Retinal pigment epithelial cells in epiretinal membranes: an immunohistochemical study

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SUMMARY Immunohistochemical techniques were used to identify cells containing cytokeratins in sections or tissue-culture monolayers from ocular (reference) tissues and also from 22 epiretinal membranes obtained during closed microsurgery for macular pucker or massive preretinal retraction. Results of cytokeratin immunostaining in reference tissues indicated that this is a valuable means of determining the contribution and distribution of epithelial cells in epiretinal membranes, and that the epithelial cells in the membranes were probably derived from the retinal pigment epithelium. Epithelial cells were identified in 17 of the 22 epiretinal membranes, but they did not usually constitute the predominant cell type. We concluded that the fibroblasts or fibroblast-like cells thought to be responsible for the contraction of epiretinal membranes are seldom of retinal pigment epithelial origin. Biomicroscopic pigmentation of a membrane was shown to be a poor guide to its epithelial cell population.

Epiretinal membranes (ERMs) are cellular proliferations on the retinal surface which sometimes complicate the natural history or surgical treatment of rhegmatogenous retinal detachment. Contraction of an ERM localised to the posterior retina produces macular pucker, while more widespread ERM contraction causes massive preretinal retraction (MPR) or proliferative vitreoretinopathy. Retinal pigment epithelial (RPE) cells have been implicated as major contributors to such proliferations and as progenitors of the fibroblasts or fibroblast-like cells responsible for membrane contraction.¹⁻⁵

Histopathological analysis has suggested that metaplastic cells derived from a variety of sources may be present in ERMs,⁴⁻¹¹ but morphological and ultrastructural criteria are an unreliable means of demonstrating the precise origins of component cells. Tissue culture and immunohistochemical techniques have recently been employed to elucidate further their cellular derivation,¹²⁻¹⁵ but so far only the glial cell contribution to ERMs has been comprehensively investigated.¹⁵ In view of the possible importance of RPE cells in the pathophysiology of ERMs, we have used immunohistochemical markers to two of the

cytoskeletal proteins of epithelial cells—prekeratin and keratin—to demonstrate the contribution and distribution of epithelial cells in ERMs. Sections of surgically excised ERMs, together with cells derived from ERMs in tissue culture, were investigated following closed intraocular microsurgery for macular pucker and MPR mainly complicating rhegmatogenous retinal detachment.

Material and methods

STANDARDS

To confirm the specificity of immunostaining within the ERMs, and to provide intensity standards for each immunohistochemical procedure, a variety of tissues (Table 1) and tissue culture monolayers (Table 2) were assessed for prekeratin and keratin content. These materials were derived from section or dissection of the following globes: 4 adult albino rabbit eyes, 2 bovine eyes, 2 adult owl monkey eyes, an albino human eye (20 weeks gestation), and 3 adult human eyebank globes. In addition 4 human eyes containing retinal detachments were studied.

EPIRETINAL MEMBRANES

Excised ERMs were obtained from 22 eyes undergoing closed intraocular microsurgery for macular

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Table 1 Tissue standards for immunohistochemical staining

		<i>Intensity of immunostaining</i>	
	<i>F/P</i>	<i>Prekeratin</i>	<i>Keratin</i>
<i>Corneal epithelium</i>			
Adult albino rabbit	F & P	+++	NP
Adult bovine	F	+++	+++
Adult owl monkey	P	+++	+++
Fetal albino human	P	+++	+++
Adult human	F & P	++	+++
<i>Corneal endothelium</i>			
Adult albino rabbit	F & P	+	NP
Adult bovine	F	—	—
Adult owl monkey	P	—	+/-
Fetal albino human	P	—	—
Adult human	F & P	+/-	+/-
<i>Lens epithelium</i>			
Adult albino rabbit	F & P	+/-	NP
Adult owl monkey	P	—	—
Fetal albino human	P	—	—
Adult human	F & P	—	—
<i>Iris epithelium</i>			
Adult albino rabbit	F & P	+/-	NP
Fetal albino human	P	+	+
Adult human	F & P	+/-	+/-
<i>Ciliary epithelium</i>			
Adult albino rabbit	F & P	—	NP
Adult human	F & P	—	—
<i>Neuroretinal tissue</i>			
Adult owl monkey	P	—	—
Fetal albino human	P	—	—
Adult human	F & P	—	—
<i>Retinal pigment epithelium</i>			
Adult albino rabbit	F & P	+	NP
Fetal albino human	P	+	+
Adult human	F & P	+	+
Metaplastic adult human	P	++	++

F=immunofluorescent technique. P=immunoperoxidase technique. NP=not performed.

pucker (14 eyes) or MPR (8 eyes); aetiological factors and clinical circumstances surrounding the surgery were recorded. A standard proforma documenting the biomicroscopic features of each membrane was also completed by the surgeon; in this investigation particular attention was paid to the presence or absence of visible pigmentation in the ERM prior to its removal from the retinal surface.

Sixteen of the peeled ERMs were embedded directly for histopathological and immunohistochemical analysis (Table 3). Monolayers of cells were grown in tissue culture from the remaining 6 ERMs by a method previously described,¹⁴ (Table 4). Only ERMs with a prominent cellular component (as distinct from late collagenous scars) were included in this study.

SPECIMEN PREPARATION

Eyes and epiretinal membranes. Globes to be used as

Table 2 Tissue culture standards for immunohistochemical staining

	F/P	Intensity of immunostaining	
		Prekeratin	Keratin
Bovine corneal epithelium	F	+++	+++
Human lens epithelium	F	NP	+
Bovine retinal pigment epithelium	F	++	+
Human retinal pigment epithelium	F & P	++	++

F=immunofluorescent technique. P=immunoperoxidase technique. NP=not performed.

standards and eight of the ERMs were fixed in 10% formol-saline, dehydrated in graded alcohols and embedded in paraffin wax. In an attempt to enhance visualisation of immunostaining, globes containing heavily pigmented tissues were bleached by various bleaching methods. Of these, only 0.25% potassium permanganate followed by oxalic acid preserved tissue integrity and produced effective bleaching. Portions of bovine cornea and the remaining eight ERMs were placed in OCT embedding media (Lab-Tek Products, Illinois) and immediately frozen in liquid nitrogen. At least four wax or frozen sections from each tissue were then prepared for immunoperoxidase or immunofluorescent staining as previously described.¹⁵ Sections were also taken for conventional (haematoxylin and eosin) staining.

Tissue culture monolayers. Monolayers to be used as reference standards and primary monolayers growing from ERM explants were thoroughly washed in phosphate-buffered saline (PBS, pH 7.6) to remove culture medium. They were then air-dried for 20 minutes before immunohistochemical staining.

IMMUNOHISTOCHEMISTRY FOR KERATINS

Cytokeratins differ both biochemically and immunologically between various epithelia,¹⁶⁻¹⁸ so we used two different antibodies, each with a wide spectrum of reactivity, in order to minimise false-negative staining: (a) antiprekeratin (Miles Scientific; Slough, England) raised in guinea-pigs with fetal bovine hoof and diluted between 1:10 and 1:50 in TRIS-buffered saline (TBS, pH 7.6). (b) antikeratin (Dako Corp., California, USA) raised in rabbits with human stratum corneum from the foot and diluted between 1:30 and 1:200 in TBS.

Immunoperoxidase demonstration of prekeratin was conducted by a standard indirect peroxidase technique as outlined by Mukai and Rosai.¹⁹ For keratin, immunoperoxidase staining was achieved with a peroxidase-antiperoxidase method previously

Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1d

Fig. 1e

Fig. 1f

Fig. 1g

Fig. 1h

Fig. 1 Dewaxed 7 μ m sections stained by immunoperoxidase methods to demonstrate prekeratin and keratin; all sections were counterstained with haematoxylin. (a) Normal human RPE stained for prekeratin; the reaction product is brown ($\times 820$). (b) Control section as in (a), but excluding the primary antibody; no reaction product is evident ($\times 820$). (c) Human RPE cells grown in vitro and stained to demonstrate keratin; a distinctive brown reaction product can be seen in the cytoplasm of the cells ($\times 165$). (d) Human corneal epithelium stained to demonstrate keratin; a dark brown reaction product is present in all layers ($\times 245$). (e and f) ERMs stained for keratin; there are many positive cells in (e), but epithelial cells are virtually absent in (f) ($\times 330$). (g and h) ERMs stained for prekeratin; in (g) the positive cells scattered within the membrane are kite-shaped like fibroblasts, whereas in (h), the epithelial cells are cuboidal and virtually restricted to a surface monolayer ($\times 165$ and $\times 330$).

Fig. 2 Immunofluorescent staining for prekeratin and keratin in frozen sections and cell monolayers. (a and b) Frozen sections from two ERMs stained for prekeratin; in (a) epithelial cells abound, whereas in (b) epithelial cells are rare ($\times 130$). (c and d) Frozen sections from two ERMs stained for keratin; in (c) epithelial cells are prominent, exhibiting either moderate or intense fluorescence, whereas in (d) only a small focus of positively staining cells can be identified ($\times 530$). (e) Frozen section from an ERM stained for prekeratin; note the elongated spindle shape of one of the positively staining cells ($\times 530$). (f) Monolayer growing from an ERM in tissue culture stained for keratin; the epithelial cells have positive cytoplasmic and negative nuclear fluorescence; note the filamentous arrangement of the cytokeratins ($\times 660$).

Table 3 Immunohistochemistry of 16 embedded peeled epiretinal membranes

No.	MP/ MPR	Aetiology	Biomicroscopic pigmentation	F/P	% cells prekeratin positive	% cells keratin positive	Intensity prekeratin staining	Intensity keratin staining	Distribution of epithelial cell component
1	MP	Multiple surgery for large tear and RRD; postop. uveitis	Non-pigmented	P	90%	90%	+++	+++	Focus/diffuse
2	MP	Surgery for multiple large breaks and RRD;	Non-pigmented	P	60%	40%	+++	+++	Focus/layer/ diffuse
3	MP	Multiple surgery for multiple breaks and RRD; vitreous haemorrhage	Pigmented	F	40%	40%	++	+++	Layer/diffuse
4	MP	Surgery for multiple tears and RRD; postop. vitreous haemorrhage	Non-pigmented	P	25%	10%	++	+	Diffuse
5	MP	Vitreous haemorrhage after blunt trauma; no retinal breaks	Non-pigmented	P	25%	20%	++	++	Diffuse
6	MP	Silicone oil exchange for recurrent RRD; later peel under oil	Non-pigmented	F	20%	20%	++	++	Focus
7	MP	Posterior penetrating injury; retinal incarceration and vitreous haemorrhage	Non-pigmented	F	10%	NP	+	NP	Diffuse
8	MP	Multiple surgery for posterior penetrating injury and RRD; retinal incarceration	Pigmented	F	5%	NP	+	NP	Diffuse
9	MP	Silicone oil exchange for giant tear and total RRD; later peel under oil	Non-pigmented	P	0%	0%			
10	MP	Central retinal vein occlusion and vitreous haemorrhage	Pigmented	F	0%	0%			
11	MPR	Surgery for aphakic RRD; postop. vitreous haemorrhage	Pigmented	F	50%	50%	++	+++	Focus/diffuse
12	MPR	Surgery for large break and RRD in high myopia	Pigmented	P	20%	20%	+	++	Focus/diffuse
13	MPR	Acute retinal necrosis syndrome; multiple breaks and RRD	Non-pigmented	P	15%	10%	++	++	Diffuse
14	MPR	Surgery for multiple posterior breaks and RRD	Pigmented	F	10%	10%	++	+	Focus/diffuse
15	MPR	Multiple surgery for multiple breaks and RRD	Non-pigmented	P	0%	0%			
16	MPR	Multiple surgery for aphakic RRD with vitreous haemorrhage and endophthalmitis	Non-pigmented	F	0%	0%			

MP=macular pucker. RRD=rhegmatogenous retinal detachment. MPR=massive preretinal retraction. NP=not performed. P=immunoperoxidase technique. F=immunofluorescent technique.

described.¹⁵ To highlight immunostaining in pigmented tissues we employed two staining substrates yielding different coloured reaction products: 3,3-diaminobenzidine tetrahydrochloride (DAB) or 3-amino-9-ethylcarbazole (AEC), which gave brown and pink reaction products respectively. Immuno-

fluorescent staining for both prekeratin and keratin on sections and cell monolayers was performed by the indirect immunofluorescent technique previously employed.^{14 15} Control sections or monolayers were processed in each immunostaining procedure with omission or substitution of the primary antibody.

Table 4 Immunofluorescence of six tissue culture monolayers from peeled epiretinal membranes

No.	MP/ MPR	Aetiology	Biomicroscopic pigmentation	% cells positive	Intensity of prekeratin staining	Intensity of keratin staining
1	MP	Multiple surgery for large tear and RRD	Non-pigmented	25%	NP	+++
2	MP	Multiple surgery for large dialysis and RRD after blunt trauma	Pigmented	10%	NP	++
3	MP	'Idiopathic' epimacular membrane and peripheral retinoschisis without breaks	Non-pigmented	10%	+++	+++
4	MP	Extensive cryotherapy for small flat horseshoe tear	Non-pigmented	0%		
5	MPR	Silicone oil exchange for giant tear and RRD; later peel under oil	Pigmented	50%	++	+++
6	MPR	Multiple surgery for dialysis and RRD after blunt trauma; retinal incarceration	Non-pigmented	50%	+++	++

MP=macular pucker. RRD=rhegmatogenous retinal detachment. MPR=massive preretinal retraction. NP=not performed.

Using 'in situ' and cultured corneal epithelium as reference standards for prekeratin and keratin labelling, we evaluated staining of the various tissues and cultured monolayers taking into account the antibody dilution at which the tissue or cells stained. For both immunoperoxidase staining ('P' in Tables 1, 2, and 3) and immunofluorescence ('F' in Tables 1, 2, 3, and 4), intensity of staining was assessed as follows:

- no staining
- /+ inconsistent staining
- + weak staining
- ++ moderate staining
- +++ intense staining.

If intensity of staining varied between cells within a specimen, the mean result was recorded. In the peeled ERMs or their monolayers the percentage of cells staining positively for prekeratin or keratin was also recorded. These semiquantitative assessments were made by two observers (P.S.H. and I.G.) independently and subsequently averaged.

Results

STANDARDS

RPE cells in situ stained positively for cytokeratins (Table 1; Figs. 1a, b): the staining patterns for keratin and prekeratin were similar but more consistent results were obtained with the prekeratin marker. Metaplastic RPE cells beneath detached human retina and bovine and human RPE cells in vitro showed more impressive (moderate) staining for keratins than did normal RPE cells (Tables 1 and 2; Fig. 1c). The only cells to show intense staining for the cytokeratin markers, either in the globes or in tissue culture, were corneal epithelial cells (Tables 1 and 2; Fig. 1d). All the other cells examined were either weakly positive, negative, or variable in staining pattern (Tables 1 and 2).

EMBEDDED ERMS

Twelve of the 16 embedded ERM-specimens contained epithelial cells as shown by immunohistochemical staining for keratin and prekeratin (Figs. 1e-h; 2a-e). The estimated epithelial cell contribution in the ERMs ranged from approximately 90% to 5% (Table 3). In the majority of ERMS, however, only 25% or less of the cells were positively staining for prekeratin and keratin, and only one specimen had a predominant epithelial cell component.

In most ERMs equal numbers of cells stained for prekeratin and for keratin; in four ERMs, however, cells staining for prekeratin outnumbered cells staining for keratin (Table 3). Overall the intensity of prekeratin staining paralleled that of keratin, and with both antibodies some variation in staining in-

tensity was observed between cells in individual specimens (Fig. 2c).

The distribution of epithelial cells within the 12 positively staining ERMs was variable, but three patterns (diffuse, foci, and layers) were recognised occurring either singly or in combination (Table 3). Foci were defined as circumscribed aggregates of cells (Fig. 2d) and were present in six of the 12 ERMs. A layer of cells forming a single lamina on a surface of the ERM occurred in two specimens (Fig. 1h). In 11 ERMs, however, isolated cells scattered throughout the membrane were recognised (Figs. 1e, g; 2a, c). Irrespective of their pattern of distribution the epithelial cells usually had a cuboidal or oval shape (Figs. 1h, 2c); the remainder of the positively staining cells looked like fibroblasts, that is, they were either kite-shaped or spindle-shaped (Figs. 1g, 2e). However, such fibroblast-like epithelial cells were present in only four ERMs, and the great majority of fibroblast-like cells were negatively staining. In only one membrane did fibroblast-shaped epithelial cells predominate (Fig. 1g; specimen 1 in Table 3).

MONOLAYERS FROM ERMS

Five of the six tissue culture monolayers growing from ERM explants contained cells which stained positively for cytokeratins, sometimes in substantial numbers (Table 4). The proportions of cells fluorescing after antiprekeratin and antikeratin preparation were judged to be equal in each monolayer (as shown in a single column in Table 4). However, these results may not represent the proportion of epithelial cells in the ERM explant because the culture medium may selectively enhance or repress the growth of individual cell types. As in pure monolayers of RPE cells, the melanin content of staining cells decreased with progressive cell division in culture and the staining intensity for epithelial markers was more pronounced than that seen in RPE cells in situ. The positively staining cells grown from ERMs adopted a flattened oval shape and showed a bright filamentous pattern in their cytoplasm which was particularly strong around the nucleus (Fig. 2f). The negatively staining cells in the monolayers had the morphological and locomotory characteristics of inflammatory cells, glial cells, and/or fibroblasts.

CLINICO-IMMUNOHISTOCHEMICAL CORRELATION

The presence of clinically recognisable pigmentation in an ERM (eight specimens in Tables 3 and 4) correlated well with subsequent immunohistochemical demonstration of an epithelial cell component in the embedded ERM or tissue culture monolayer. The only exception (specimen 10 in Table 3) was a pigmented ERM removed during microsurgery for a vitreous haemorrhage in which haemosiderin laden

macrophages were responsible for the ERM pigmentation. Conversely, the absence of biomicroscopically apparent pigmentation in an ERM provided no clue to the epithelial cell content; 10 of the 14 non-pigmented membranes had a significant epithelial cell component (Tables 3 and 4), and the embedded ERM with the greatest proportion of positively staining cells (specimen 1 in Table 3) was clinically non-pigmented. Identification of the reasons for the presence or absence of biomicroscopic pigmentation in an ERM was attempted. Most melanin-containing cells were positive for epithelial markers, but not all. Substantial numbers of cells without melanin in their cytoplasm were positively staining, and extracellular melanin granules were abundant in some specimens. Thus such factors as incorporation of melanin by non-epithelial cells, progressive melanin-loss from RPE cells after each cell division, cocooning of cells in collagen and clumping of melanin-containing cells all tend to make biomicroscopic estimation of pigmentation a poor guide to epithelial cell content in ERMs. Our histochemical estimates of epithelial cell contribution in ERMs showed that those classified as 'pigmented' had on average a 21% epithelial cell content, whereas non-pigmented ERMs were not dissimilar with a 22% epithelial cell content.

Overall, epimacular membranes removed from eyes with traction detachment localised to the posterior pole (MP) contained a somewhat greater proportion of epithelial cells than did ERMs peeled from bullously elevated immobile retina (MPR) (Table 3). Fourteen of the 17 ERMs with an epithelial cell component in sections or monolayers were removed from eyes with a history of (or in the presence of) a rhegmatogenous retinal detachment (Tables 3 and 4). Of the remaining three ERMs which contained or grew positively staining cells two (specimen 5 in Table 3; specimen 3 in Table 4) were obtained from eyes with no clinically identified retinal break, and the origin of this epithelial cell component is therefore obscure. Four of the five ERMs with no positively staining cells in sections or monolayers were removed from eyes which contained a retinal break (Tables 3 and 4), so the presence of exposed RPE cells on Bruch's membrane did not necessarily ensure an epithelial cell component in the associated ERM.

Discussion

When RPE cells are implanted into the vitreous cavity of rabbits and monkeys, their morphology changes so they may no longer resemble RPE cells 'in situ.'^{20 21} Human RPE cells can similarly change in their appearance if they are displaced on to the retinal surface,⁵ with consequent difficulties in cell identifica-

tion in ERMs and potential confusion with fibrous astrocytes.⁸ Conversely, the cytokeratins of epithelial cells are retained in long-term tissue culture and during neoplastic transformation²²⁻²⁶ and frequently become more prominent—for example, keratin synthesis demonstrated during squamous metaplasia of RPE cells in organ culture.²⁷ We have shown that, both in tissue culture and during metaplasia in the subretinal space, RPE cells stain more intensely for cytokeratins than normally despite their altered morphology. Thus, while the reliability of the ultrastructural criteria which can be used to identify RPE cells *decreases* with cellular transformation, the reliability of immunohistochemical identification may well *increase*. Furthermore, light microscopic immunohistochemistry on multiple sections of ERM specimens permits more representative tissue sampling for analysis compared with electron microscopy. For these reasons we consider immunostaining to be a powerful technique in the elucidation of the contribution and distribution of epithelial cells in ERMs.

Apart from penetrating injuries, the clinical circumstances surrounding the development of ERMs suggest a limited number of sources from which epithelial cells could gain access to the retinal surface. Because of the well established association of retinal break formation, RPE cell dispersion into the vitreous cavity, and development of ERMs, it appears likely that most epithelial cells in ERMs would be of RPE cell origin. Furthermore, of all the intraocular epithelia 'in situ' in our study, RPE cells stained for keratins the most consistently (albeit weakly), and showed marked enhancement of immunofluorescent staining in tissue culture. For these reasons we believe that positively staining cells in our ERMs were derived from the RPE (though a ciliary or iris epithelial origin could not be entirely excluded).

SIGNIFICANCE OF RPE CELLS IN ERMS

RPE cells in ERMs may adopt the morphology of macrophages or fibroblasts, while others resemble normal RPE.²⁻⁴ In this study we identified oval cells, spindle-shaped cells, and cuboidal cells which were positively staining for epithelial markers. Moreover, the staining characteristics in embedded specimens and monolayers were broadly comparable for both prekeratin and keratin, so it is unlikely that the use of additional markers to cytokeratins would demonstrate a further complement of epithelial cells in the ERMs. For these reasons, and bearing in mind our careful use of controls and standards, we consider that cells staining negatively for prekeratin and keratin in the ERMs and monolayers were *not* of RPE origin.

For the first time, it has been possible *specifically* to

identify fibroblast-like cells of epithelial origin in ERMs. Indeed, in one embedded specimen the epithelial component (including fibroblast-like cells) was so prominent as apparently to confirm the 'RPE cell hypothesis' of ERM formation and contraction.²⁻⁵ However, in a majority of our ERMs (most of which were associated with rhegmatogenous retinal detachment) epithelial cells were either absent or formed only a minor component comprising cells scattered diffusely throughout the specimen. These positively staining but isolated cells might represent wandering macrophages derived from RPE cells² or RPE cells incidentally caught up within developing ERMs following dispersion from Bruch's membrane, showering throughout the retrohyaloid space, and settling on to the retina.

Most fibroblast-like cells in our ERMs were not of epithelial cell origin (as judged by negative staining for prekeratin and keratin). The origin of these non-staining fibroblasts remains obscure. We have previously shown that they are also unlikely to be of glial origin on the basis of anti-GFAP staining,¹⁵ neither are they derived from vascular endothelial cells as judged by negative staining with anti-factor VIII related antibody.¹⁴ Thus, while accepting that bio-microscopic pigmentation of an ERM strongly suggests an RPE cell component, and while conceding that many non-pigmented ERMs also have a RPE cell component, *we consider that the fibroblasts or fibroblast-like cells generally held to be responsible for ERM contraction are seldom of RPE origin.*

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