Cytokeratins and retinal epithelial cell behaviour

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Summary

The expression of cytokeratins 18 and 19 by human retinal pigment epithelial cells (HRPE) has been suspected of being associated with HRPE proliferation. We have investigated the involvement of these cytokeratin subtypes in the proliferative and migratory behaviour of cultured HRPE. Cell proliferation markers (bromodeoxyuridine and proliferating cell nuclear antigen) and the cytokeratins were identified using immunohistochemical techniques.

In vitro, cytokeratins 18 and 19, as detected by the monoclonal antibodies RGE 53 and K4.62, were expressed in a subset of HRPE and this subset was significantly less likely to be proliferating.

Micro-chemotaxis chambers were used to study migrating cells and immunohistochemical staining for cytokeratins 18 and 19 revealed that actively migrating cells always expressed these two cytokeratins, whereas stationary cells did not label for these cytokeratin subtypes. It was apparent that cytokeratins 18 and 19 were not markers of proliferation, but were involved in the mobility of HRPE in vitro. Cytokeratins 18 and 19 may be useful indicators of simple epithelial cell migration in tissues.

Key words: cytokeratin, migration, epithelium.

Introduction

Retinal pigment epithelial cells (RPE) of mammals and amphibians express a range of cytokeratins in vivo and in vitro (Hitchins and Grierson, 1988; McKechnie et al. 1988; Kaspar et al. 1988; Owaribe et al. 1988) that do not seem to be present in the chick (Docherty et al. 1984). The cytokeratins, of the human RPE (HRPE) at least, are generally a selection of the intermediate and low molecular mass forms and, on the basis of the classification scheme introduced by Moll (Moll et al. 1982), cytokeratins 5,6,7,8,14,15,16,17,18 and 19 are either suspected or have been shown to be present. Cytokeratin intermediate filaments may also be co-expressed with vimentin in HRPE (McKechnie et al. 1988).

There seem to be differences between immunohistochemical staining patterns in the intact tissue monolayer and those in culture (McKechnie et al. 1988). Cytokeratin 18 is faintly positive if present at all and cytokeratin 19 is absent from the intact monolayer in situ, but in tissue culture subpopulations of RPE are identified that exhibit intense immunoreactivity for both cytokeratins while adjacent cells can be completely negative. It is suspected that these cytokeratins may be markers for proliferating RPE. RPE normally do not proliferate in the adult eye where they function as sedentary phagocytes but they can be stimulated to do so in pathological conditions such as complex retinal detachment. In complex detachment, the retinal scar tissue that forms is rich in mobile proliferating RPE (Grierson et al. 1987) and many of these cells stain prominently with antibodies to cytokeratin 18 (McKechnie et al. 1988; Hiscott et al. 1991). It would appear that immunohistopathological observations are consistent with the concept that the expression of acidic low molecular mass cytokeratins is a feature of subsets of RPE involved in altered behavioral activity. We undertook the present study to obtain further evidence concerning proliferative and migratory activity of human RPE and the expression of cytokeratins 18 and 19, employing the two monoclonal antibodies RGE 53 and K4.62.

Materials and methods

Tissue culture

Eyes up to but not exceeding 48 hours post-mortem were obtained from the local Eye Bank. Human RPE were isolated following the procedures outlined by Edwards (1981) and modified by Boulton et al. (1982). Briefly, the eyes were soaked for 10 minutes in phosphate buffered saline (PBS) containing 100 units of penicillin and streptomycin. The anterior segment was removed and discarded and thereafter the vitreous humour was dislodged by gently shaking the posterior eye cup upside down. The neural retina was teased away from the RPE and the exposed RPE was washed three times in PBS with 0.02% EDTA to remove adherent photoreceptor debris.

The posterior eye cup was divided into three segments and
areas of the RPE monolayer were isolated by brass cloning rings (Boulton et al. 1982). The wells formed by the cloning rings were filled with a mixture of 0.25% trypsin, 0.02% EDTA and PBS, and incubated at 37°C for not more than 1.5 hours. Dissociated RPE cells were aspirated from Bruch's membrane and seeded into 25 cm² tissue culture flasks (Gibco Europe Ltd, Scotland). The RPE were fed with Ham's F-10 culture medium, 20% foetal calf serum, 0.003 g/ml glucose, 2.5 mg/ml amphotericin B and 100 units/ml of penicillin and streptomycin (Gibco Europe Ltd, Scotland). The cultures were maintained at 37°C in an atmosphere of 5% CO₂ and air.

Primary cultures were fed once a week until cellular outgrowth was stabilized, at which point feeding was increased to twice a week. The RPE reached confluence within 2-3 weeks, were split at a ratio of 1:3 and subcultures were maintained at 37°C in an atmosphere of 5% CO₂ and air.

Cytokeratin staining of slides

Human RPE grown on LabTek slides were fixed in pre-cooled (—20°C) methanol (5 minutes) and acetone (2 minutes), then incubated with monoclonal antibodies: anti-cytokeratin 8.13 (clone K8.13, ICN Biomedicals Ltd., UK), which labels cytokeratins 1,5,6,7,8,10,11 and 18 (Gigi et al. 1982); keratin RGE 53 (clone RGE 53, Bio-nuclear Services Ltd., UK), shown to be exclusive to cytokeratin 18 (Raemaekers et al. 1983); and anti-cytokeratin 4.62 (clone K4.62, ICN Biomedicals Ltd., UK), a cytokeratin 19 marker. The monoclonal antibodies were used at a 1:20 dilution, for broad-spectrum cytokeratin markers and at a 1:10 dilution for the single keratin markers. Following washing in PBS, the cells were exposed to the particular antibody for 45 minutes. The cells were then washed in PBS and immersed in either anti-mouse peroxidase conjugate (1:50, DAKO) or anti-mouse FITC conjugate (1:40, SIGMA) for 45 minutes. The peroxidase was developed in AEC, giving a red reaction product to the cytokeratins. Slides were mounted in glycerol (peroxidase) or Fluorostab (fluorescence; Eurodiagnostics BV Holland) and viewed by bright-field and DIC microscopy or observed under epi-illumination optics (Polyvar, Reichert-Jung).

Staining for both cytokeratins 18 and 19 was done using a cocktail of the two monoclonals (1:10). Combined cytokeratin (18 or 19) and anti-BrdU (bromodeoxyuridine) or anti-PCNA (proliferating cell nuclear antigen) staining was done on appropriate slides and counted in the manner described above.

Cytokeratin staining of tissue culture flasks

Human RPE grown on tissue culture flasks were fixed for 10 minutes with pre-cooled methanol (—20°C) and then stained for cytokeratin 18 as described above. The RPE were counter-stained with haematoxylin for 15 minutes, washed in tap water, mounted in glycerol and viewed by bright-field microscopy.

Vimentin staining of HRPE

Human RPE grown on LabTek slides were fixed in methanol and acetone as described above (see Cytokeratin staining of slides). Using the same technique used to stain for cytokeratins, cells were stained for vimentin using the monoclonal VIM 13.2 (1:200 SIGMA).

Proliferation evaluations

Human RPE were seeded onto LabTek slides at a concentration of 7.5 × 10³ cells/cm². Immunohistochemical localization of cell cycle activity involved the identification of nuclear incorporated bromodeoxyuridine or of proliferating cell nuclear antigen/cyclin. BrdU is a thymidine analogue that is incorporated at S-phase of the cell cycle, whereas PCNA peaks in concentration during S-phase but is a more general indicator of cell cycle activity (Kurki et al. 1988).

For BrdU labelling, the culture medium was exchanged for fresh medium containing 1:1,000 BrdU (Amersham UK). The cells were then incubated at 37°C in 5% CO₂ for a range of time periods between 15 minutes and 6 hours to identify the optimum labelling period. After incubation, the cells were washed in PBS, fixed in methanol (—20°C) for 5 minutes, acetone (—20°C) for 2 minutes and air dried. The S-phase nuclei having incorporated BrdU were detected with an anti-BrdU monoclonal antibody (Amersham, UK). The reaction product was dark brown as revealed using the ‘Biotin-Avidin’ peroxidase technique (Grocco, 1994) and diaminobenzidine (DAB) substrate (BDH, UK).

Human RPE cells prepared and fixed in the same way as with BrdU were treated with an anti-PCNA monoclonal antibody (Boehringer Mannheim Biochemica, Germany), again the ‘Biotin-Avidin’ procedure was adopted and the labelling was visualised as either a brown (DAB) or a red (3-amino-9-ethylcarbazole, AEC) reaction product.

The cells and their stained or unstained nuclei were visualised using DIC optics (Polyvar, Reichert-Jung, Austria). Cells in the proliferation experiments were counted in 15 random fields (x400) per well.

A double immunoperoxidase staining technique was used to identify both cell cycle activity and expression of either cytokeratin 18 or 19. HRPE were grown on LabTeks and treated as described above for labelling with either BrdU or PCNA. However, nuclei were visualised using DAB. Then either cytokeratin 18 or 19 was identified as described earlier and visualised using AEC.

Migration assay

RPE migration was conducted in 48-well micro-chemotaxis chambers (Neuro Probe, Cabin John, USA). Bovine fibronectin (SIGMA) was used as the chemoattractant because previous studies had shown that it was a potent inducer of human RPE migratory activity (Campochiaro et al. 1984). Bovine fibronectin was checked for purity by gel electrophoresis. Contaminants were at the limit of resolution with human RPE migratory activity (Campochiaro et al. 1984). Bovine fibronectin was checked for purity by gel electrophoresis. Contaminants were at the limit of resolution with human RPE migratory activity (Campochiaro et al. 1984). Bovine fibronectin was checked for purity by gel electrophoresis. Contaminants were at the limit of resolution with human RPE migratory activity (Campochiaro et al. 1984). Bovine fibronectin was checked for purity by gel electrophoresis. Contaminants were at the limit of resolution with human RPE migratory activity (Campochiaro et al. 1984). Bovine fibronectin was checked for purity by gel electrophoresis. Contaminants were at the limit of resolution with human RPE migratory activity (Campochiaro et al. 1984). Bovine fibronectin was checked for purity by gel electrophoresis. Contaminants were at the limit of resolution with human RPE migratory activity (Campochiaro et al. 1984).
Actin staining of migration membranes

for actin in a similar manner to that outlined above. UK) at a 1:100 dilution. Cells on the membrane were stained was examined in order to identify cells with patterns

The data were evaluated using the paired Mest, Mann

Statistical analysis

were placed in ethanol for 15 seconds and then air dried. Membranes were stained with haematoxylin for 30 minutes, washed in cold water and mounted on glass slides. Attached cells on the upper surface and migrated cells on the lower surface of the membrane were counted in 20 high-power fields (×1,000) per well using conventional bright-field microscopy (Zeiss, Germany).

Scanning electron microscopy (SEM) of migration membranes

Membranes at the end of a migration run were washed in PBS in the usual manner, then the cells were fixed in 2.5% glutaraldehyde in Sorenson's phosphate buffer for 4 hours. The specimens were postfixed in 1% buffered osmium tetroxide, dehydrated through graded ethanol, critical point dried (Polaron, UK) and sputter coated with gold (Polaron, UK). After drying, the membranes were orientated on stubs so that both the upper and lower surfaces could be examined on the same specimen. Examination was in a Hitachi S520 scanning electron microscope to identify the phenotypes of motile cells.

Cytokeratin staining of migration membranes

For routine evaluation, cytokeratin 18 and 19 intermediate filaments were visualised using indirect immunofluorescence with the monoclonal antibodies RGE 53 and K4.62. The methanol-fixed membrane was rehydrated in PBS for 5 minutes, immersed in 5% normal goat serum in PBS for 10 minutes, floated onto primary antibody and 1 ml of primary antibody was added to the upper surface. After 30 minutes the membrane was turned over and fresh antibody was applied for a further 30 minutes. The membrane was rinsed in PBS and then incubated with the secondary antibody, goat anti-mouse FITC (1:40, SIGMA), for 30 minutes on each side. Finally the membrane was washed, mounted under a coverslip with Fluorostab and observed under epi-illumination optics (Polyvar, Reichert-Jung).

Actin staining of migration membranes

The actin-staining pattern of HRPE on migration membranes was examined in order to identify cells with patterns associated with motility. Actin was identified using an anti-actin monoclonal antibody (clone C4, ICN Biomedicals Ltd., UK) at a 1:100 dilution. Cells on the membrane were stained for actin in a similar manner to that outlined above.

Results

General cytokeratin staining characteristics in cultured HRPE

In general, the cytokeratin staining characteristics were similar in HRPE grown on glass and plastic at equivalent culture times and seeding densities. All cells stained positively for vimentin with monoclonal VIM 13.2 (Fig. 1A).

The fifth to seventh passage human RPE exhibited a positive staining response for the monoclonal antibody K8.13 (labels 1,5,6,7,8,10,11 and 18) (Fig. 1B). Since all cells stained with this antibody, this confirmed the purity of the cultures under investigation. Possible contaminants include Muller glia, astrocytes, vascular endothelium, pericytes and fibroblasts, none of which exhibits positive staining for cytokeratin markers. The wide-spectrum antibody (K8.13) delineated a fine filamentous pattern in the cytoplasm of well-spread cells, with more-pronounced staining in the perikaryon than in the periphery of the cells (Fig. 1C).

Within the same culture not all HRPE labelled with the cytokeratin 18 (K18) and cytokeratin 19 (K19) antibodies. Indeed, at confluence, the K18 and K19 (as recognised by RGE 53 and K4.62) populations represented 47% and 15% of the total number of cells present, respectively, and double labelling for K18 and K19 did not increase the labelling index above 47%, indicating that the K19 population was not a separate group of cells but a subset of the K18 population (Fig. 2). The K18 and K19 staining pattern in the HRPE were distinguishable from that associated with the more broad spectrum cytokeratin antibody by the presence of distinct intensely stained filaments curled around the nucleus with finer filaments extending into the perikaryon. In recently settled cells that were still in the process of spreading (up to 4 h post-seeding), the K18 and K19 pattern was one of an intense-staining non-filamentous aggregate in the cytoplasm, usually adjacent to the nucleus (Fig. 1D).

Effects of post-seeding time and seeding density on the K18 population

The proportion of K18 positive (K18+ve) cells was noted to vary with post-seeding time and seeding density. At 4 hours, the K18+ve cells represented over 50% of the total. Thereafter, there was a steady decrease in the percentage of K18+ve cells up to post-confluence at 72 h by which time the population had decreased to 10% or less. From the raw data it was clear that the decrease in the percentage of K18+ve cells resulted from the K18+ve cell population remaining more or less stable while the overall population was proliferating (Fig. 3). These findings were true for both flask and LabTek cultures. Likewise, at the higher seeding densities needed for subsequent migration experiments (5 × 10^5 cells/cm^2 for membranes compared to 7.5 × 10^5 cells/cm^2 for flasks), the incidence of K18+ve cells at 2 hours was 24% and at 5 hours was only 11%.

Immunoreactivity for cytokeratins 18 and 19 is not associated with RPE proliferation

The apparent stability of the K18+ve population in culture with time suggested that K18 was not, after all, associated with HRPE proliferation and this was further supported by proliferation experiments. Between 25 and 35% of the cells labelled with the S phase marker BrdU following an optimal period of exposure of 120 minutes and approximately 60% expressed PCNA (Fig. 4). Labelling preconfluent cultures on LabTek slides with BrdU and K18 showed that the BrdU+ve/K18+ve subset was extremely sparse.
Fig. 1. Immunofluorescence (A) and immunoperoxidase (B, C and D) staining of cytokeratin intermediate filaments in cultured HRPE. (A) All cells stained positively for vimentin intermediate filaments. (B) All cells stained with the wide-spectrum cytokeratin monoclonal antibody K8.13. (C) Cytokeratin staining with K8.13 reveals a fine filamentous network within the perikaryon that extends to the periphery of the cell. (D) Keratin 18 staining appeared as an aggregate, rather than filamentous, in recently settled cells in the process of spreading. Bars: A, D, 30 μm; B, 50 μm; C, 25 μm.

and by far the largest group was BrdU−ve/K18−ve, which would be expected from the previous population analysis. The incidence of BrdU+ve cells in the K18+ve group was less than 11% whereas the BrdU+ve cells in the K18−ve population was almost 35%. Chi-squared analysis showed the difference to be highly significant (P<0.001). Similarly, with BrdU and K19 labelling a similar trend was evident. The BrdU+ve cells were over 14% in the K19+ve group, compared to almost 33% in the K19−ve group and again the difference was highly significant (P<0.001) (Fig. 5). PCNA also had a much lower K18 and K19 labelling index in the +ve compared to the −ve cells, there being three to four times as many K18 and K19+ve cells in the PCNA−ve than the PCNA+ve group.

The morphology of migrating HRPE cells
Dose response curves (5 μg/ml to 50 μg/ml) showed that human RPE cells migrated optimally to a 10 μg/ml concentration of fibronectin, and at this level fibronectin was almost four times more effective than a 1% solution of FCS with over 5% of the population migrating through the permeable membrane over the period of the assay. As a consequence, fibronectin at 10 μg/ml was used as the positive chemoattractive stimulus in all subsequent experiments.

On the upper 'settlement' side of the membrane the cells varied from a rounded to a flattened appearance as seen by SEM (Fig. 6A). The flattened cells had obvious surface microvilli and well-developed cytoplasmic processes (Fig. 6B). Cells were seen with processes extending into pores (Fig. 6C) and in more advanced stages of migration only a rounded knot of cytoplasm remained on the upper surface of the membrane (Fig. 6D). Examination of the pores on the lower 'migration' side of the membrane showed some that contained the cytoplasm of RPE in transit through the membrane. The earliest stage was a small bulbous projection of cytoplasm, which made contact with the membrane surface via numerous microspikes (Fig. 7A). Other cells exhibited more advanced extension out of the pore (Fig. 7B), until eventually the perikaryon was evident
Fig. 2. A comparison of the keratin 18 and keratin 19 populations in cultured HRPE. Cultures of HRPE were stained for keratin 18 alone, keratin 19 alone and both keratin 18 and 19. Cells that stained were counted and represented as a percentage of the total cells in each culture. Cells expressing keratin 19 were found to be a subset of the keratin 18 cell population.

Fig. 3. The expression of keratin 18 in cultured HRPE with post-seeding time. Cells were seeded and cultured up to 72 hours. During this time the number of cells increased, but the keratin 18 subset remained constant throughout.

Fig. 4. Identification of proliferating HRPE in pre-confluent cultures using BrdU and PCNA. Cells were labelled with two equally good markers of proliferation. BrdU, an analogue of thymidine, specifically labels cells in the S-phase of the cell cycle, whereas PCNA, shown to be identical to cyclin and the auxiliary protein of DNA polymerase-p, accumulates during G1, peaks in S-phase and decreases during G2 and M phase. Therefore more cells in the cell cycle are labelled with PCNA than with BrdU.

Fig. 5. The expression of keratins 18 and 19 is not related to proliferation in HRPE. Using indirect double immunoperoxidase staining the subsets of keratin 18+ve and 19+ve cells were identified as well as proliferating cells. The incidence of BrdU+ve cells in the keratin 18+ve subset was lower than in the keratin 18-ve cells. Similarly, the keratin 19+ve subset had far less BrdU+ve cells than the keratin 19-ve cells. (Fig. 7C). The cells coming out and just leaving the pores had an elongated kite shape and were smooth surfaced except for microspikes at their periphery (Fig. 7A and B).

The RPE that had completed the migration through the pores were extremely flattened so that their nuclei were prominent. Processes and surface microvilli were more abundant than on those cells that were still obviously in transit. The cells were larger, uniform in shape and more epithelioid than those seen on the upper surface (Fig. 7A and D).

Actin in migrating HRPE

Actin staining of cells on the settlement side of the membrane revealed a variable pattern. Rounded and poorly spread cells had diffuse cytoplasmic staining as did those in passage through pores. Spread cells had either a reticular or a filamentous pattern but well-developed stress fibres were rare (Fig. 8A). Actin staining of cells on the migrated side showed up clear differences. The diffuse staining of cytoplasm in pores was again evident. A reticular pattern was present in the elongated kite-shaped RPE (Fig. 8B) but the most obvious feature was abundant stress fibres in the thin extended epithelioid phenotype (Fig. 8C).

Cytokeratins 18 and 19 are associated with migration in HRPE

Subsets of RPE stained up for K18 and K19 on both sides of the membrane and, in addition, +ve cells were identified in transit through the pores. It was apparent even from cursory examination that there were more +ve cells on the migrated than on the settlement side of the membrane. Subsequent counts, totalling 28,976 cells, showed that just over 8% of these were K18+ve on the settlement side whereas 45% were +ve on the migrated side (total of 1144 migrated cells counted). Only 2.5%, from a total of 468,215 cells, were K19+ve on the settled side while over 21% were positive on the migrated side (total of 1396 migrated cells counted;
Fig. 6. SEM of migrating HRPE on the upper surface of a migration membrane. (A) Cells on the upper surface of the membrane had phenotypes varying from rounded (short arrow) to flattened (long arrow). (B) The flattened cells had well-developed cytoplasmic processes and surface microvilli. (C) Some cells appeared to be in the initial stages of migration with cell processes extending into a pore. (D) Other cells were in a more advanced stage of migration where only a remnant of cytoplasm remained on the upper surface of the membrane. Bars: A, 10 μm; B, 5 μm; C, 2 μm; D, 1 μm.
Fig. 7. SEM of HRPE migrating onto the lower side of the membrane. (A) Small bulbous projections of cytoplasm protruding from a pore indicated a cell emerging onto the lower side of the membrane (arrow). (B) Other cells showed more advanced stages of emergence with spread cytoplasm extending onto the lower surface. (C) In the final stages of migration the perikaryon was evident on the lower surface (arrow). (D) Cells that had completed migration were flattened epithelioid cells with abundant surface microvilli (see also foreground of A). Bars: A,D, 2 μm; B,C, 1 μm.
Fig. 8. HRPE on the upper and lower side of a membrane were stained for actin and visualised with immunofluorescence. (A) On the upper side of the membrane rounded poorly spread cells had a diffuse actin staining pattern. Whereas, spread cells had a filamentous staining pattern. (B) The filamentous staining pattern was also seen in cells on the lower side of the membrane. (C) The flattered epithelioid cells had abundant actin stress cables radiating throughout the cytoplasm. Bars: A, B, 50 μm; C, 20 μm.

Fig. 9. The expression of keratins 18 and 19 in migrating HRPE. Keratins 18 and 19 were visualised in cells on the upper and lower sides of the migration membrane using the immunofluorescence staining technique. The subset of keratin positive cells on either the upper or lower side of the membrane was expressed as a percentage of the total number of cells on that side of the membrane. The keratin 18 and 19 subset of cells was larger on the migrated (lower) side of the membrane than on the settled (upper) side of the membrane.

Fig. 9. The differences were significant in both cases using chi-squared analysis and the Mann-Whitney U-test (P<0.001). (The value of 8.2% K18+ve cells on the settlement side of the membrane may appear to be considerably lower than that of 47% in a confluent culture. Indeed, it could be predicted that the frequency of K18+ve cells should be over 40% not 8% (Fig. 3). However, this discrepancy is due to the differences in keratin expression during time in culture and seeding densities (see above).)

A field-by-field examination of both sides of the membranes was conducted under the ×100 oil-immersion objective of the microscope. Initially we used fluorescence; however, only the keratin +ve cells were visualised. So subsequently, we concentrated on the membranes that had been stained using the peroxidase method, because with haematoxylin counter-staining non-decorated (keratin —ve) cells could be seen clearly. On the upper surface of the membrane, the rounded cells were K18 and K19 negative but some of the partially spread cells had foci of positive staining adjacent to the nucleus (though they were relatively infrequent). On the other hand, the flattened RPE that were positive for keratin exhibited a fibrillar staining pattern and, unlike their corresponding keratin nega-
Fig. 10. Immunoperoxidase staining of keratin 18 in HRPE on the upper (A) and lower (B) sides of a migration membrane. Cells were counter-stained with haematoxylin. (A) Upper surface: rounded cells were keratin 18 negative (short white arrow) and spreading cells had aggregates of keratin 18 positive staining adjacent to the nucleus (long white arrow). Cells in the initial stages of migration were keratin 18 positive and had a fibrillar staining pattern. These cells often had a cell process extending into a pore (short black arrow). Spread stationary cells were keratin 18 negative (long black arrow). (B) Lower surface: keratin 18 positive cells were small polarised cells often with some of their cytoplasm still within a pore (short white arrow). Keratin negative cells were large flat non-polarised epithelioid cells (long white arrow). Keratin 18 positive cytoplasm could be seen extending out of a pore and spreading onto the lower surface (short black arrow) (comparable to Fig. 7B). This spreading cytoplasm comes from keratin 18 positive cells on the upper surface, which, because the micrograph is focused on cells on the lower side of the membrane, appears as a diffuse red shadow (long black arrow). N.B. the density of cells on the lower side of the membrane is the same as that in Fig. 8B.
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Fig. 11. (A and B) A migrating HRPE stained for keratin 18 and captured on film at two levels of focus. (A) On the lower side of the membrane a filamentous array of keratin filaments has emerged from the pore (arrow) and spread onto the lower surface (comparable to Fig. 7B and C). (B) While on the upper surface the cytoplasm within the pore, that remaining on the upper surface and a cell process stain diffusely for keratin 18 (comparable to Fig. 6B).

tive cells, they all had cell processes extending into an adjacent pore (Fig. 10A). The large flattened non-polarised epithelioid cells on the migration side of the membrane were negative in the main. The vast majority of the positive cells were smaller bipolar cells, often with cytoplasm still within a pore (Fig. 10B).

All cells in passage through the pores were K18+ve and K19+ve and no exceptions were found in over 300 wells examined. The pattern was particularly clear by fluorescence microscopy and when the cells were predominantly on the settlement side the cytoplasm on that side had a fibrillar pattern whereas the staining of the cytoplasmic processes in and through the pores was diffuse (Fig. 11A). With cells that had a major component of their cytoplasm on the migration surface of the membrane (see Figs 6C, 7B and C) the staining pattern was reversed. The cytoplasm spread on the membrane had a fibrillar or filamentous pattern while the remnant in the pore and on the settlement side was diffuse (Fig. 11B).

Using BrdU-labelled cell proliferation on the upper and lower sides of the migration membrane was investigated. Probably because of the short incubation period of the migration experiments (i.e. 4 hours) no positively BrdU-labelled nuclei were found.

Discussion

For over a decade it has been known that the cytokeratin organisation in epithelial cells alters with general changes in cell behaviour but is particularly evident during cell division (Aubin et al. 1980; Horwitz et al. 1981; Lane et al. 1982; Franke et al. 1982, 1983). The suggestion has been put forward that cytokeratins may be modulated to permit radical shape change during mitosis (Horwitz et al. 1981; Lane et al. 1982).

McKechnie et al. (1988) identified a subset of cultured human RPE that expressed cytokeratin 18 with the monoclonal antibody RGE 53 and suggested that the expression may be cell cycle-related. Indeed the possibility arose that K18 could be a specific marker for the detection of proliferating RPE in culture and in pathological tissues such as retinal scars. RPE play a key role in retinal scar formation but they are difficult to identify on phenotype alone (Kampik et al. 1981). However, our investigations, far from establishing an association between K18 expression and replication, demonstrated that K18 (and for that matter K19) positive HRPE were less likely to be replicating than K18 negative HRPE. Indeed, there are implications from recent literature that K19 is not functionally involved in cultured keratinocyte cell proliferation (Lindberg and Rheinwald, 1991).

From the present study it appeared that HRPE expressing K19 were a subset of the K18 population and the incidence of K18 steadily decreased in the culture flask as HRPE numbers increased from sparse to confluent. This would seem on the face of it to have been an anomalous finding, given that their expression was not proliferation related. On the other hand, as cells approach confluence not only does their division rate decrease but their mobility is impaired. Mobility is also compromised at high seeding density and in this circumstance K18 positive cells made up a significantly lower percentage of the population than in cultures seeded at lower density when comparable time periods were evaluated.

Much stronger evidence was provided by our chemotraction assays, which clearly showed that a five times greater percentage of K18 positive HRPE were on the lower, migrated side of the permeable membranes than on the upper (settlement or non-migrated) side. Furthermore, there was an even greater (10-fold)
Fig. 12. A model for the involvement of actin, keratin 18 and keratin 19 in the migration of HRPE in vitro. (1) A rounded cell with diffuse actin staining is keratin 18 and 19 negative. (2) A spread cell with diffuse or filamentous actin, but is still keratin 18 and 19 negative. (3) A well-spread flattened cell with a cell process placed in a pore, has filamentous actin and is always keratin 18 and 19 positive. (4) A cell actively migrating through a pore has filamentous actin and is keratin 18 and 19 positive. (5) This is identical to stage 3 although the cell is on the lower surface rather than the upper surface and has a foot process within the pore. (6) A large flattened migrated cell with actin stress cables and negative for keratin 18 and 19.

increase in the K19 population on the migrated side of the membranes. The enhancement of the K18 and K19 populations from upper to lower sides of the membranes occurred because all cells caught in transit through the pores in the membrane were positive for these two cytokeratins. In other words, K18 and K19 were expressed by HRPE involved in active migration.

Six stages in the migration process were identified and summarised in Fig. 12. Stage 1 consisted of the rounded cells on the upper surface of the membrane, which were settled but not spread. These exhibited a diffuse actin staining pattern and were invariably K18 and 19 negative. Partially spread cells, which by SEM had numerous microvilli, were called stage 2 and these were mostly negative for the two cytokeratins, although some cells had the intense staining non-filamentous aggregate adjacent to the nucleus seen in recently settled cells (see Fig. 1E). A third stage consisted of well-spread cells with cytoplasm invading the pores. These cells had prominent processes seen by both light microscopy and SEM, a filamentous network of actin in the cytoplasm, were always K18 positive and were usually K19 positive. HRPE in transit through pores were invariably K18 and K19 positive and these we called stage 4. Stage 4 cells had a distinctive actin network in the cytoplasm spread on the under side of the membrane but the cytoplasm in the pore and the remnant on the upper surface exhibited a diffuse staining pattern. Stage 5 cells were identical to stage 3 except that they were on the bottom of the membrane rather than on the top. Stage 6 cells were extremely large and flat with well-developed actin cables or stress fibres in their cytoplasm. These cells were negative for both cytokeratins. Clearly HRPE committing (stage 3) and committed (stage 4) to migration became more spread and mobile, and exhibited K18 and K19 staining whereas HRPE on the upper surface, which were not yet involved in migration, were negative. In the post-migrational phase on the lower surface of the membrane, staining was progressively lost (stages 5 and 6). The post-migrational cells (stage 6) were well spread and flat but were probably immobile, given the presence of stress fibres in their cytoplasm (Herman et al. 1981). Stress fibres are found in many tissue culture systems and these large actin microfilament bundles are thought to be a feature of stationary cells (Herman et al. 1981). Stress fibres abound in cells with strong adhesion to their substrata (Willingham et al. 1977). They are contractile (Kreis and Birchmeier, 1980) and may maintain tension between the cells and their rigid substratum (isometric forces) (Grierson et al. 1988; Heath and Dunn, 1978). It has been said that cytokeratins help cultured epithelium to remain flat and spread (Aubin et al. 1980), but this would not be the case with K18 and K19, which are present during spreading, which involves locomotion, but cannot be detected in spread or sedentary HRPE.

The expression of vimentin intermediate filaments in HRPE would not interfere with their mobility. Vimentin, a rigid cytoskeletal element, has recently been shown to have unique viscoelastic properties, demonstrating a flexible nature under low stress; this suggests a role that vimentin may play in maintaining cell integrity during events such as locomotion (Janmey et al. 1991).

A current area of research interest has been the altered expression of cytokeratin subtypes with the emergence of premalignant or malignant epithelial lesions (Smedts et al. 1990; Cintorino et al. 1990; Schaalma et al. 1990; Nagle et al. 1991; Markey et al. 1991). It has been suggested that the expression of K8, K18 or K19 may be upregulated in premalignant or malignant lesions of various tissues including the cervix, prostate, tongue or skin. In addition, infiltrating cells of transitional cell carcinomas were shown to be strongly immunoreactive for K18 (Schaalma et al. 1990). Furthermore, it has been shown in transfected mouse L cells that expression of K8 and K18 significantly increased their invasive ability (Chu et al. 1990) and that K8 and K18 are characteristic of invasive squamous cell carcinomas (Markey et al. 1991). Invasive epithelia would be expected to be highly motile, but in addition an association between cell flexibility and K18 expression has been shown (Schaalma et al. 1990; Markey et al. 1991). Undoubtedly, HRPE in transit through pores would need to be highly flexible and to some extent this was born out by our SEM studies, which demonstrated the complex distortion of cell shape required to negotiate successfully the pore channels through the membrane.
Although this study has pinpointed an association of keratins 18 and 19 with RPE cell migration it would be interesting to investigate the expression of keratins 8 and 7 in this migration model.

Our observation of an apparent specific association between RPE locomotion and immunoreactivity of RPE cytoskeletal proteins for particular monoclonal antibody(s) in vitro suggests that the migratory behaviour of RPE may be detected by immunocytochemical means in tissue sections. Indeed, we have already documented the presence of a subset of K18 positive RPE in situ in some retinal scars (Hiscott et al. 1991) and it is also possible that K18 may be useful in the detection of migratory activities by other simple epithelia.

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References


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