of three different epithelia grown in cell culture express a large number of differentiation markers with either no differences or relatively small differences, depending on the species. Much of the distinctive phenotype of these epithelia in vivo *must be due to external modulation and relatively little, at least in the case of the human, to permanent intrinsic divergence during development.*

THE corneal and conjunctival epithelia and the epidermis are stratified squamous epithelia. Cells in the basal layer of all three seem similar, but differentiation in the superficial layers is obviously different. The corneal and conjunctival epithelia do not possess the granular cell layer and anucleate stratum corneum typical of epidermis. Since all three epithelia rise embryologically from closely related precursors, it is not surprising that they should share some common properties; but as these epithelia later become very different, they might be thought to have diverged markedly and irreversibly during embryogenesis.

With proper fibroblast support, human epidermal cells (keratinocytes) can be grown serially in cell culture[1,2], where they show many of the differentiation markers characteristic of this cell type *in vivo*. Single cells give rise to colonies, each forming a stratified squamous epithelium containing multiplying and differentiating cells. The cells synthesise keratins (T.-T.S. and H.G. unpublished) and grow in size, eventually forming cornified (cross-linked) envelopes[3,4]. The final stages of epidermal differentiation are promoted by placing the cells in suspension—a condition which does not permit the cells to grow; instead they become permeable to Trypan blue, their keratin filaments become detergent insoluble, they form cross-linked envelopes, and with the aid of serum plasminogen, their nuclei are digested[5].

We describe here the behaviour of corneal and conjunctival epithelial cells when grown in the same conditions. They can multiply and carry out the same programme of differentiation as the epidermal cells, including some aspects not observed *in vivo*. All three cell types are clearly keratinocytes and, at least in the case of the human, their behaviour in common culture conditions is so nearly the same that we are not able to distinguish them. In the case of the rabbit, there are some persistent, though small, differences between the keratinocytes of different origin.

Fibroblast dependence and effect of EGF

In earlier studies of cultured corneal epithelial cells, fibroblast support was not provided[6–8], but epidermal keratinocytes have since been found to require the support of fibroblasts in order to form colonies[1]. This support was usually provided in the form of lethally-irradiated 3T3 cells at a density of about 20,000 cm^{-2} inoculated together with or before the keratinocytes. In these conditions, the keratinocytes form expanding colonies, displacing the 3T3 cells from the vessel surface. The epidermal cells are serially cultivable and grow through many cell generations. In the human, epidermal cells do not transform into established lines and retain the diploid chromosome number[1].

This culture system was applied to epithelial cells of cornea and conjunctiva. Human corneas were obtained from local eye banks and after the endothelial layer and Descemet's membrane were removed with jeweller's forceps, the remaining tissue (epithelium plus a small part of the stroma) was minced to approximately 1–2 mm^3 and digested with 0.25% trypsin and 0.002% EDTA at 37 °C for 30–45 min. The disaggregated single cells were then plated with irradiated 3T3 cells. Multiplication of human fibroblasts was inhibited by the 3T3 cells, but if necessary, human fibroblasts were selectively removed with 0.02% EDTA after the epithelial colonies grew to appreciable size[1,3].

Figure 1 shows subcultures containing corneal epithelial and epidermal colonies 11 d after inoculation of 5×10^4 cells together with irradiated 3T3 cells. The colonies stained red with Rhodanile Blue and were of similar size and appearance. In the absence of 3T3 cells, neither epithelial cell type formed colonies; as there were no fibroblast colonies either, the cultures did not contain appreciable numbers of living human fibroblasts.

In the presence of epidermal growth factor[9] (EGF), the cells of large colonies of epidermal keratinocytes sustain their growth rate much better than in its absence[2]. Corneal epithelial cells were similarly responsive, judging from the size of the colonies (Fig. 1). Such an effect of EGF is to be expected from its action on intact corneal epithelium[10]. Conjunctival epithelial cells were similar to corneal epithelial cells in their colony-forming properties and EGF responsiveness.

When examined under the phase microscope, the basal cells of colonies of human corneal epithelial cells (Fig. 2*a*) seemed similar to those of epidermal cells (Fig. 2*b*) in their shape, their mosaic-like arrangement and their ability to displace the 3T3 cells from the vessel surface. The stratified structure of the colonies, and the silver-staining properties of the superficial cells[3] were also very similar.

Four strains of corneal epithelial cells, originating from donors aged between 25 and 60, and growing in the presence of EGF, were subcultured twice and grew through a total of about 30 cell generations. Although epidermal cells of newborn donors can grow through many more generations[2], the culture lifespan of corneal epithelial cells in the present experiments seems similar to that of epidermal cells of adult donors[1].

Synthesis of keratins and other cellular proteins

The keratins of bovine epidermal stratum corneum have been characterised electrophoretically[11,12]. Those of cultured human epidermal cells and human stratum corneum have recently been found to be similar in solubility, electrophoretic behaviour, immunological reactivity and ability to assemble into filaments *in vitro* (T.T.S. and H.G., unpublished).

The proteins of cultured epidermal, corneal and conjunctival epithelial cells were compared by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate (SDS). Figure 3*a* (track 1) shows the total protein of human corneal epithelial cells extracted with 2% SDS and 1% β-mercaptoethanol. Identical patterns were obtained from epidermal and conjunctival cells. In addition to the large number of protein bands unrelated to keratins, there were present several intense bands of keratins with a molecular weight range from slightly greater than that of actin (42,000) to slightly greater than that of tubulin (55,000). The non-

© 1977 Nature Publishing Group

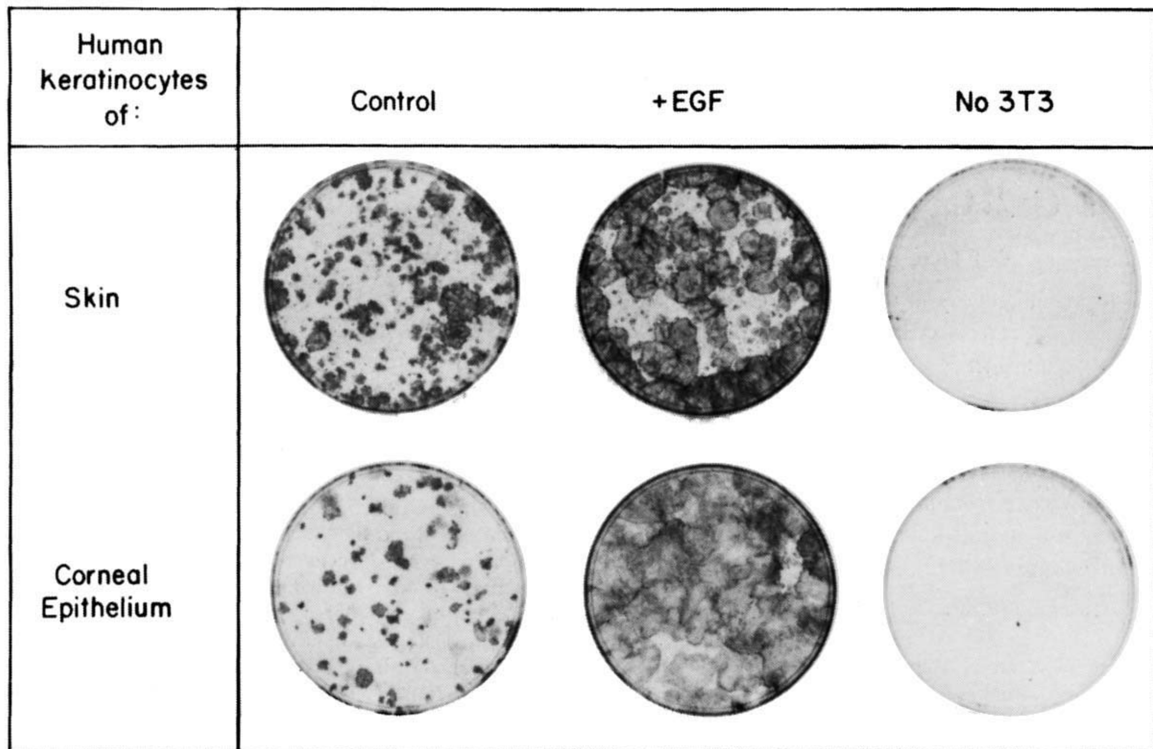
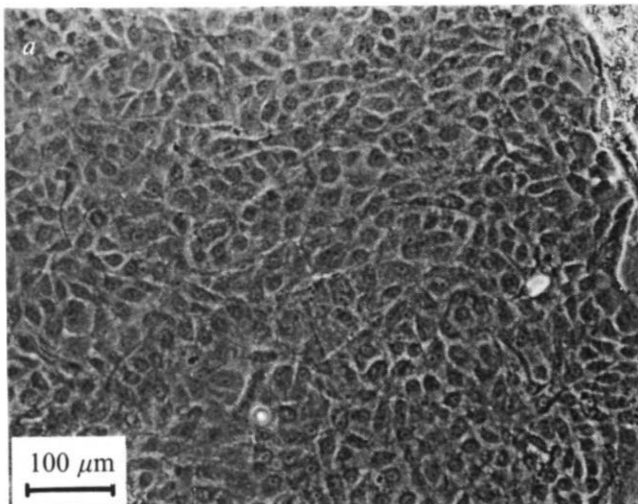
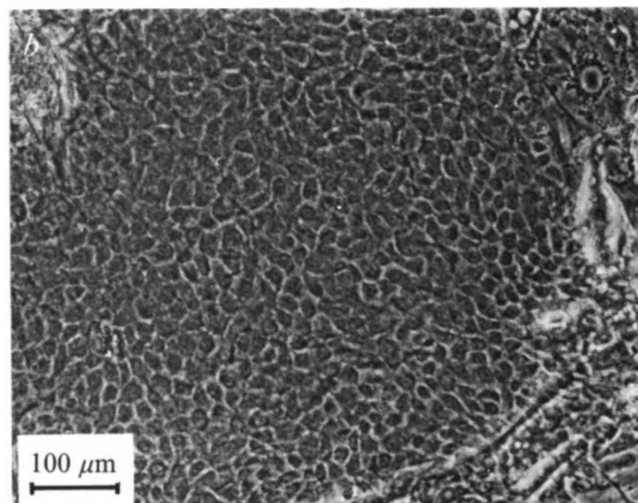


Fig. 1 Colony formation, fibroblast dependence and the effect of EGF. Human corneal epithelial cells (5×10^4, strain B, donor aged 25 yr, secondary culture) and epidermal cells (5×10^4, strain M, newborn donor culture) were plated in 60-mm dishes with or without 4×10^5 lethally-irradiated 3T3 cells. To some dishes, epidermal growth factor (EGF) was added to a final concentration of 15 ng ml^{-1}, starting on the third day after plating[2]. After a total of 11 d, the cultures were fixed with 10% formalin and stained with Rhodanile Blue[19].





Single colonies of corneal epithelial and epidermal cells. Primary colony of corneal epithelial cells of strain B (*a*) and secondary colony of epidermal cells of strain M (*b*) 11 d after inoculation. The cultures were fixed with buffered 10% formalin and photographed directly without staining. One margin of each colony appears in the right hand side of the pictures. Phase-contrast microscopy.

keratin proteins can be removed selectively by extraction in dilute buffer, leaving substantially only the keratins to be extracted with SDS and reducing agent (Fig. 3*a*, tracks 2–5). Tracks 2, 3 and 4 show no consistent differences in the position or relative intensity of the keratin bands of corneal and conjunctival epithelial cells and of epidermal cells. No keratins were detected in fibroblast extracts (track 5).

In order to compare the keratins of the different cell types further, antiserum specific for epidermal keratins of human stratum corneum was prepared and tested in double diffusion experiments against an extract of the total protein of each cell type. It was found that the proteins of cultured epithelial cells of epidermis, cornea and conjunctiva reacted strongly with the antiserum and showed a precipitin band in common with one of the bands produced by the keratins of stratum corneum (Fig. 4). A second precipitin band produced by the keratins of stratum corneum was not present in any of the cultured cell types and may have been due to a 63,000-molecular weight protein known to be present in stratum corneum, but not in corneal epithelium or any cultured keratinocytes (unpublished). No visible precipitin band was produced by the proteins extracted from human fibroblasts.

Increase in cell size and formation of cross-linked envelope

During differentiation in culture, human epidermal cells increase their cell size and protein content[3] as they do *in vivo*[13]. Figure 5*a* shows that, like epidermal cells, trypsin-disaggregated corneal epithelial cells adopted an approximately spherical shape and could be seen to vary greatly in size. The smallest cells, which are probably the basal cells[3], had a diameter of about 12 μm. The

©1977 Nature Publishing Group

largest cells, probably the superficial cells of the epithelium[3], exceeded 35 μm in diameter. Some of the large cells possessed cross-linked envelopes similar to those made by terminally differentiated epidermal keratinocytes. The presence of these envelopes was demonstrated by heating a trypsinised cell suspension in the presence of 5% SDS and 1% β-mercaptoethanol, a procedure which dissolves entirely cells without such envelopes but only the intracellular contents of cells with envelopes[3] (Fig. 5*b*). The insolubility of the envelopes is due to proteins cross-linked by ε-(γ-glutamyl)-lysine bonds[4]. As in the case of epidermal cells, usually 5–10% of the cells in surface cultures of corneal epithelial cells possessed cross-linked envelopes.

Cross-linked envelopes were also found in human corneal epithelium *in vivo*. Samples were scraped from the surface of excised corneas with a knife, and treated directly with detergent and reducing agent. Microscopic examination showed that envelopes were abundant. These envelopes preserved the flattened shape of the cells *in vivo*, as they do even in cultures of epidermal keratinocytes when the cells are treated with the reducing agent and detergent without previous trypsinisation[3].

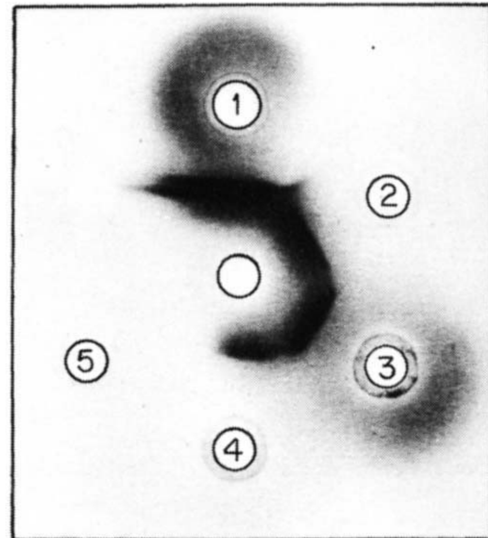
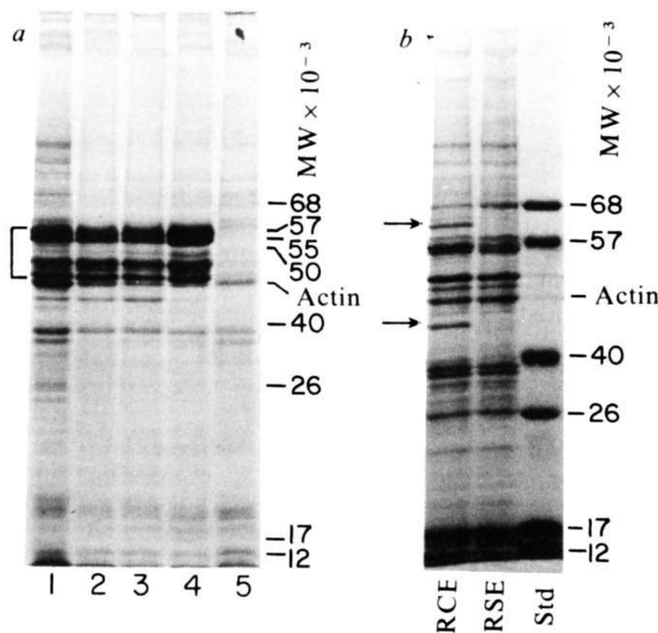


Fig. 4 Immunological cross-reactivity between keratins of human stratum corneum and proteins of cultured human epithelial cells. Stratum corneum was minced and pre-extracted with 8 M urea to remove non-keratin proteins. The keratins were then dissolved in 8 M urea containing 10 mM dithiothreitol. Rabbits were immunised at multiple sites with a total of 20 mg protein over a period of 2 months. Antiserum thus obtained was placed in the centre well of an Ouchterlony plate and tested against 80–120 μg of total cell protein of different cell types extracted with a solution containing 1% SDS, 10 mM dithiothreitol and 10 mM Tris-HCl (*p*H 7.4). 1, Keratins of stratum corneum (30 μg); 2, extract of cultured epidermal keratinocytes; 3, extract of cultured corneal epithelial cells; 4, extract of cultured conjunctival epithelial cells; 5, extract of cultured human dermal fibroblasts. The double diffusion test was performed in an agar plate containing 0.1% SDS, 0.5% Triton X-100, 0.01% Thimerosal, in addition to phosphate buffer and 1% Agarose, basically according to Yen *et al.*[20].



The proteins of cultured epithelial cells of cornea, conjunctiva and epidermis. *a*, Human. 3T3 cells were removed from nearly confluent epithelial cultures with isotonic buffer containing EDTA (refs 1, 3). The epithelial cells were collected in a small volume of the same buffer with the aid of a rubber policeman. Half of the cells were extracted directly with a solution of 2% SDS and 1% β-mercaptoethanol at 100 °C for 5 min. The extract was analysed by 12.5% disc polyacrylamide gel electrophoresis in the presence of SDS. The other half of the cells were first extracted with several changes of 100 vol 20 mM Tris-HCl (*p*H 7.4) and the insoluble proteins were then dissolved in detergent and β-mercaptoethanol as before and subjected to electrophoresis: protein (50–100 μg) was applied to each slot. The direction of electrophoresis was from top to bottom. Numbers on the right hand side of the gel denote molecular weights of standard proteins including human serum albumin (68,000), tubulin (57,000 and 55,000), the heavy chain of human gammaglobulin (50,000), and actin (42,000). The bracket at the left hand side of the gel indicates the mobility range of the keratin proteins. Samples in different tracks are: 1, total proteins of cultured human corneal epithelial cells; 2, insoluble proteins of the same cultured corneal epithelial cells; 3, insoluble proteins of cultured conjunctival epithelial cells; 4, insoluble proteins of cultured epidermal keratinocytes; 5, insoluble proteins of cultured human foreskin fibroblasts. Any apparent difference in band intensity between the extracts of the three epithelial cell types was not reproducible. *b*, Rabbit, 12-d-old primary cultures of corneal epithelial cells and epidermal cells derived from the same 1-yr-old animal were collected. Total cellular protein was extracted and analysed as for the human. Arrows show two non-keratin proteins present only in the corneal epithelial cells. The keratin bands, which lie between actin and molecular weight 57,000, differ slightly in mobility from those of the human and may be less abundant. RCE, Rabbit corneal epithelial cells; RSE, rabbit epidermal cells; Std, molecular weight standards.

Terminal differentiation of suspended cells

When human epidermal cells are suspended as single cells in medium stabilised with methylcellulose (methocel), they develop disulphide-stabilised keratin filaments and become insoluble in SDS solutions. These conditions also greatly favour the formation of cross-linked envelopes. Eventually, the cell nuclei are destroyed[5].

By all three criteria, human corneal epithelial cells were found to behave similarly. Within a few days in suspension, all the cells became insoluble in detergent, and about 50% developed cross-linked envelopes (Fig. 6). About 80% of the cell nuclei were destroyed within 9 d.

Properties of rabbit keratinocytes in culture

In order to compare the same cell types from another species, experiments were carried out on epidermal and corneal epithelial cells of the rabbit (newborn to 1 yr old). Both formed stratified squamous epithelial colonies in surface culture and developed detergent-insoluble keratin filaments and cross-linked envelopes in suspension. Both cultured cell types were shown to possess desmosomes and tonofilaments on electron microscopic examination, and both contained keratins demonstrable by electrophoresis (Fig. 3*b*). It is clear that, as in the human, both cell types are keratinocytes; the two cell types of the rabbit could, however, be distinguished in culture. For example, although the corneal epithelial cells formed cross-linked envelopes in methocel suspension, they did so with lower frequency than epidermal cells and did not form envelopes at all in surface culture. This is perhaps related to the fact that, unlike that of the human, the corneal epithelium of the rabbit *in vivo* does not possess cells with cross-linked envelopes. Rabbit corneal epithelial cells grew poorly on subculture in comparison with the epidermal cells. Finally, the

©1977 Nature Publishing Group

cultured rabbit corneal epithelial cells contained two water-soluble non-keratin proteins not present in epidermal keratinocytes (Fig. 3*b*).

External modulation or intrinsic divergence of differentiated state

By the criteria already described, the cultured cells of all three epithelia are keratinocytes (to be distinguished from fibroblasts of corneal stroma, sometimes referred to as keratocytes). In the human cell cultures we have been unable to distinguish the three cell types by any criterion. The same differentiation markers can be expressed in culture by epidermal and corneal epithelial cells of the rabbit, although the culture phenotypes of the two cell types are not identical. These results suggest that an important part of the differences between these epithelia *in vivo* results from differences in the local environment in which the cell type is found.

One obvious local difference is related to the adhesiveness of the superficial cells of the epithelium. A dry-surfaced epithelium (skin) invariably possesses an anucleate stratum corneum, whereas wet-surfaced epithelia frequently do not. Whether an anucleate cell layer is present or not may simply be a matter of the adhesiveness of the superficial cells, which in turn could be affected by the degree of hydration. If adhesiveness is low, detachment may occur before differentiation is complete. This occurs in surface cultures of epidermal keratinocytes; nuclear destruction is completed, for the most part, after detachment of the squames[5], rather than before, as in intact skin. Similarly, we have shown here that nuclear destruction takes place in corneal keratinocytes suspended in methocel much as it does in epidermal keratinocytes, although corneal epithelium does not normally possess a stratum corneum.

A second and perhaps more important difference is related to the nature of the cells located beneath the epithelium. Instructive effects of the underlying connective tissue are known to specify keratinocyte behaviour in adults[14] as well as in embryonic life[15]. In our cultures of the various epithelia, the fibroblast population consists mainly of (irradiated) 3T3 cells. For the reasons mentioned, fibroblasts of the type associated with the epithelium *in vivo* were virtually absent from the cultures studied. The very similar phenotype exhibited by the cultured keratinocytes might be explained by either absence of specific fibroblast instruction or the presence of common instruction provided by the 3T3 cells. The possibility exists that any one of these keratinocyte types, especially in the human, placed in the *in vivo* site of one of the other, even after birth, would conform in phenotype to the other keratinocyte type and generate the site-specific epithelium[14-16].

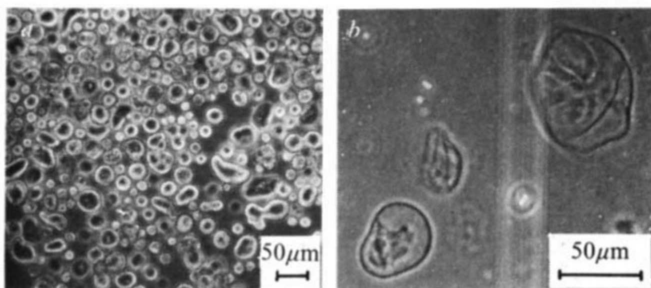


Fig. 5 Cell enlargement and formation of cross-linked envelopes by cultured human corneal epithelial cells. *a*, Heterogeneity of cell size in the growing colonies. 3T3 cells and any contaminating living fibroblasts were removed selectively from a nearly confluent 17-d primary culture with EDTA. The remaining corneal epithelial cells were trypsinised and an aliquot of the single-cell suspension examined in a haemocytometer chamber under the phase microscope. Note the presence of cells of variable diameter from 12 μm to > 35 μm. *b*, Cross-linked envelopes formed in surface culture. To a trypsinised single-cell suspension of cultured human corneal epithelial cells, SDS and β-mercaptoethanol were added to final concentrations of 5% and 1% respectively and the solution was heated to 100 °C for 3 min. The photograph taken under phase microscopy shows several of the large envelopes remaining. All small cells are dissolved.

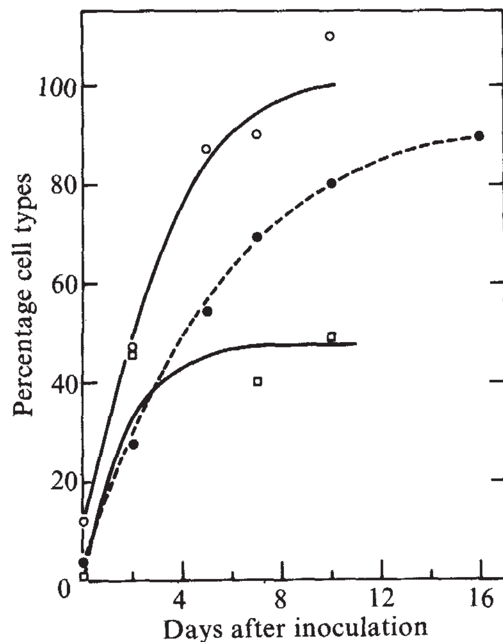


Fig. 6 Terminal differentiation of human corneal epithelial cells in methocel-stabilised suspension. Thirteen-day tertiary cultures of human corneal epithelial cells grown in the presence of EGF were trypsinised and resuspended at 2×10^5 cells ml^{-1} in medium containing 20% foetal calf serum and 1.2% methylcellulose. Aliquots were removed at intervals, diluted in isotonic buffer and the cells centrifuged. Cells insoluble in detergent (○) alone possess disulphide-stabilised keratin filaments. Cross-linked envelopes (□) were scored by insolubility in ionic detergent plus reducing agent. Cells were also deposited on filters, fixed and stained with haematoxylin and eosin, and the proportion of cells possessing nuclei was determined[5]. ●, Anucleate cells.

3T3 cells are known to be fibroblasts[17], but it is not known from what organ they originated, as the line was evolved from mixed mouse embryo cell cultures[18]. This may not be important insofar as ability to support proliferation is concerned, since human diploid fibroblasts of non-dermal as well as of dermal origin support the growth of epidermal keratinocytes[1,19]. The experiments described here also show that the ability of 3T3 cells to support keratinocyte multiplication is not specific for the type of keratinocyte. Although the fibroblast products necessary for the support of keratinocyte growth have not yet been identified, they may be different from those involved in instructive effects.

Whatever the special conditions that may modulate their differentiated state *in vivo*, the three cell types can, in the human, show a common phenotype in culture in which all the properties examined, including the differentiated ones, are expressed equally. In the case of the rabbit, although all the differentiated properties can also be expressed in culture by corneal and epidermal cells, the differences between the two cell types grown in identical culture conditions show that conversion to a common phenotype is not complete. Thus in this species, the intrinsic differences may make a significant contribution to the phenotypic differences between the two epithelia *in vivo*.

These investigations were aided by grants from the National Cancer Institute. Human corneas were provided from local eye banks by Drs Beatrice Y. J. T. Yue, and Jules L. Baum and Miss Rasma Niedra.

Received 20 June; accepted 4 August 1977.

1. Rheinwald, J. G. & Green, H. *Cell* **6**, 331–344 (1975).
2. Rheinwald, J. G. & Green, H. *Nature* **265**, 421–424 (1977).
3. Sun, T.-T. & Green, H. *Cell* **9**, 511–521 (1976).
4. Rice, R. H. & Green, H. *Cell* **11**, 417–422 (1977).
5. Green, H. *Cell* **11**, 405–415 (1977).
6. Allman, M. I. *et al. Invest. Ophthal.* **15**, 666–668 (1976).
7. Newsome, D. A., Takasugi, M., Kenyon, K. R., Start, W. F. & Opelz, G. *Invest. Ophthal.* **13**, 23–32 (1974).
8. Yanoff, M. *Documenta ophth.* **41**, 157–204 (1976).
9. Cohen, S., Carpenter, G. & Lembach, K. J. in *Advances in Metabolic Disorders* **8**, (ed. Lust, R. & Hall, K.) 265–284 (Academic, New York, 1975).

© 1977 Nature Publishing Group

10. Savage, C. F., Jr & Cohen, S. *Expl Eye Res.* **15**, 361–366 (1973).
11. Steinert, P. M. *Biochem. J.* **149**, 39–48 (1975).
12. Baden, H. P., Lee, L. D. & Kubilus, J. *J. invest. Derm.* **67**, 573–576 (1976).
13. Yardley, H. J. & Goldstein, D. J. *Br. J. Derm.* **95**, 621–626 (1976).
14. Billingham, R. E. & Silvers, W. K. *J. exp. Med.* **125**, 429–446 (1967).
15. Sengel, P. *Morphogenesis of Skin* (Cambridge University Press, Cambridge, 1976).
16. Van Scott, E. J. & Reinertson, R. P. *J. invest. Derm.* **36**, 109–131 (1961).
17. Goldberg, B. *Cell* **11**, 169–172 (1977).
18. Todaro, G. J. & Green, H. *J. Cell Biol.* **17**, 299–313 (1963).
19. Rheinwald, J. G. & Green, H. *Cell* **6**, 317–330 (1975).
20. Yen, S.-H., Dahl, D., Schachner, M. & Shelanski, M. L. *Proc. natn. Acad. Sci. U.S.A.* **73**, 529–533 (1976).

letters to nature

Rapid fluctuations of radio flux and polarisation in quasar 3C273

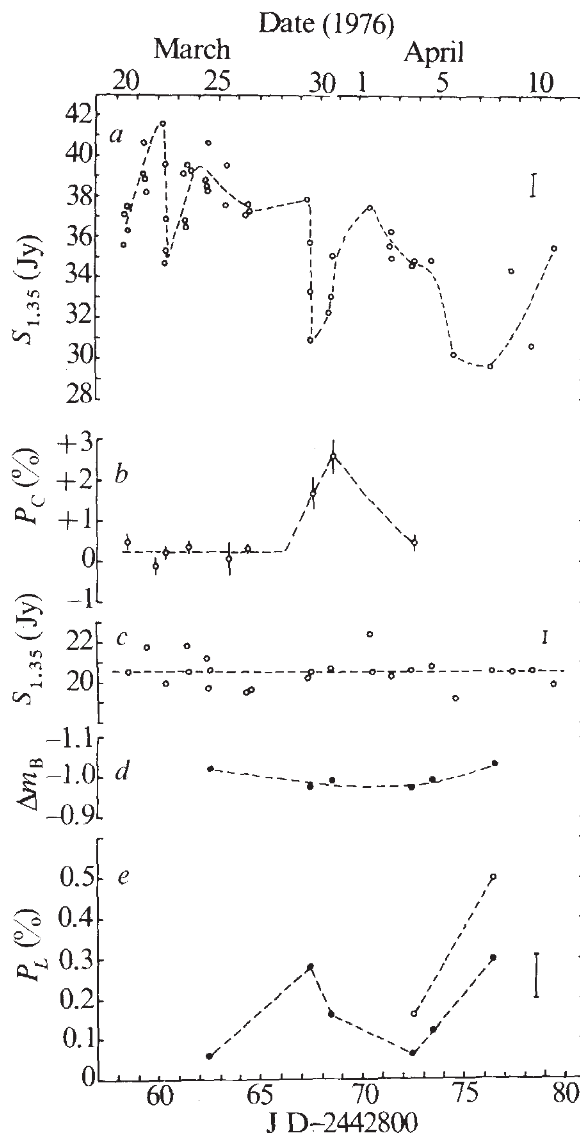
QUASAR 3C273, one of the brightest and most studied quasars, is variable over a wide wavelength range. The changes of its luminosity may occur on a timescale of a few years or of several days in both the radio[1] and optical[2,3] range. There are variations of linear and circular polarisation in the radio domain[4] and sometimes the optical emission is circularly polarised[5]. The data from our simultaneous radio and optical observations show rapid variations of radio flux and circular polarisation at $\lambda = 1.35$ cm, and of optical linear polarisation of 3C273 within one day or several hours. These variations are possibly more rapid than those previously reported.

Total flux I and intensity V of circular polarised emission at 1.35 cm of the quasar 3C273 were measured during March–April 1976 using the 22-m radio telescope of the Crimean Astrophysical Observatory. The angular resolution of the radio telescope was $2.5' \times 2.6'$ and the sensitivity was about 0.7 Jy with a time constant of 1 s using a switched receiver with a maser as a high-frequency preamplifier. Beam switching in the azimuth plane was used to reduce the influence of fluctuations of atmospheric radio emission. The quarter-plate analyser was placed behind the central circular feed. The main and reference beams had orthogonal polarisation and measurements of flux I were made by an 'on-off' method similar to that described in ref. 6. A minicomputer operating on-line processed the output signals of the receiver and checked its amplification[7]. Flux density was measured relative to the standard sources Saturn (brightness temperature 128 ± 5 K) and the thermal source DR21 (flux density 19.5 ± 0.6 Jy). The water line at 1.35 cm lies in the received band, so the atmospheric attenuation may be variable. Therefore, to eliminate the influence of the atmospheric extinction variations the standard sources and the control source 3C274 were measured just before or after 3C273 and at about the same zenith distances. The corrections for extinction in this case did not exceed a few per cent. The r.m.s. error of the total flux determined with the aid of 3C274 does not exceed 2.5%. Zero level for parameter V was found by observation of DR21 which is expected to have zero circular polarisation; it showed no fluctuations in excess of the r.m.s. errors (its mean value found here is -0.35 ± 0.07% where minus corresponds to left-handed polarisation).

Concurrently with radio observations of 3C273 optical observations were carried out with a polarimeter[8] at the Cassegrain focus of the 2.6-m Crimean reflecting telescope. The optical intensity and the linear polarisation were recorded in B and V spectral bands with $\lambda_{eff} = 430$ and 540 nm respectively. The optical flux was measured with respect to the comparison star 50″ to the West of 3C273 and polarisation was calibrated with the aid of standard stars[8].

The results of radio and optical measurements are shown in Fig. 1. They sometimes show rapid intensity fluctuations

Fig. 1 *a*, Variations of the radio flux of 3C273 at 1.35 cm; *b*, the degree of circular polarisation 3C273 at 1.35 cm; *c*, the radio flux of control source 3C274; *d*, the optical brightness 3C273 at the *B* band in magnitudes; *e*, the degree of linear polarisation of optical radiation in the B (●) and V (○) bands. Vertical bars show the value of r.m.s. error.



©1977 Nature Publishing Group