In-vitro development of corneal epithelial cells on a new hydrogel for epikeratoplasty

F. MAURY*, J. HONIGER‡, D. PELAPRAT§, M. BAUDRIMONT#, V. BORDERIE*, W. ROSTENE§ and L. LAROCHE*
Department of Ophthalmology and Pathology# INSERM Research Unit 339§ and Bioengineering Laboratory‡, Hôpital Saint-Antoine, Paris, France*

This study demonstrates the adhesion and growth of bovine corneal epithelial cells on the surface of a new hydrogel. The hydrogel, containing 78% of water and 22% of AN-69 polymer (poly(acrylonitrile-sodium methallyle sulfonate)), was obtained by phase inversion of polymer–dimethylformamide solution in physiological saline (0.9% NaCl). Experiments were also carried out using hydrogel treated with arginine, human albumin and collagen IV. Covering of hydrogel samples by epithelial cells was completed within ten days, with good cell viability. The epithelial cells spread out and formed a consistent cell layer, confirmed by immunocytochemistry experiments against cytokeratins. Transmission electron micrographs showed numerous desmosomes between cells and the presence of some membrane differentiations at the cell/hydrogel interface. This study suggests therefore that the hydrogel might be suitable for the development of artificial epikeratoplasty grafts.

1. Introduction

The widespread application of new alloplastic materials used in ophthalmology poses the problem of their biological acceptance. These devices need to be rather unique in their optical and surface characteristics. The peculiar reactivity displayed by the biological sites of the eye globe in contact with ocular implants has to be analysed and taken into account for their biological evaluation. Epikeratoplasty is a refractive surgery technique derived from the Barraquer [1] lamellar procedure, which is theoretically simple, effective and reversible. However, donor tissue presents problems in terms of availability, sterilizability and optical predictability, and research is presently focused on the development of alloplastic materials which could replace human corneal grafts [2–7]. The long-term success of such an ocular prosthesis requires that the biomaterial supports the adhesion and proliferation of corneal epithelial cells, and heals securely to the surrounding cornea by natural anchorage. A great deal of interest has centred upon medical application of artificial hydrogels since the pioneering work of Wichterle and Lim [8] some twenty years ago. Our aim is to assess the cytocompatibility of a specific hydrogel and to study the capacity of epithelialization of this material. Based on an *in vitro* tissue culture of corneal epithelium, we present in this paper the development of corneal epithelial cells on a hydrogel polymer designed for alloplastic epikeratoplasty.

2. Materials and methods

Hydrogel samples (diameter 14 mm) were cut from hydrogel film (thickness 0.11–0.12 mm), prepared by phase inversion of a solution containing 9.0% (in weight) of polymer AN-69, which is a poly(acrylonitrile-sodium methallyle sulfonate), obtained from HOSPAL R. & D. Int. France, 85% of dimethylformamide and 6.0% of physiological saline solution (0.9% NaCl). The solution was cast on a plate-glass mould and dipped into physiological saline at 2 °C. After about 5 min, the gel was removed and immersed into distilled water at room temperature for a sufficient time to allow total substitution of dimethylformamide by water, then decontaminated with peracetic acid solution and stocked in physiological saline.

Some experiments were also carried out using surface-modified hydrogel: the hydrogel sample was submerged in saline containing either 0.01% of arginine (PROLABO France) for 1 min or for 5 min, or 5% of human albumin (fraction V.-SIGMA) for 5 min, or 1% of type IV collagen (SIGMA) for 1 min.

2.1. Culture of epithelial explants

Bovine corneas were obtained from the Meaux slaughter-house (France). The anterior portion of bovine eyes, cornea and limbal zones, were aseptically removed. Corneal epithelium was separated with forceps from the stroma, and explants of 1 to 2 mm^2, corresponding to an epithelio-limbal fragment, were prepared as described by Ebato [9].

Five to ten explants were taken from each cornea, and the corneas of ten animals were used in each experiment. One drop of fetal calf serum (GIBCO) was carefully placed deep down plastic wells before the laying of hydrogel lenticles, in order to allow biomaterial attachment. Each explant was then layered

0957–4530

basal side facing the biomaterial to be tested, or the bottom of tissue culture wells, in a 24-well plate (COSTAR), and gently pressed down with forceps to ensure good contact between tissue and the support (biomaterial or culture well). The tissue was allowed to dry for 10 min, and 0.3 ml of culture medium was carefully placed upon each explant. Cultures were incubated overnight at 37 °C in moist atmosphere containing 5% CO_2. The medium was then removed, and another 0.3 ml of medium was added to each well. Medium was replaced with fresh medium once every three days. Culture medium consisted of Dulbecco Modified Eagle Medium (Boehringer Mannheim) and Ham F12 (BOEHRINGER Mannheim) (1:1) supplemented with 5% fetal calf serum (GIBCO), 5 μg/ml insuline (ORGANON), 10 μg/ml Gentamicin (Pharmacie Centrale des Hôpitaux, Paris, France), 0.5% dimethylsulfoxide (BRUNEAU, France) and 10 μg/ml Epidermal Growth Factor (BOEHRINGER Mannheim)

2.2. Cell layer observation and measurements

At different times in culture, vital dye fluorescein diacetate (FDA) was used for cell layer observations. FDA was stored at −20 °C at a concentration of 5 mg/ml in acetone. This stock solution was diluted 1/250 in PBS. A few drops of the diluted FDA solution were laid on hydrogel samples for 1 to 2 min. Cell layers were observed thereafter under an inverse phase microscope, and photographs were taken. The surfaces occupied by the neoformed tissues were then quantified with an image analysis system Histo-Rag (BIOCOM). When evaluating the treated hydrogel samples, tissue cultures underwent quantitative analysis after 4 days.

2.3. Immunofluorescence staining

Monoclonal anti-cytokeratin hybridoma culture supernatants AE1/AE3 and AE5 were obtained from Cliniscience. Culture supernatant AE1 recognizes a group of acidic keratins including keratin 16, which is an acidic protein of 48 kDa found in hyperproliferative epithelium [10]. AE3 reacts with a number of basic keratins which are found in all epithelial cells [11]. AE5 is specific for keratin 3, a 64 kDa protein found in differentiated corneal epithelial cells [12]. Before staining, the medium was aspirated, and cultures were rinsed with Phosphate Buffered Saline (PBS) and fixed in ice-cold acetone: methanol (1:1) for 10 min. After rehydration in PBS/3% Bovine Serum Albumin (BSA), cultures were incubated with 300 μl/well of antibody AE1/AE3 (1:200 in 1% BSA) for 60 min or AE5 antibody (1:50 in 1% BSA) at room temperature. After two 15 min washes in PBS, fluorescein isothiocyanate conjugated goat antimouse antibody (1:100 in 1% BSA) was added to each well, and incubated for 60 min at room temperature. After three washes, tissue cultures on hydrogel surfaces were mounted between a glass slide and coverslip using glycerol/gelatin (Sigma). Cell layers were viewed with an EM 10 Zeiss electron microscope.

2.4. Tissue preparation for ultrastructural analysis

For electron microscopy, cultures were directly fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at 4 °C, rinsed in 0.1 M cacodylate buffer, and then post-fixed in 1% osmium tetroxide for 1 h. After rinsing in cacodylate buffer, fragments were dehydrated in a graded alcohol series and embedded in epoxyresin (Epon). Semithin sections (0.5 μm) were stained with toluidine blue. Ultrathin sections (60 nm) were contrasted with uranyl acetate and lead citrate, and examined with an EM 10 Zeiss electron microscope.

3. Results

3.1. Cell viability

The cell viability in the culture was followed by using the fluorescent vital dye fluorescein diacetate (FDA) (Fig. 1). Fig. 1a presents the outgrowth of cells from bovine corneal explants plated on hydrogel samples after 3 days in culture. Migration of cells from the explants could be observed within the first 24 h when cultures were carried out on the hydrogel sample, whereas it was only evident on the second day when cultures were directly carried out on the bottom of the plastic dishes (not shown). The morphology of the cells is presented at higher magnification in Fig. 1b and 1c after 7 days in culture. As seen, the cells displayed a typical cobblestone morphology associated with epithelia. Cultures were followed for 10 days, and good cell viability was observed throughout this period.

Fig. 2 compares the spreading of epithelial cells from explants plated on hydrogel samples and on the bottom of the wells as a function of the time in culture. As seen, covering of the support was almost completed within 7 days in both cases (surfaces of culture wells and hydrogel lenticles were 201 and 154 mm^2, respectively). However, the initial growth of cells appeared to be somewhat slower on the bottom of the wells and increased abruptly between days 6 and 7, whereas a more constant increase could be observed on the hydrogel.

In order to demonstrate the production of different cytoskeletal elements by cells grown on the hydrogel surface, immunocytochemistry experiments were carried out after 10 days in culture using anti- AE1/AE3 and anti-AE5 antibodies (Fig. 3). Both AE1/AE3 (Fig. 3a, 3b) and AE5 (Fig. 3c, 3d) immunoreactivities could be observed in the cultures, indicating the epithelial (AE3), hyperproliferative (AE1) and corneal (AE5) nature of the cells in the neoformed tissue. Cytokeratin staining was concentrated in the cytoplasm, with a distinct network aspect of the labelling. No staining was observed in the absence of the primary antibodies (not shown).

3.2. Transmission electron microscopy

Transmission electron micrographs of cell layers grown on the hydrogel surface have been performed in an attempt to further analyse epithelial development

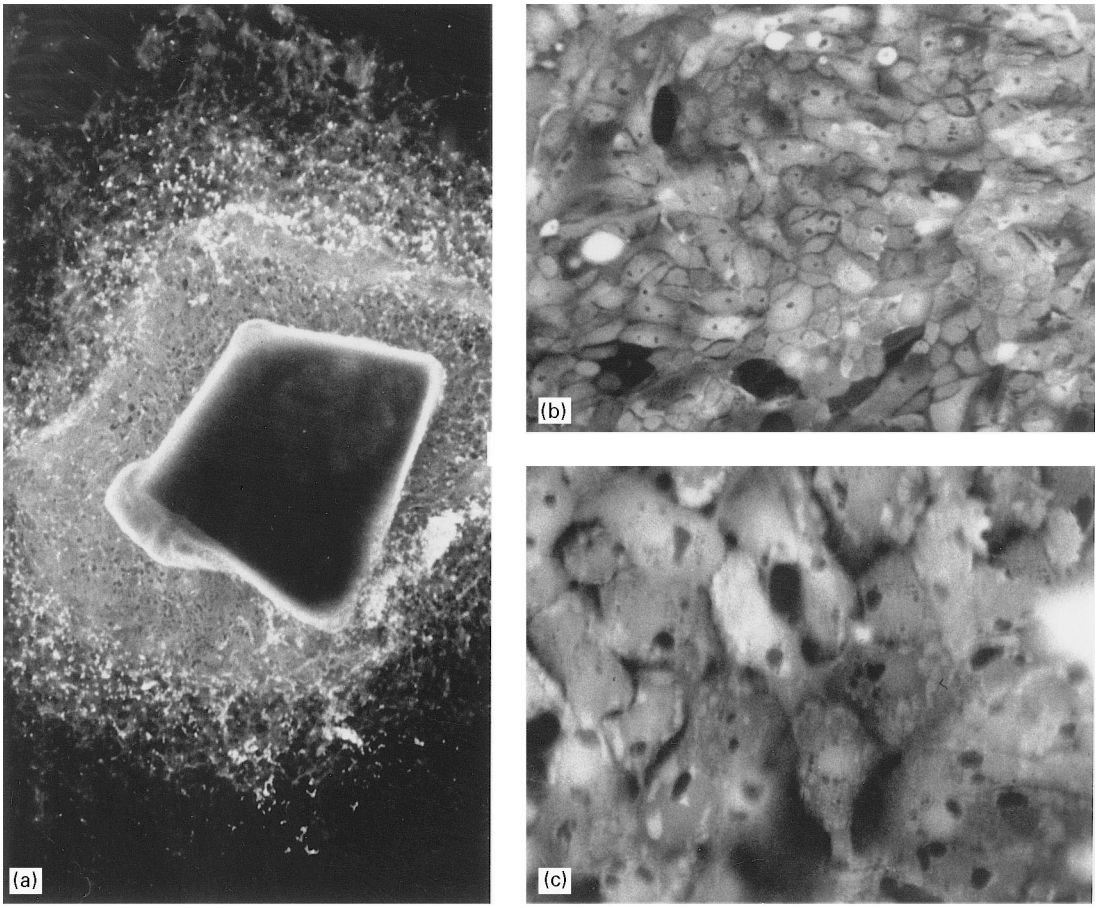

Figure 1 Corneal epithelial cultures from epithelio-limbal explants on hydrogel surface after 3 days in culture (a), or after 7 days (b) and (c). Cells were visualized by the vital dye fluorescein diacetate, as described in the text. Note the outgrowth of cells from the explant (a), and the organization of the cell layer at higher magnification (b) and (c). Magnification: (a) × 20; (b) × 250; (c) × 500.

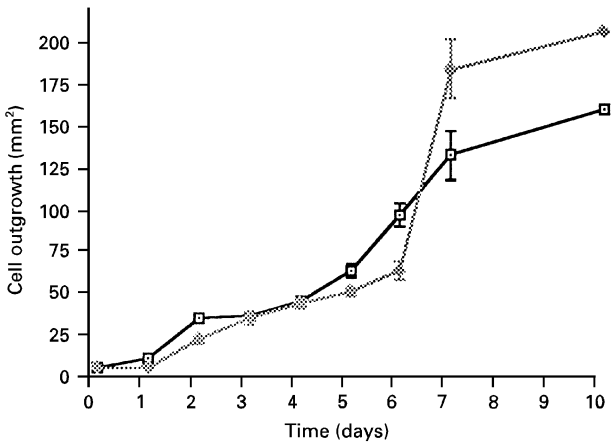

Figure 2 Corneal epithelial cell outgrowth as a function of time on hydrogel and on culture wells. Cells were grown for different times in culture, and the surface area covered by the cell layer was measured by image analysis system (Histo-Rag) as described in the text. –⊡–Hydrogel An-69; –◆–culture wells.

(Fig. 4). An epithelial cell monolayer was formed after 4–5 days in culture with cytoplasmic folds, as seen in the transmission electron micrographs (Fig. 4a and 4b). These interdigitalized membranes presented numerous well-defined desmosomes (Fig. 4b, 4c and 4d). After 10 days in culture, these squamous epithelial cells became organized in multilayers and presented cytoplasmic elements such as mitochondria, granular endoplasmic reticulum and cytoskeletal elements (Fig. 4c). The latter were bundled keratofibrils which were distributed throughout the anterior and posterior epithelial cytoplasm. Attenuated microplicae and microvilli were sparsely distributed across the corneal surface (Fig. 4a and 4c). Secretory vesicles, filled with different types of granular material, and numerous glycogen particles could also be seen (Fig. 4d).

3.3. Hydrogel treatments

Fig. 5 shows the extent of cell outgrowth obtained after 4 days in culture, with the various hydrogel surface treatments: arginine treatment for 1 and 5 min, albumin for 1 min and type IV collagen. When compared with untreated hydrogel, a 1 min treatment with arginin or a treatment with albumin did not alter the cell outgrowth. However, weak cell outgrowth was observed on the surface coated for 5 min with arginin and with collagen.

4. Discussion

In this work, we have studied the development of corneal epithelium cultures on the surface of a new hydrogel material. Several epithelial cell culture models have been used to study corneal epithelial cell

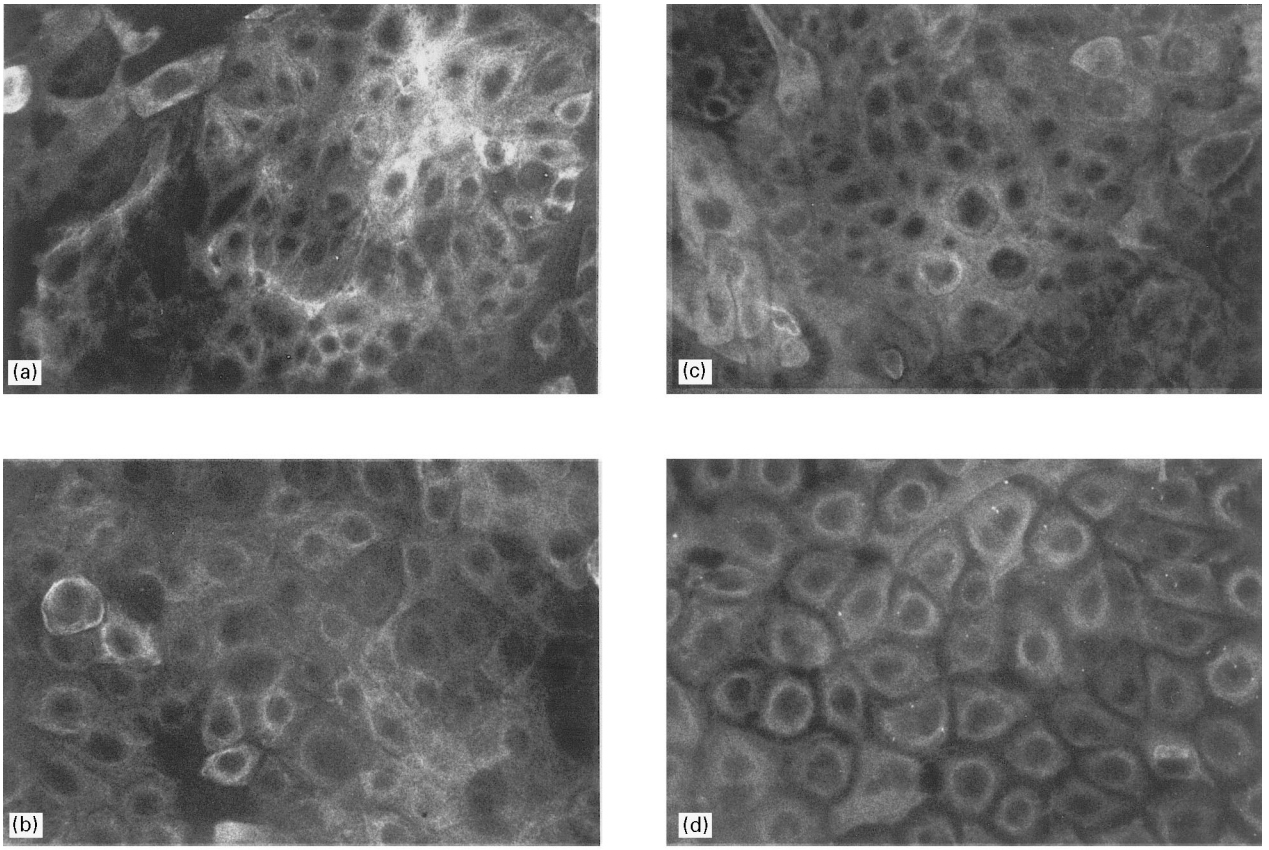

Figure 3 Detection of cytokeratins by immunocytochemistry in cultures of corneal epithelial cells. (a), (b) cytokeratins AE1/AE3. (c), (d) cytokeratins AE5. Magnification: (a), (c) × 250; (b), (d) × 500. Cells were taken after 7 days in culture.

development *in vivo* [13, 14] or *in vitro* [15] and cell-substratum interactions on synthetic supports [16, 17] or extracellular matrix [18]. Capecchi *et al.* [19] and Trinkaus-Randall *et al.* [20] developed criteria for predicting the capacity of various materials to support cell growth *in vitro* and *in vivo*, and selected a polyvinylalcohol copolymer hydrogel coated with proteins which supported cell growth *in vitro* and *in vivo*. For the epikeratophakia procedure, a synthetic graft material has to allow epithelial cell outgrowth, proliferation and adhesion. Indeed, on the hydrogel surface, corneal epithelial cells in culture spread out from the corneal explant, migrate and adhere to form an epithelial layer, demonstrating the cytocompatibility of this material. The cells were polygonal, strongly cohesive and formed a consistent cell layer.

One of the well-studied phenotypic traits of corneal epithelium is its synthesis of corneal-specific cytokeratins. Cytokeratins are a family of at least 20 intermediate filament proteins found in epithelial cells. The expression of various cytokeratins depends on the type and state of differentiation of the epithelia. The 10 nm intermediate filaments containing these cytokeratins form a cytoskeletal network thought to provide mechanical integrity to the cell in the context of its tissue [21]. The types of synthesized keratins are specific to both the developmental stage and the phenotype of the cell [22]. In the cornea, AE1 is found in hyperproliferative epithelial cells, AE3 immunoreactivity is seen in all epithelial cells, whereas AE5 immunoreactivity is observed in all but the basal cells of the limbus [12]. In our tissue culture model, AE1/AE3 staining confirms the epithelial nature of the cell layers, while AE5 staining, representing corneal cells in an advanced stage of differentiation, substantiates the corneal nature of this cell outgrowth.

Various treatments have been previously tested in order to find a suitable procedure for complete covering of biomaterials by corneal epithelial cells, and for good cell adhesion [23]. Some extracellular matrix proteins, such as fibronectin, laminin and type IV collagen, strongly influence differential adhesion and directed migration, which are main features for the repair of corneal epithelium [3, 23]. These proteins, neutralizing positive charges, stimulate adherence of the cells to the support, and the intensity of this property differs according to the protein. Cells must maintain adhesion control over their support during wound healing, to promote migration while preserving contact with the underlying substrate. In the present study, we have tested the influence of coating the hydrogel with arginin, type IV collagen or albumin, on the outgrowth of the corneal epithelial cells. None of these treatments gave results superior to the untreated material. Moreover, the low cell outgrowth observed on arginine- or collagen-treated hydrogel could reflect excessively strong adhesion to the substrate, preventing cell migration, and leading to cell death.

The corneal epithelium adheres to the underlying stroma in part by an adhesion complex consisting of hemidesmosomes, filaments that traverse the lamina lucida region of the basal lamina, and of anchoring fibrils [24, 25]. Anchoring fibrils are extracellular matrix structures that arise at the lamina densa of the

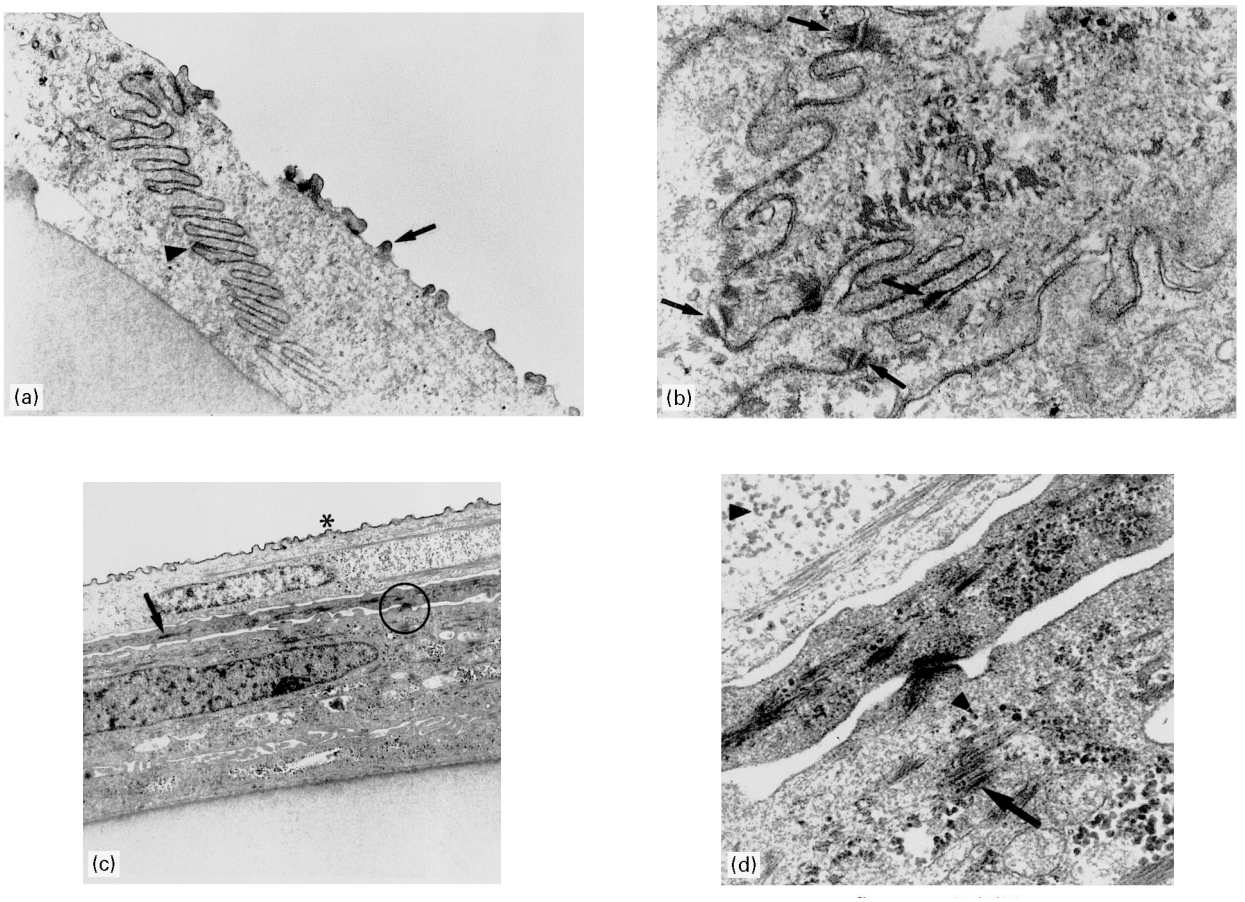

Figure 4 Transmission electron micrographs of the corneal tissue cells cultured on the hydrogel surface. (a) Micrograph taken after 5 days of culture, showing the presence of microplicae (arrows) at the cell surface. Cells establish interdigitalized connections (arrowhead). Magnification: ×8000. (b) These interdigitalized connections show the presence of numerous desmosomes (arrows) along these cell junctions. Magnification: ×25 000. (c) Micrograph taken after 10 days of culture shows corneal cell development in multilayers. The apical surface shows attenuated microplicae (asterisks). Bundled keratofibrils appear throughout the cytoplasm of these multilayer cells (arrows), as well as desmosomes (circle). Mitochondria are filled with several different types of granular material. Magnification: ×6300. (d) Enlargement from the circle in 4c, showing a desmosome along the cell-to-cell junction. Secretory vesicles (arrows) and glycogen particles (arrowheads) can also be seen. Magnification: ×40 000.

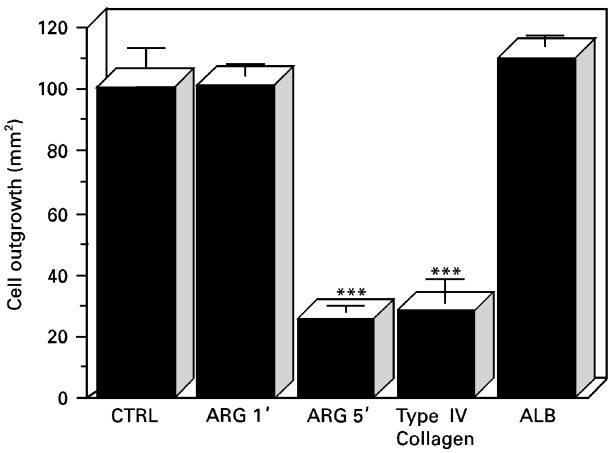

Figure 5 Spreading of epithelial cells on untreated or treated hydrogel. The biomaterial was either untreated (control, CTRL), or treated with arginin for 1 (ARG 1′) or 5 min (ARG 5′), or with human albumin (ALB) or type IV collagen. Epithelial cells were grown for 4 days in culture, and the surface occupied by the cell layer on each support was measured as described in the text. Data are presented as the percentage of surface covered by the cell layer on the untreated hydrogel (100% was equal to 36 ± 4 mm^2), and are expressed as means $\pm$ SEM from three separate experiments performed in triplicate. ***: $p < 0.001$ versus control (Dunnett's test).

basal lamina and splay out on the stroma side opposite sites where hemidesmosomes are present on basal cells [26, 27]. These anchoring structures are composed of type VII collagen, and form an important network binding the basal membrane of corneal epithelium to the underlying connective tissue [28]. According to Azar *et al.* [29], degradation of components of these adhesion structures could be involved in corneal wound formation. Abnormal adhesion between the corneal epithelium and the underlying basement membrane and stroma may account for ocular surface problems, such as recurrent erosions and persistent epithelial defects, and lack of adhesion structures may lead to corneal ulceration.

Using electron microscopy, cell membranes appear highly interdigitalized, increasing intercellular surface contact. At the level of intercellular junctions, numerous desmosomes are conspicuous, demonstrating the existence of tight junctions between cells. Under our experimental conditions, we did not observe hemidesmosomes at the cell/hydrogel interface. However, we detected the presence of some membrane differentiations which could correspond to nascent anchoring

structures between basal membranes of the epithelial cells and the hydrogel (data not shown).

In conclusion, we have studied the development of corneal epithelial cultures on a hydrogel surface. Previous studies had shown that hydrogel materials might be suitable for ophthalmologic applications [30], owing to the softness and elasticity of these biomaterials. By quantitative imaging methods and vital dye, we have demonstrated that the new hydrogel promotes corneal epithelial cell growth, a necessary step for the re-epithelialization of synthetic corneal grafts in epikeratoplasty.

Acknowledgements

The authors would like to thank A. Vallée for her skillful technical assistance, and J. Demeurie and Y. Issoulié for their excellent photographic work. This work was supported by a grant from Transphyto (Clermont-Ferrand, France) to F. Maury.

References

1. J. L. BARRAQUER, *Int. Ophthalmol. Clin.* **6** (1966) 53.
2. D. K. PETTIT, A. S. HOFFMAN and T. A. HORBETT, *J. Biomed. Mater. Res.* **28** (1994) 685.
3. J. COLIN, F. MALLET and P. MADER, *J. Fr. Opthalmol.* **14** (1991) 21.
4. K. P. THOMPSON, K. D. HANNA and P. GRAVAGNA, *Invest. Ophthalmol. Vis. Sci.* **31** (Suppl) (1990) 301.
5. P. ALBINET, J. P. ROMANET and M. MOUILLON, *J. Fr. Ophthalmol.* **13** (1990) 109.
6. J. V. AQUAVELLA, G. N. ROA, A.C. BROWN and J. K. HARRIS, *Ophthalmology* **89** (1982) 655.
7. J. J. BARNHARM and M. J. ROPER-HALL, *Bir. J. Ophthalmol.* **67** (1983) 468.
8. O. WICHTERLE and D. LIM, *Nature* **185** (1960) 117.
9. B. EBATO, J. FRIEND and R. H. THOFT, *Invest. Ophthalmol. Vis. Sci.* **28** (1987) 1450.
10. T. T. SUN, R. EICHNER, W.-G. NELSON, S. C. G. TSENG, R. A. WEISS, M. JARVINEN and J. WOODCOCK-MITCHEL, *J. Invest. Dermatol.* **81** (1983) 109.
11. T. T. SUN, S. C. G. TSENG, A. J. W. HUANG, D. COOPER, M. H. LYNCH, R. A. WEISS and A. SCHERMER, *New York Acad. Sci.* **455** (1985) 307.
12. A. SCHERMER, S. GLAVIN and T.-T. SUN, *J. Cell. Biol.* **103** (1986) 49.
13. M. MENASCHE, L. ROBERT, P. PAYRAU, R. HAMADA and Y. POULIQUEN, *Path. Biol.* **36** (1988) 781.
14. M. JUMBLATT and A. M. NEUFELD, *Invest. Ophthalmol. Vis. Sci.* **27** (1986) 8.
15. J. D. CAMERON, R. R. WATERFIELD, M. W. STEFFES and L. T. FURCHT, *ibid.* **30** (1989) 2407.
16. P. R. BERGETHON, V. TRINKAUS-RANDALL and C. FRANZBLAU, *J. Cell. Sci.* **92** (1989) 111.
17. V. TRINKAUS-RANDALL, A. NEWTON and C. FRANZBLAU, *Invest. Ophthalmol. Vis. Sci.* **29** (1988) 1800.
18. S. NAKAGAWA, T. NISHIDA, Y. KODAMA and M. ITOIN, *Cornea* **90** (1990) 125.
19. J. CAPECCHI, V. TRINKAUS-RANDALL, A. VADASZ, H. LEIBOWITZ and C. FRANZBLAU, *Invest. Ophthalmol. Vis. Sci.* **28** (Suppl.) (1987) 160.
20. V. TRINKAUS-RANDALL, J. CAPECCHI, A. NEWTON, A. VADASZ, H. LEIBOWITZ and C. FRANZBLAU, *ibid.* **29** (1988) 393.
21. P. A. COULOMBE, M. E. HUTTON, R. VASSAR and E. FUCHS, *J. Cell. Biol.* **115** (1991) 1661.
22. P. M. STEINERT and D. R. ROOP, *Ann. Rev. Biochem.* **57** (1988) 593.
23. V. TRINKAUS-RANDALL, J. CAPECCHI, A. NEWTON, A. VADASZ, H. LEIBOWITZ and C. FRANZBLAU, *Cell. Tissue Res.* **251** (1988) 315.
24. I. K. GIPSON, S. J. SPURR-MICHAUD and A. S. TISDALE, *Invest. Ophthalmol. Vis. Sci.* **28** (1987) 212.
25. I. K. GIPSON, S. J. SPURR-MICHAUD, A. TISDALE and M. KEOUGH, *ibid.* **30** (1989) 425.
26. L. A. STAEHELIN, *Int. Rev. Cytol.* **39** (1974) 191.
27. I. K. GIPSON, S. M. GRILL, S. J. SPURR and S. J. BRENNAN, *J. Cell. Biol.* **97** (1983) 849.
28. E. L. STOCK, M. A. KURPAKUS, B. SAMBOL and J. C. R. JONES, *Invest. Ophthalmol. Vis. Sci.* **33** (1992) 304.
29. D. T. AZAR, S. J. SPURR-MICHAUD, A. S. TISDALE, M. B. MOORE and I. K. GIPSON, *Arch. Ophthalmol.* **109** (1991) 1279.
30. M. B. McDONALD, *Ref. Corneal. Surg.* **4** (1988) 158.
31. G. H. SHIUE, S. D. LEE, C. C. WANG, M. HUNG-SHIEU and P. CHUEN-TSUEI CHANG, *Biomaterials* **14** (1993) 591.
32. K. P. THOMPSON, K. HANNA, G. O. WARING, J. GIPSON, Y. LIU, R. P. GAILITIS, B. JOHNSON-WINT and K. GREENT, *Refractive & Corneal Surgery* **7** (1991) 240.
33. T. V. CHIRILA, S. VIJAYASEKARAN, R. HORNE, Y. C. CHEN, P. D. DALTON, I. J. CONSTABLE and G. J. CRAWFORD, *J. Biomed. Mater. Res.* **28** (1994) 745.

Received 19 April
and accepted 24 May 1996