The Immunohistochemical Characterisation of an SV40-Immortalized Human Corneal Epithelial Cell Line

Anne Huhtala[1], Sami K. Nurmi[1], Hanna Tähti[1], Lotta Salminen[1,2], Päivi Alajuuma[3], Immo Rantala[4], Heikki Helin[4], and Hannu Uusitalo[1,5]

1 University of Tampere, Medical School, FIN-33014 University of Tampere, Finland
2 Tampere University Hospital, Department of Ophthalmology, FIN-33521 Tampere, Finland
3 Santen Oy, P.O. Box 33, FIN-33721 Tampere, Finland
4 Tampere University Hospital, Department of Pathology, FIN-33521 Tampere, Finland
5 Kuopio University Hospital, Department of Ophthalmology, FIN-70211 Kuopio, Finland

Summary

Alternatives to the Draize rabbit eye irritation test are currently being investigated. Because of morphologic and biochemical differences between the rabbit and the human eye, continuous human cell lines have been proposed for use in ocular toxicology studies. Single cell-type monolayer cultures in culture medium have been used extensively in ocular toxicology. In the present study, an SV40-immortalised human corneal epithelial (HCE) cell line was characterised immunohistochemically, using 13 different monoclonal antibodies to cytokeratins (CKs), ranging from CK3 to CK20. The results from the monolayer HCE cell cultures were compared with those from the corneal epithelium of human corneal cryostat sections. Previous studies have shown that the morphology of the HCE cell line is similar to that of primary cultured human corneal epithelial cells, and that the cells express the cornea-specific CK3. In the study reported here, we show that the cell line also expresses CKs 7, 8, 18 and 19. These CKs are typically expressed by simple epithelial cells, and are not found in human cornea *in vivo*. Therefore, the monolayer HCE cell line grown in culture medium does not express the CK pattern that is typical of human corneal epithelium. This should be taken into consideration when using HCE cell cultures in similar single cell-type experiments for ocular toxicology.

Key words: cell line, cytokeratin, corneal Epithelium, eye.

Introduction

The relevance of the Draize rabbit eye irritation test, which was developed for human ocular safety testing (1), was criticised almost from its inception, because of morphological and biochemical differences between the rabbit and the human eye (2-4). The reliability of the test has also been questioned (2-8). Furthermore, the use of laboratory animals in modern drug development and for the toxicological testing of new drug molecules is economically challenging. Due to the technical, economical, and ethical problems associated with the use of the Draize eye irritation test, many

alternatives to the animal model have been proposed. Human corneal organ culture techniques have been developed (9,10), and donor-derived tissue cultures of human corneal epithelium have been used to model the ocular surface *in vitro* (11,12). However, these systems become senescent after several passages *in vitro*. In addition, the availability of donor corneal material is limited.

Models involving continuous cell lines have been proposed for ocular toxicology studies. These models include the widely used SIRC rabbit corneal cell line, which has a fibroblastic morphology (13). Several corneal epithelial cell lines have been developed, including cell lines originating from the rabbit (14-16), rat (17,18), human (19,20), and hamster (21). Cell immortalisation techniques have led to the development of corneal epithelial cell clones that can withstand continual passage, and show excellent revitalisation after storage, as well as ease of handling in culture (20). The present study was undertaken because of the widespread use of single cell-type corneal epithelial monolayer cell cultures for high troughput toxicity screening in ocular toxicity, for example to study the adverse effects of topically applied ocular drugs. A key question that arises when an immortalised corneal epithelial cell line is used in these kinds of single cell-type culture conditions is how closely the cells resemble the epithelial cells *in vivo*. Thus, it is important that a detailed characterization of these cell lines is carried out, under several different culture conditions.

In the present study, we have investigated the expression of cytokeratins (CKs) in the SV40-immortalised human corneal epithelial (HCE) cell line that was established by Araki-Sasaki *et al.* (20), by using an immunohistochemical monoclonal antibody (mAB) analysis. A panel of 13 commercially available mABs to CK were used to characterise the epithelial nature of the HCE cells grown in culture medium, and to compare the cell cultures with the human corneal epithelium *in vivo.*

Materials and methods

Materials for cell culture

Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (1:1), Hank's balanced salt solution (HBSS), trypsin-EDTA solution, L-glutamate, fetal bovine serum (FBS), and antibiotic/antimycotic solution (penicillin G sodium, streptomycin sulphate, amphotericin B) were obtained from Gibco (Paisley, UK). Human epidermal growth factor and insulin were from Sigma (St. Louis, MO, USA). Chamber slides and culture flasks were from Nunc (Roskilde, Denmark).

Materials for immunohistochemistry

Tris[hydroxymethyl]aminomethane (Tris) and bovine serum albumine (BSA) were from Sigma. Rabbit anti-mouse immunoglobulins, alkaline phosphatase - anti-alkaline phosphatase (APAAP) immunoglobulins, alkaline phosphatase substrate, and aqueous mounting medium (Aquamount) were obtained from Dako (Carpinteria, CA, USA). The non-ionic surfactant, Triton X-100, was obtained from Bio-Rad Laboratories (Richmond, CA, USA). Pap Pen, a water-repelling pen, was from Daido Sangyo Co (Tokyo, Japan).

mABs to CKs 4, 6, 7, 8, 10, 13, 14, 16, 17, 18, 19 and 20 were obtained from Neomarkers (Fremont, CA, USA), and mAB to CK3 (clone AE5) was obtained from ICN Biochemicals (Aurora, OH, USA). mABs to CKs 3, 6, 7, 8, 10, 14, 16, 17, 18 and 19 were produced by the mouse ascites method, whereas mABs to CKs 4, 13 and 20 were produced by using cell culture techniques.

Cell line culture technique

We characterised the immortalized HCE cell line that was established by Araki-Sasaki and colleagues (20). In brief, the cell line was established by infecting primary cultured corneal epithelial cells with the SV40-adenovirus vector. The cells were then cloned three times to obtain a continuously growing cell line. The HCE cells were grown in a culture medium comprising Dulbecco's Modified Eagle's Medium and Ham's nutrient mixture F12 (1:1), supplemented with 15% (v/v) FBS, 1% (v/v) antibiotic/antimycotic solution, 2mM L-glutamine, 5 µg/ml insulin, and 10ng/ml human epidermal growth factor. The cells were grown in 80cm^2 T-flasks and serially passaged at a split ratio of 1:5, twice per week.

For immunohistochemistry, the cells were trypsinised with trypsin-EDTA solution, counted under a microscope by using a haemocytometer, and seeded on 8-well chamber slides, at a density of 30,000cells/well. The slides were incubated in a humidified atmosphere at 37ºC, in 5% CO_2-95% air for 2, 4 and 14 days. The culture medium was changed every 2 days. After the incubation period, the cells were washed with HBSS and fixed in methanol-acetone (1:1) for 10 min. They were then air-dried and stored at –20ºC prior to immunohistochemical analysis.

Human corneal cryostat sections

Human corneas were obtained from cadaver donors, without any known eye diseases, for corneal transplantation at Helsinki University Hospital (Finland). The centres of the corneas were used for transplantation. The remaining peripheral corneal specimens were snap-frozen and stored at -80ºC. The specimens were cut into 6µm thick sections, which were placed on Vectabond slides (two sections per slide), and stored at -20ºC prior to immunohistochemical analysis.

Immunohistochemistry

The cultured HCE cells and the human corneal cryostat sections were characterised in a similar manner, using 13 mABs to CKs, ranging from CK3 to CK20. The chamber slides containing the fixed HCE cells were placed at room temperature for 2 hours prior to the immunohistochemical staining. The slides containing the frozen, unfixed corneal cryostat sections were placed at room temperature 1 hour before the staining procedure. The cryostat sections were then fixed in acetone at -20ºC for 10 minutes, and air-dried. Each section was outlined, by the Pap Pen, to avoid any mixing of the primary antibodies between different sections on a single slide. The slides containing the HCE cells and the cryostat sections were then placed into a buffer bath containing 0.5% (v/v) Triton X-100

for 10 minutes, to enhance the penetration of the antibodies. Triton X-100 residues were removed by washing the slides with normal Tris-buffered saline (TBS; pH 7.6) containing 0.05M Tris and 0.15M NaCl. In order to minimise any non-specific binding of the antibodies, the slides were then incubated in 1% (w/v) BSA for 20 min. All the antibodies used were diluted in TBS containing 1% (w/v) BSA. The APAAP staining method used in the present study comprises a three-step staining procedure, in which the tertiary antibody is conjugated with APAAP molecules (22). The cells were first incubated with the primary antibody (anti-CK mAbs) for 30 minutes, then with the secondary antibody (link) for 30 min, followed by a 30-min APAAP incubation. In the controls the primary antibody was omitted. All incubations were performed in a humidified chamber. The cells were then incubated for 20 minutes in alkaline phosphatase substrate containing naphtol AS-MX phosphate, Fast Red TR, and levamisole. A positive alkaline phosphate reaction was indicated by a reddish color. Finally, the slides were thoroughly washed with water and mounted in Aquamont for light microscopy.

Results

Growth characteristics

The HCE cell line was cultures for more than 50 passages in our laboratory. The cells retained the cobblestone-like appearance that is typical of corneal epithelial cells exhibiting. The HCE cells in the 8-well chamber slides became confluent after 3 days of culture, and grew as an evenly spread monolayer. After 4 days in culture, stratified piles of cells began to form. This stratification process continued to occur to some extent, but, even after 14 days of culture, the pattern of stratification was similar to that of the 4-day culture.

Immunohistochemistry

Three different growth phases of the HCE cell line was studied: pre-confluent (2-day culture), confluent (4-day culture) and post-confluent (14-day culture). The HCE cells that were cultured for 2 days showed intensive staining with the mABs to CKs 7, 8, 18 and 19 (Table I, Figure 1F). The staining pattern observed with each mAB was identical: cytoplasmic and smooth, not granular. When the cells were incubated with the remaining mABs, no reaction was observed. In the 4-day-old culture, intensive staining was observed with the mABs to CKs 3, 7, 8, 18 and 19, indicating the expression of these CKs (Table I, Figures 1C, 2A and 2D). Both the monolayer and the stratified parts of the culture reacted with the mABs. When the cells were incubated with the remaining mABs, no positive reactions were observed. In the 14-day-old culture, an intensive staining reaction was observed with the mABs to CKs 3, 7, 8, 18 and 19 (Table I and Figure 2B). Incubation with the remaining panel of CK mABs did not result in any positive staining reactions.

In the cryostat sections of the human cornea, the whole corneal epithelium stained intensively when the mAB to CK3 was used (Table I, Figure 1A). With the mAB to CK4, only the suprabasal cells of the corneal epithelium showed a positive reaction (Figure

1D). The limbal cells of the corneal epithelium showed a positive staining reaction when the mAB to CK19 was used, whereas the cells in the rest of the corneal epithelium did not react (Figure 2C). No positive staining reactions were observed with the remaining mABs.

Discussion

The CKs are a part of the intermediate filament family, and are characteristic of epithelial cells (23,24). At least 20 different CKs have been identified in humans, with molecular masses ranging from 40kDa to 67kDa (23,25). CKs are often referred to according to their Moll catalogue numbers, ranging from 1 to 20 (23). Specific CK classes can be used as markers for distinguishing different types of epithelial cells (25-28). According to Moll *et al.* (23): CKs 1 and 2 are usually characteristic of human keratinising epidermis; CK3 is specific to the corneal epithelium; CKs 4-6 characterise non-keratinising stratified epithelia; CKs 7 and 8 are characteristic of simple epithelia; CKs 9-11 are expressed in the epidermis; and CK12 is specific to the corneal epithelium; CK13 is characteristic of non-cornified stratified squamous epithelia; CKs 14-17 occur in the epidermis and in non-cornified stratified epithelia; CK's 18 and 19 are characteristic of simple epithelia (although CK19 is also found as a minor component in many stratified epithelia, including the corneal limbus; 29, 30); and CK20 is expressed by gastric and intestinal epithelium, urothelium, and Merkel cells (31).

The cytokeratin dimer CK3-CK12 has been designated as a marker for corneal-type differentiation, and is cornea-specific in human tissues (23,27). It has been shown to exist in combination as a CK pair (32). CK3 is a 64kDa basic keratin, whereas CK12 is a 55kDa acidic keratin (23). Immunological studies with mABs have demonstrated that CK3 and CK12 are expressed by all the cell layers of the corneal epithelium, but not by conjunctival epithelial cells (33). CK12 was not included in the present study, since mABs to CK12 were not commercially available. The human corneal epithelium may also express minor CK components, such as CKs 4, 5, 13, 14, 15 and 16 (29,34,35) CKs 8 and 18 are expressed in fetal corneas (34) and in the conjunctival epithelium (34), and CK19 is expressed in the peripheral corneal epithelium (29).

The HCE cell line used in this study has been characterised previously, but only by using the primary antibodies, AE1 and AE5 (20). AE1 detects the acidic CKs 10, 14-16 and 19, while AE5 detects the basic CK3 (28). The results of the study of Araki-Sasaki *et al.* (20) indicated that the HCE cell line expressed CK3 after 7 days and 14 days of culture. A positive immunofluorescence was also reported for mAB AE1 on days 7 and 14, indicating the expression of one or more of CKs 10, 14-16 and 19. Araki-Sasaki *et al.* (20) also reported that cultured human primary corneal epithelial cells did not express the cornea-specific CK3 after 7 days of culture, but that, after 14 days of culture, cell stratification began to occur and CK3 was expressed.

According to our results, the epithelium of the corneal cryostat sections showed a positive reaction to CK3. This was to be expected, since CK3 is cornea-specific and is reported to be present in adult human corneas (23,29,36). We also found that, in the corneal cryostat sections, CK4 was expressed in the suprabasal cells of the corneal epithelium, and CK19

was expressed in the peripheral/limbal cells of the corneal epithelium. Similar results have also been reported previously by Kasper *et al.* (29).

Our results show that CK3 (AE5) was expressed in the HCE cell line on days 4 and 14, but not on day 2. The monolayer HCE cells do not express CK3, but after stratified cell layers began to form, CK3 began to be expressed. This is consistent with the CK3 expression by human primary corneal epithelial cells reported by Araki-Sasaki *et al.* (20), since expression of CK3 also occurred as stratified cell layers began to be formed. In this respect, the HCE cell line resembles the human primary corneal epithelial cells. Our results further indicate that CK4 was not expressed by the HCE cell line, even though it was expressed by the suprabasal corneal epithelial cells in the corneal cryostat sections. However, CKs 7, 8, 18 and 19 were expressed by the HCE cells. This is of importance, since CKs 7, 8, 18 and 19 are normally expressed by simple epithelial cells that have a free luminal surface and are in contact with the basal lamina, and not by either human cornea *in vivo* or cultured human corneal epithelial cells *in vitro* (23,37).

Carcinoma cell lines have often been reported to express CKs that are different from those of their cell type of origin (23,38,39). Primary cells may also produce other CKs, in addition to or as an alternative to the CKs of their tissue of origin (40-43). The expression of the simple epithelium-specific CKs 7, 8, 18 and 19 has been reported in the SV40-immortalised human keratinocyte cell lines originating from epidermis (44-47). We found that the stratified HCE cell lines expresses cornea-specific CK3, and thus resembles human corneal epithelial cells *in vivo,* but the HCE cell line also expresses simple epithelium-specific CKs 7, 8, 18 and 19.

The synthetic plastic substrate that widely used for *in vitro* culture forces cells to adjust to an artificially flat and rigid surface (48). However, in the authentic environment *in vivo,* corneal epithelial cells grow on a complex three-dimensional extracellular matrix (ECM). The cells are influenced by various complicated cell-to-cell and cell-to-ECM interactions. HCE cells cultured on a collagen membrane at the air-liquid interfase to form a three-dimensional, stratified multilayered epithelium, similar to that found in the human cornea (49), may express a CK pattern that is more like that of the normal human cornea *in vivo*. Moreover, if the HCE cell line is grown as a multilayered epithelium on a stromal support with fibroblasts, or more precisely with stromal keratocytes (50), it could be expected to behave more like the normal human cornea, with a CK pattern that is similar to that found in the normal intact human cornea. *In vivo*, the membrane-spanning heterodimeric receptors, integrins, have been found to mediate the adhesion of cells to the ECM and to other cells (48,51). They are also involved in transducing extracellular signals into the cell. The $\alpha_6\beta_4$ integrin, which is a receptor for various laminin isoforms, has a crucial role in the assembly of hemidesmosomes and their linkage to the CK filament system, and in the adhesion of epithelial cells to the underlying basement membrane (51).

The differences between the patterns of CK expression in the corneal epithelium *in vivo* and in the HCE cell line grown in culture medium may be due to the SV40-immortalisation process, or possibly to the culture conditions used. In the normal human cornea *in vivo,* corneal epithelial cells form a multilayered epithelium that is supported by a stromal layer. When corneal epithelial cell cultures are used in ocular toxicology for high-throughput toxicity screening, the changes in the CK pattern may not have a great impact on the results. However, when these cells are used in more-detailed studies, it is

important that the cells, and their culture conditions, as much as possible resemble the normal *in vivo* situation of a multilayered epithelium and a stromal support.

Conclusions

The use of primary cultures as a source of cell material for ocular studies requires a good resource of original tissue. In the case of human corneas, the availability of donor corneas for human primary corneal epithelial cultures is often poor. Since the HCE cell line with an infinite life span provides a virtually unlimited source of cells, it is a very convenient source of cells for ocular studies. The cell morphology is similar to that of cultured human primary corneal epithelial cells. The stratified HCE cells also express the cornea-specific CK3, and thus they resemble primary cultured human corneal epithelial cells. However, the expression of other CKs in the HCE cells grown as monolayer cultures in culture medium is clearly different from that of a normal cornea *in vivo*. This must be taken into account when using the presently studied HCE cell line as a single cell-type monolayer culture, in culture medium. HCE cells cultured at the air-liquid interfase to form a three-dimensional epithelium on a stromal support could be expected to express a CK pattern that was more like that of a human cornea *in vivo*.

Acknowledgements

This study was supported by the European Union; the Finnish Ministry of Agriculture and Forestry; the Medical Research Fund of Tampere University Hospital, Finland; Sokeain ystävät r.y., Finland; and the Eye and Tissue Bank Foundation, Finland. The authors would also like to thank Marja-Leena Koskinen, Paula Helpiölä, Maija Koskela, Tarja Toimela, Hanna Mäenpää and Markku Pelto-Huikko for their support in making this study possible.

References

1. Draize, J.H., Woodard, G., Calvery H.O. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucus membranes. *Journal Pharmacology and Experimental Therapeutics* **82**, 377-390.

2. Weil, C.S. and Scala, R.A. (1971). Study of intra- and interlaboratory variability in the results of rabbit eye and skin irritation tests. *Toxicology and Applied Pharmacology* **19**, 276-360.

3. Sharpe, R. The Draize test--motivations for change (1985). *Food and Chemical Toxicology* **23**, 139-143.

4. Swanston, D.W.(1985). Assessment of the validity of animal techniques in eye-irritation testing. *Food and Chemical Toxicology* **23**, 169-173.

5. Frazier, J.M., Gad, S.C., Goldberg, A.M., McCulley, J.P. (1987). *A Critical Evaluation of Alternatives to Acute Ocular Irritation Testing. Vol. 4.* pp. 21-29. New York: Mary Ann Liebert, Inc.

6. Earl, L.K., Dickens, A.D., Rowson M.J. (1997). A critical analysis of the rabbit eye irritation test variability and its impact on validation of alternative methods. *Toxicology in Vitro* **11**, 295-304.

7. York, M, Steiling, W. (1998). A critical review of the assessment of eye irritation potential using the Draize rabbit eye test. *Journal of Applied Toxicology* **18**, 233-240.

8. Wilhelmus, K.R. (2001). The Draize Eye Test. *Survey of Ophthalmology* **45**, 493-515.

9. Doughman, D.J. (1980). Prolonged donor cornea preservation in organ culture long-term clinical evaluation. *Transactions of the American Ophthalmological Society* **78**, 567-628.

10. Richard, N.R., Andersson, J.A., Weiss, J.L., Binder, P.S. (1991). Air/liquid corneal organ culture: a light microscopic study. *Current Eye Research* **10**, 739-749.

11. Sun, T.T., Green, H. (1978). Immunofluorescent staining of keratin fibers in cultured cells. *Cell* **14**, 469-476.

12. Ebato, B., Friend, J., Thoft, R.A. (1987). Comparison of central and peripheral human corneal epithelium in tissue culture. *Investigative Ophthalmology and Visual Science* **28**, 1450-1456.

13. Niederkorn, J.Y., Meyer, D.R., Ubelaker, J.E., Martin, J.H. (1990). Ultrastructural and immunohistological characterization of the SIRC corneal cell line. *In Vitro Cellular and Developmental Biology* **26**, 923-930.

14. Araki, A., Ohashi, Y., Sasabe, T., Kinoshita, S., Hayashi, K., Yang, X.Z., Hosaka, Y., Aizawa, S., Handa, H. (1993). Immortalization of rabbit corneal epithelial cells by a recombinant SV40 adenovirus vector. *Investigative Ophthalmology and Visual Science* **34**, 2665-2671.

15. Castro-Munozledo, F. (1994). Development of a spontaneous permanent cell line of rabbit corneal epithelial cells that undergoes sequential stages of differentation in cell culture. *Journal of Cell Science* **107**, 2343-2351.

16. Okamoto, S., Ohji, M., Kosaku, K., Sundarraj, N., Hassell, J.R., Thoft, R.A., Pipas J.M. (1995). Characterization of immortalized rabbit corneal epithelial cells with SV 40 large T antigen. *Japanese Journal of Ophthalmology* **39**, 323-333.

17. Araki, K., Sasabe, T., Ohashi, Y., Yasuda, M., Handa, H., Tano, Y. (1994). Immortalization of rat corneal epithelial cell line by the SV40-adenovirus recombinant vector. *Nippon Ganka Gakkai Zasshi* **98**, 327-333.

18. Jozwiak, J., Skopinski, P., Komar, A., Wojcik, A., Malejczyk, J. (2001). Characterization of epithelial cell line from rat cornea. *Eye* **15**, 82-88.

19. Kahn, C.R., Young, E., Lee, I.H., Rhim, J.S. (1993). Human corneal epithelial primary cultures and cell lines with extended life span: *in vitro* model for ocular studies. *Investigative Ophthalmology and Visual Science* **34**, 3429-3441.

20. Araki- Sasaki, K., Osashi, Y., Sasabe, T., Hayashi, K., Watanabe, H., Tano, Y., Handa, H. (1995). An SV40-immortalized human corneal epithelial cell line and its characterization. *Investigative Ophthalmology and Visual Science* **36**, 614-621.

21. Halenda, R.M., Grevan, V.L., Hook, R.R., Riley, L.K. (1998). An immortalized hamster corneal epithelial cell line for studies of the pathogenesis of *Acanthamoeba* keratitis. *Current Eye Research* **17**, 225-230.

22. Mason, D.Y., Abdulaziz, Z., Falini, B., Stein, H. (1983). Double immunoenzymatic labeling. In *Immunocytochemistry, Practical Applications in Pathology and Biology* (eds. J.M. Polak, S. van Noorden), pp. 113-128. Bristol, England: John Wright and Sons.

23. Moll, R., Franke, W.W., Schiller, D.L., Geiger, B., Krepler, R. (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **31**, 11-24.

24. Osborn, M. (1983). Intermediate filaments as histologic markers: an overview. *Journal of Investigative Dermatology* **81**, 1045-1049.

25. Tseng, S.C., Jarvinen, M.J., Nelson, W.G., Huang, J.W., Woodcock-Mitchell, J., Sun, T.T. (1982). Correlation of specific keratins with different types of epithelial differentiation: Monoclonal antibody studies. *Cell* **30**, 361-372.

26. Sun, T.T., Eichner, R., Schermer, A., Cooper, D., Nelson, W.G., Weiss, R.A. (1984). Classification, expression, and possible mechanisms of evolution of mammalian keratins: a unifying model. In *Cancer Cells Vol I. The Transformed Phenotype.* (Eds. A. Levine, W. Toop, G. Van de Woude, J.D. Watson), pp. 169-176. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

27. Cooper, D., Schermer, A., Sun, T.T. (1985). Classification of human epithelia and their neoplasms using monoclonal antibodies to keratins: strategies, applications, and limitations. *Laboratory Investigation* **52**, 243-256.

28. Schermer, A., Galvin, S., Sun, T.T. (1986). Differention-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *Journal of Cell Biology* **103**, 49-62.

29. Kasper, M., Moll, R., Stosiek, P., Karsten, U. (1988). Patterns of cytokeratin and vimentin expression in the human eye. *Histochemistry* **89**, 369-377.

30. Barnard, Z., Apel, A.J., Harkin, D.G. (2001). Phenotypic analyses of limbal epithelial cell cultures derived from donor corneoscleral rims. *Clinical and Experimental Ophthalmology* **29**, 138-142.

31. Moll, R., Lowe, A., Laufer, J., Franke, W.W. (1992). Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies. *American Journal of Pathology* **140**, 427-447.

32. Chaloindufau, C., Pavitt, I., Delorme, P., Dhouailly, D. (1993). Identification of keratin 3 and keratin 12 in corneal epithelium of vertebrates. *Epithelial Cell Biology* **2**, 120-125.

33. Kurpakus, M.A., Stock, E.L., Jones, J.C. (1990). Expression of the 55-kD/64-kD keratins in ocular surface epithelium. *Investigative Ophthalmology and Visual Science* **31**, 448-456.

34. Kasper, M., Stosiek, P., Lane, E.B. (1992). Cytokeratin and vimentin heterogeneity in human cornea. *Acta Histochemica* **93**, 371-381.

35. Nishida, K., Adachi, W., Shimizu-Matsumoto, A., Kinoshita, S., Mizuno, K., Matsubara, K., Okubo, K. (1996). A gene expression profile of corneal epithelium and the isolation of human keratin 12 cDNA. *Investigative Ophthalmology and Visual Science* **37**, 1800-1809.

36. Wiley, L., SundarRaj, N., Sun, T.T., Thoft, R.A. (1991). Regional heterogeneity in human corneal and limbal epithelia: an immunohistochemical evaluation. *Investigative Ophthalmology and Visual Science* **32**, 594-602.

37. Ramaekers, F., van Niekerk, C., Poels, L., Schaafsma, E., Huijsmans, A., Robben, H., Schaart, G., Vooijs, P. (1990). Use of monoclonal antibodies to keratin 7 in the differential diagnosis of adenocarcinomas. *American Journal of Pathology* **136**, 641-655.

38. Wu, Y.J., Parker, L.M., Binder, N.E., Beckett, M.A., Sinard, J.H., Griffiths, C.T., Rheinwald, J.G. (1982). The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. *Cell* **31**, 693-703.

39. van der Velden, L.A., Schaafsma, H.E., Manni, J.J., Ramaerkers, F.C.S., Kuijpers, W. (1993). Cytokeratin expression in normal and (pre)malignant head and neck epithelia: an overview. *Head & Neck* **15**, 133-146.

40. Franke, W.W., Schiller, D.L., Moll, R., Winter, S., Schmid, E., Engelbrecht, I., Denk, H., Krepler, R., Platzer, B. (1981). Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. *Journal of Molecular Biology* **153**, 933-959.

41. Franke, W.W., Denk, H., Kalt, R., Schmid, E. (1981). Biochemical and immunological identification of cytokeratin proteins present in hepatocytes of mammalian liver tissue. *Experimental Cell Research* **131**, 299-318.

42. Franke, W.W., Mayer, D., Schmid, E., Denk, H., Borenfreund, E. (1981). Differences of expression of cytoskeletal proteins in cultured rat hepatocytes and hepatoma cells. *Experimental Cell Research* **134**, 345-365.

43. Su, R.T., Chang, Y.C. (1989). Transformation of human epidermal cells by transfection with plasmid containing simian virus 40 DNA linked to a neomycin gene in a defined medium. *Experimental Cell Research* **180**, 117-133.

44. Banks-Schlegel, S.P., Howley P.M. (1983). Differentiation of human epidermal cells transformed by SV40. *The Journal of Cell Biology* **96**, 330-337.

45. Bernard, B.A., Robinson, S.M., Semat, A., Darmon, M. (1985). Re-expression of fetal characters in simian virus 40-transformed human keratinocytes. *Cancer Research* **45**, 1707-1716.

46. Brown, K.W., Gallimore, P.H. (1987). Malignant progression of an SV40-transformed human epidermal keratinocyte cell line. *British Journal of Cancer* **56**, 545-554.

47. Kamalati, T., McIvor, Z., Howard, M., Green, M.R., Brooks, R.F. (1989). Expression of markers of differentiation in a transformed human keratinocyte line induced by coculture with a fibroblast line. *Experimental Cell Research* **185**, 453-463.

48. Cukierman, E., Pankov, R. and Yamana, K.M. (2002). Cell interactions with three-dimensional matrices. *Current Opinion in Cell Biology* **14**, 633-639.

49. Ward, S.L., Walker, T.L., and Dimitrijevich, S.D. (1997). Evaluation of chemically induced toxicity using an *in vitro* model of human corneal epithelium. *Toxicology In Vitro* **11**, 121-139.

50. Orwin, E.J., Hubel, A. (2000). *In vitro* culture characteristics of corneal epithelial, endothelial, and keratocyte cells in a native collagen matrix. *Tissue Engineering* **6**, 307-319.

51. Rezniczek, G.A., de Pereda, J.M., Reipert,, S., and Wiche, G. (1998). Linking integrin $\alpha_6\beta_4$-based cell adhesion to the intermediate filament cytoskeleton: Direct interaction between the β_4 subunit and plectin at multiple molecular sites. *The Journal of Cell Biology* **141**, 209-225.

Table I. Immunohistochemical reactions of the human corneal cryostat sections and the human corneal epithelial (HCE) cells to the monoclonal antibodies (mABs) to cytokeratins (CKs)

Cytokeratin mAB	Cryostat section	HCE 2 days	HCE 4 days	HCE 14 days
CK3	+++	-	+++	+++
CK4	++[a]	-	-	-
CK6	-	-	-	-
CK7	-	+++	+++	+++
CK8	-	+++	+++	+++
CK10	-	-	-	-
CK13	-	-	-	-
CK14	-	-	-	-
CK16	-	-	-	-
CK17	-	-	-	-
CK18	-	+++	+++	+++
CK19	+[b]	+++	+++	++
CK20	-	-	-	-

[a] Only the suprabasal layer of the corneal epithelium showed a positive reaction.
[b] Only the limbal part of the corneal epithelium showed a positive reaction.

- = no staining;
+ = positive staining reaction;
++ = more intense staining reaction;
+++ = very intense staining reaction.

HCE 2 days = pre-confluent 2-day-old culture
HCE 4 days = confluent 4-day-old culture
HCE 14 days = post-confluent 14-day-old culture

Figure 1. Immunohistochemical reactions of the human corneal cryostat sections and the human corneal epithelial (HCE) cells to monoclonal antibodies (mABs) cytokeratins (CKs)

A) Human corneal cryostat section showing the positive staining reaction of the corneal epithelium **(e)** with the anti-CK3 mAB. Staining was evident in all the cell layers of the corneal epithelium, but not in the stroma **(s)**. Magnification x70.

B) No staining was observed when pre-confluent HCE cells (2-day culture) were incubated with the anti-CK3 mAB. Magnification x210.

C) Positive cytoplasmic staining of confluent HCE cells (4-day culture) with the anti-CK3 mAB. Magnification x210.

D) Human corneal cryostat section showing the subrabasal staining of the corneal epithelium **(e)** with the anti-CK4 mAB. Magnification x70.

E). No staining was observed when post-confluent HCE cells (14-day culture) were incubated with the anti-CK4 mAB. Magnification x210.

F) Intensive staining of the cytoplasm of pre-confluent HCE cells (2-day culture) with the anti-CK7 mAB. Magnification x210.

Figure 2. Immunohistochemical reactions of the human corneal cryostat sections and the human corneal epithelial (HCE) cells to monoclonal antibodies (mABs) cytokeratins (CKs)

A) Intensive cytoplasmic staining of confluent HCE cells (4-day culture) with the anti-CK8 mAB. Magnification x210.

B) Positive staining reaction of post-confluent HCE cells (14-day culture) with the anti-CK18 mAB. Magnification x210.

C) Human corneal cryostat section showing the positive staining reaction of the limbal corneal epithelial cells **(e)** to the anti-CK19 mAB. Magnification x210.

D) Positive cytoplasmic staining of confluent HCE cells (4-day culture) with the anti-CK19 mAB. Magnification x210.

E) Human cornea negative control. No staining was evident in the corneal epithelium **(e)** or stroma **(s)**. Magnification x70.

F) Post-confluent HCE cells (14-day culture) negative control. Magnification x210.

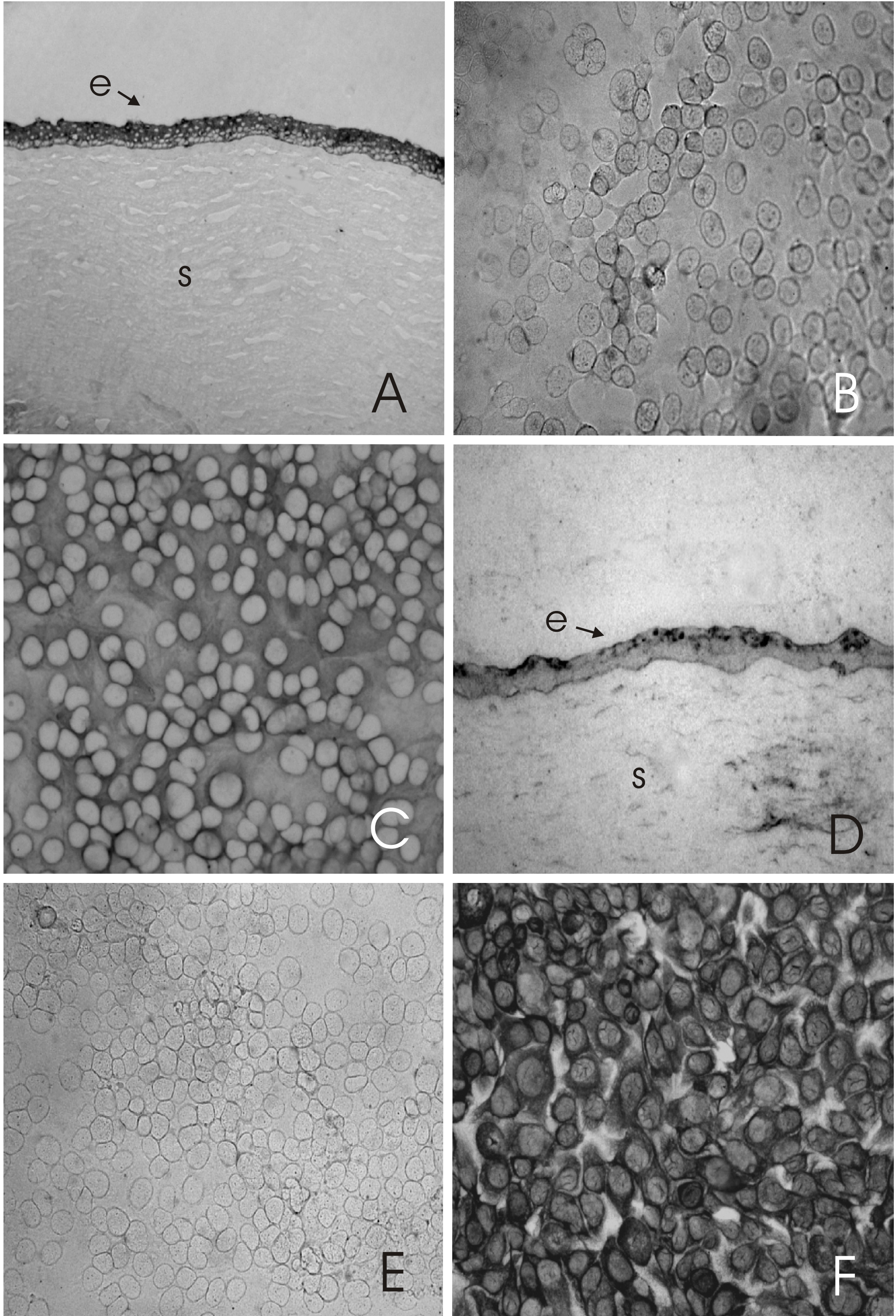

Figure 1.

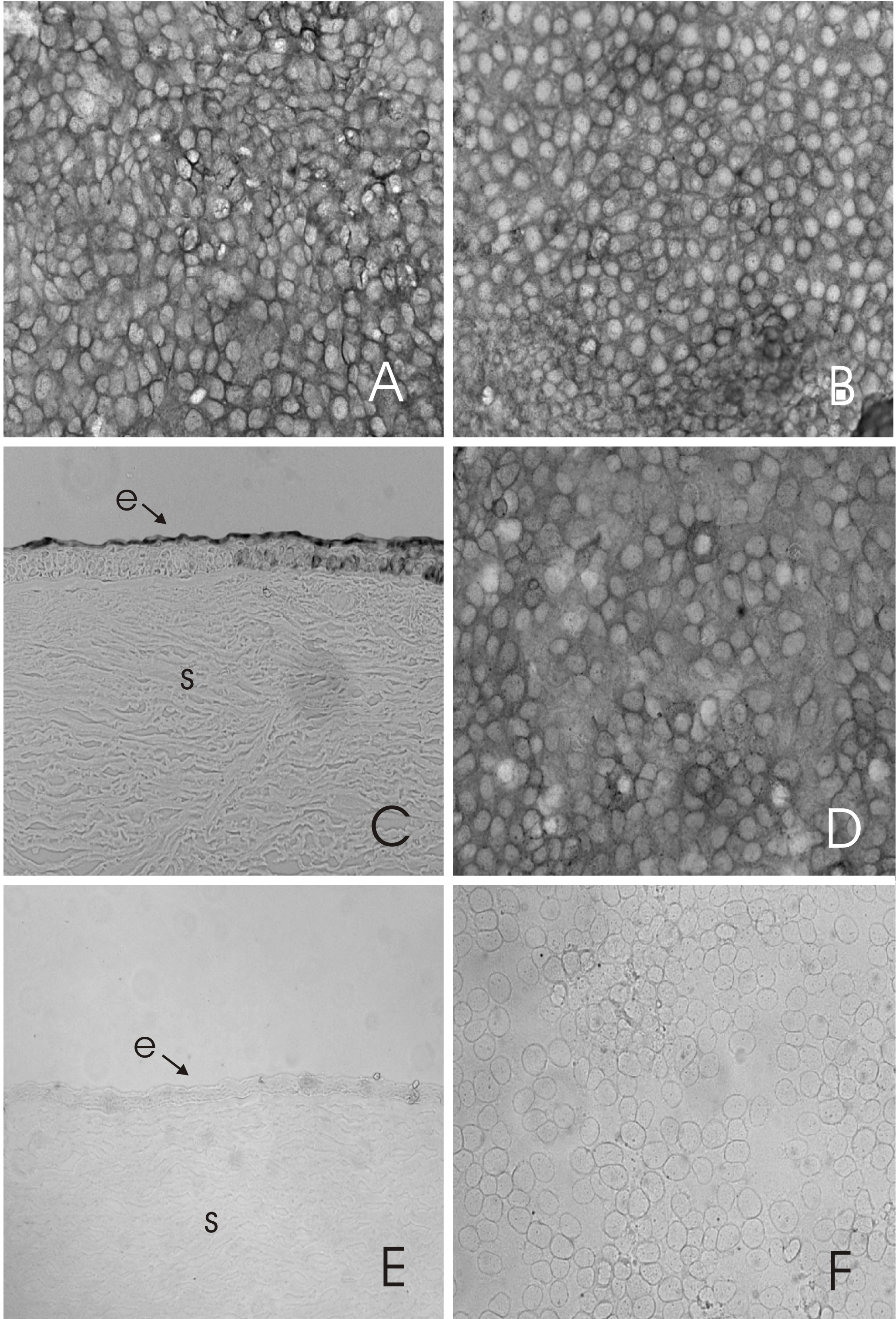

Figure 2.