NON-VASCULAR VITREORETINOPATHY: THE CELLS AND THE CELLULAR BASIS OF CONTRACTION

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SUMMARY

Background: **We consider epiretinal membranes in terms of the two repair processes of gliosis and fibrosis and look at the cellular basis of contraction.**
Methods: **Pathological material removed at surgery was examined by a range of morphological procedures. Cultures of fibroblasts, retinal pigment epithelium cells and retinal glia were subjected to bioassays which relate to behavioural activities in scar formation.**
Results and conclusions: **Our findings highlight the importance of activities such as migration and adhesion in the formation of epiretinal membranes, and also show that these activities are central to our understanding of contraction.**

The name 'proliferative vitreoretinopathy' (PVR), introduced by the Retina Terminology Committee,[1,2] and the earlier appellation of 'massive periretinal proliferation' (MPP), introduced by Robert Machemer,[3] describes the complication of retinal detachment where essentially non-vascular scar-like tissue develops on the retina, contracts, and turns a simple detachment into a complex one. Surgical intervention may be necessary to relieve the traction and allow anatomical resettlement of the neural retina onto the retinal pigment epithelium (RPE). The formation of scar tissue, usually called an epiretinal membrane (ERM), may follow trauma to the posterior segment of the eye or episodes of intraocular inflammation. The scar tissue which complicates end-stage diabetic eye disease is considered part of the vascular vitreoretinopathy process and is dealt with in another paper in this issue,[4] but the distinction between vascular and non-vascular membranes is one of degree and is rarely, if ever, an absolute.

The term MPP and its later refinement to PVR, with its classification of disease development, has been crucial in placing the emphasis on the ERMs themselves in the generation of traction – in contrast to earlier terminology which highlighted the role of the vitreous in this process. We now know that contraction is a cell-mediated event[5,6] and is not produced by the shrinkage of collagens, as was once thought. ERM formation is a form of wound healing, and cellular behaviour in healing wounds can be broken down into a number of overlapping activities which include activation, signalling, adhesion, settlement, migration, proliferation, contraction, synthesis and remodelling. The terms MPP and PVR concentrate our attention on proliferation as being the key event in ERM formation and subsequent retinal traction. On the other hand, our concept of how developing scar tissue produces contraction is changing, and emphasis now has to be placed on other activities such as adhesion and migration. Indeed, in the early stages of ERM development the absolute amount of proliferation contributing to the increase in scar tissue bulk is relatively small.

FORMATION OF ERMs

ERMs may be relatively small in terms of scars elsewhere in the body, but their formation is complex and combines two repair processes at the one site. Preretinal membranes form at the interface of 'brain and body' and merge the repair processes of both systems to form a double scar. The gliotic repair of neural tissue (in the retina the major players are the Müller cells and the astrocytes) is characterised by activation, migration and proliferation taking place in the absence of substantial contraction.[7] Synthesis

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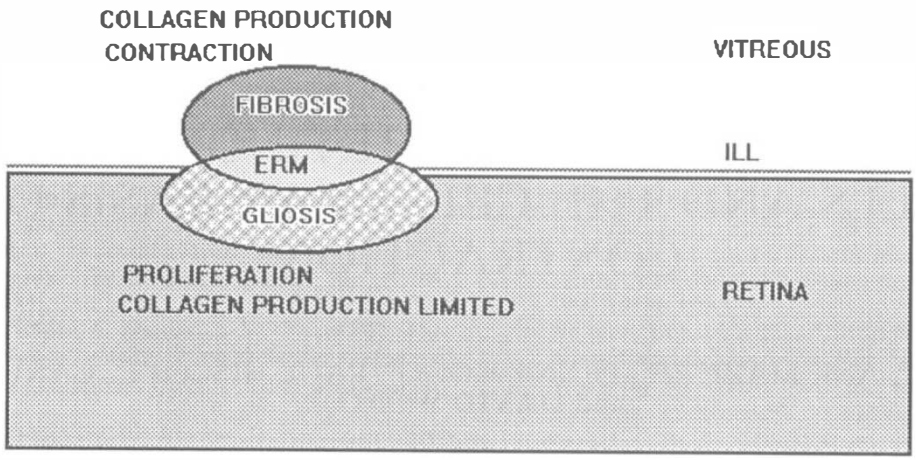

Fig. 1. *Epiretinal membrane (ERM) formation involves the coming together of two repair processes: gliosis and fibrosis. Fibrosis is characterised by strong contraction and abundant collagen production and in these it differs from gliosis. ILL, inner limiting lamina.*

of glycoproteins and glycosaminoglycans occurs, but collagen production is usually sparse[8] and remodelling virtually non-existent. On the other hand, avascular connective tissue repair (heralded by RPE behaving like fibroblasts[3,9] and the presence of true ocular fibroblasts[9]) is characterised by strong contraction and abundant collagen synthesis (Fig. 1). Thus gliosis and fibrosis go hand-in-hand, with considerable 'cross-talk' between the two processes serving to make the active phase of scar formation more protracted than is usual elsewhere.

GLIOSIS: SIMPLE ERMs

The precise stimulus necessary for the migration and proliferation of retinal glia to move out of the retina and onto the surface of the inner limiting lamina (ILL) is obscure. Among the key factors are detachment of the vitreous, hole formation in the ILL and inflammation. The presence of a defect in the ILL was thought at one time to be a prerequisite for the formation of a glial ERM but, in the rabbit at least, we have shown that glia can actively push their way through a previously intact ILL in response to inflammatory provocation (Fig. 2).[9–12] The presence of macrophages,[12,13] other inflammatory cells,[13] damaged cells and inflammatory mediators[12] in the vitreous seems to be important, but further work needs to be done to identify which specific signals are effective and which are not.

Certainly cytokines such as tumour necrosis factor alpha (TNFα) and interleukins 1 and 6 (IL-1, IL-6) are present in mature ERMs,[14] as are some of the more common growth factors[4,15] and bioactive substances such as fibronectin[16,17] and thrombospondin.[18,19] These cytokines, growth factors and glycoproteins are also present in the vitreal fluid of patients with established PVR,[4,19–22] but it remains to be determined which, if any, of these agents is active in the earliest stages of membrane formation. It was once held that the dominant cell in the glial outgrowth was the retinal astrocyte,[23] but simple glial ERMs form in species such as the rabbit which do not have an astrocyte population at the site of membrane formation.[10,11] Glial sheets form not only in a preretinal position on the ILL but also, after long-standing detachments, in a subretinal position. The glial component of subretinal ERMs is invariably of Müller cell origin. In addition the first cellular elements to protrude through gaps in the ILL, or to burst through the intact ILL, are Müller cell end feet[9–12] (Fig. 2). Experimental studies using models of PVR and retinal detachment in the owl monkey,[24] the cat[25] and the rabbit[10,11] highlight the incorporation of tritiated thymidine into the inner nuclear

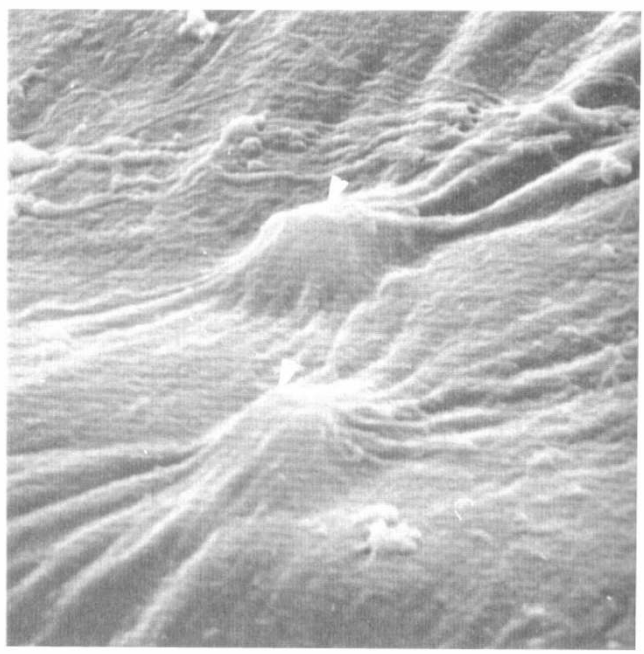

Fig. 2. *Scanning electron micrograph of the ILL in a rabbit with vitreal inflammation. The Müller cell end feet are pushed up to distort the ILL (arrowheads). (×2500)*

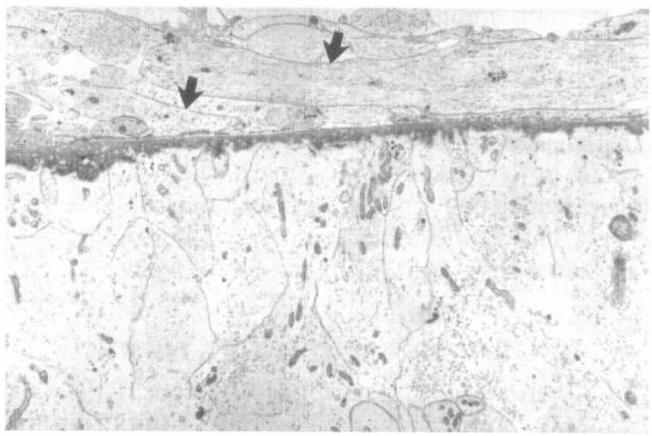

Fig. 3. *A transmission electron micrograph of a simple glial ERM on the ILL (arrows). (×6500)*

layer where Müller cells have their nuclei. It should also be said that the glial cells that characterise simple membranes (Fig. 3) have a plate-like phenotype, are remarkably similar to cultured retinal Müller cells[26,27] and look not at all like astrocytes in cell culture (which tend to vary from being fibroblastic to multi-process-bearing cells[28]).

It would seem that Müller cells are the dominant, or perhaps even the exclusive, glia of simple ERMs and contained within their domain are usually some inflammatory cells consisting of a few macrophages and lymphocytes. These simple ERMs develop in normal ageing as well as in diseased eyes, and Foos[29] identified simple membranes in about 20% of older eyes at post mortem. Clinically simple membranes are extremely difficult, if not impossible to see because the lack of associated collagen leaves them clear or at most translucent.

To say that this gliotic tissue is non-contractile is not entirely true, but by comparison with complex ERMs its contractility is marginal. Simple ERMs do produce areas of surface wrinkling when the retina is examined at high magnification,[30] but these are nothing like the full-thickness folds associated with complex ERMs. This feeble contractility was highlighted in experiments where glial cells were injected into the vitreous of rabbits. Simple membranes formed on the ILL in profusion but no retinal detachment was generated in 10 months of follow-up.[31]

FIBROSIS: COMPLEX ERMs

It is thought that complex ERMs are formed when migratory RPE and ocular fibroblasts bed down on and around the gliotic tissue of simple glial ERMs. The route taken by RPE to become part of an ERM has been carefully deduced and documented by Machemer from his studies of experimental retinal detachment in owl monkeys.[3,32,33] The RPE under a detachment eventually loses contact inhibition of division, by mechanisms we do not understand,[34] and begins to round up and divide. These proliferating cells form sheets which can develop into subretinal membranes with[35] or without[36] a major influx of glia from the detached and degenerating neural retina (see above). In addition, substantial numbers pass through a retinal hole to become what is clinically described as tobacco dust[33] in the vitreous and subhyaloid space. From here they settle onto the retinal surface and complex with the simple glial ERMs. Mobilised RPE cells do not need to use a retinal hole to gain access to the vitreal cavity and have been shown to migrate through the detached but degenerate neuroretinal tissues.[11]

RPE and glial involvement in complex ERMs is well established (Fig. 4) from both experimental investigations of animal models[10,11,24,32,33] and studies of surgically excised ERM material.[37–44] Immunohistochemistry, using antikeratin antibodies to identify RPE cells and antibodies to glial fibrillary acid protein (GFAP) to pick up both reactive Müller cells and astrocytes, is a particularly powerful tool for investigating the cell population of ERMs. Extreme variation exists between membranes, but quantitative analysis shows that, broadly speaking, glia make up about one-third of the non-inflammatory component in a complex ERM and RPE another third.[9,16] On the basis of appearance and location in the extracellular matrix of the ERMs, the remaining one-third is presumed to be fibroblasts (Fig. 4).[9,16] However, as there is no specific immunohistochemical stain for fibroblasts, their involvement in the genesis of the complex ERM remains speculative. Their origin in perforated eyes would most probably be the coats of the eye, but fibroblasts are equally prominent in the ERMs which form as complications of rhegmatogenous detachment where there is no obvious source. Metaplasia of RPE to produce the fibroblasts has been proposed[32,33] and dismissed.[9] Indeed we consider that the RPE cells do not undergo metaplasia (become fibroblasts) in PVR but merely adopt a fibroblastic appearance in

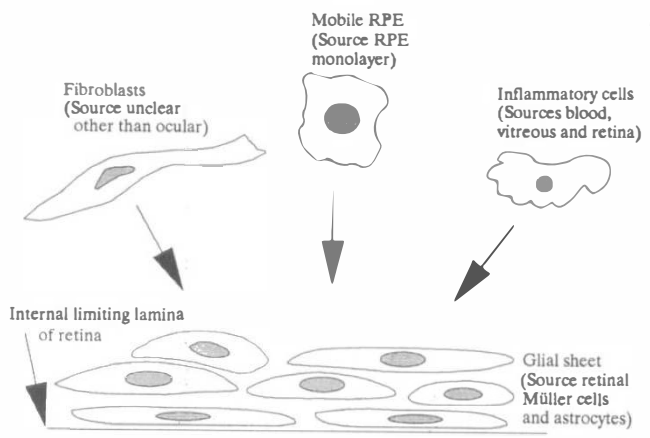

Fig. 4. *The components of a complex ERM which aggregate at the site of a glial sheet (simple ERM).*

response to the bioactive milieu, a change in behaviour and an altered substrate, i.e. they retain their essential epithelial nature (remain keratin positive).[34] Alternatively it is possible that the fibroblasts originate from the hyalocytes or a precursor located in the vitreous. That hyalocytes might be a source of fibroblasts in disease and injury has been discussed rather than studied,[45,46] but on balance hyalocytes have macrophage rather than fibroblast characteristics and one cannot differentiate into the other.[47] It has also been suggested that the fibroblasts might originate within the neural retina itself, perhaps existing as isolated fibrocytes around the retinal blood vessels.[9] That they have not been located at that site may be down to the cellular complexity of the perivascular area or simply that a fibrocyte population does not exist in the retina. The question of the origin of the fibroblasts in complex ERMs therefore remains problematical and a fertile area for future research.

The inflammatory cell component of ERMs has been highlighted in a number of studies. Macrophages are a known component of both simple and complex ERMs[12,16,33,38,39] (Fig. 4) and they have been found either in the majority[39] or all[16] the complex ERMs examined. The presence of lymphocytes has also been noted.[12] These were for the most part incidental findings in broader-based investigations, but specific studies to identify subtypes of inflammatory cell have also been undertaken. Limb's group,[48] using specific lymphocyte markers, concluded that ERMs were infiltrated by T rather than B cells and that the T cells were a mixture of $CD4^+$ and $CD8^+$. In a separate study at much the same time our own group with Charteris *et al.*[49] looked predominantly at the lymphocyte population and concluded that over 80% of complex ERMs contained T cells and that of these $CD4^+$ cells predominated. Expression of the interleukin-2 marker confirmed that a substantial proportion of the T cell population was activated. In this study B cells were not found and macrophage numbers were not impressive. However, the findings contrast with those of other studies[43,44] where, in agreement with earlier investigations,[12] macrophages were found in relative abundance. The discrepancy might be explained by interpretation of staining, sampling or perhaps the stage of ERM development, but it does underline the cellular variation that occurs in ERMs.

The absence[48,49] or near absence[44] of B lymphocytes and deposits of IgG, IgM and complement from ERMs confirms that humoral immune responses do not have a substantive role in the pathogenesis of PVR. However, the role of macrophages and activated T cells in ERM development still needs considerable study. Cytokine secretion by these cells would contribute to fibrosis in the ERMs and most ERMs stain immunohistochemically for the cytokine TNFα and some also for IL-1, IL-6 and interferon gamma (IFNα); these secreted cytokines bind to the extracellular matrix of the ERMs.[50] *In situ* hybridisation has confirmed that cells native to the ERMs express mRNA for these cytokines,[51] but it is not known which cell types are upregulated and it is fair to say that all the inflammatory and non-inflammatory cells of ERMs have the potential to be involved.

ADHESION AND MIGRATION

The mixture of glia, macrophages, T cells, RPE and fibroblasts can cause blindness because of the strong traction generated by this menacing concoction of cells when they form an ERM. Many questions arise about the pathobiology of ERMs, such as how these various cells manage to come together in the first place, how scar tissue contracts, and why complex ERMs contract and simple ones do not.

We attempted to address the question of how the cells come together to form the complex ERM by suggesting that a glial sheet (the simple ERM) was a preferential surface for cells such as RPE in particular, but also fibroblasts, to settle on. Thus the suggestion was that the key cells come together because of good adhesion to the surface of glia and poor adhesion elsewhere. Rather fancifully we have called this the 'Velcro' model and have tried to test the presumptions with bioassays (Fig. 5). Simple settlement assays were conducted which involved measuring the adhesion of cultured RPE and ocular fibroblasts to serum-coated tissue culture plastic and also to a sheet of cultured retinal glia simulating a simple ERM. Both visual analysis (Fig. 6) and scintillation counting of cells labelled with [^{14}C]adenine (see de Bono and Green[52] for technique) showed us that double the number of fibroblasts

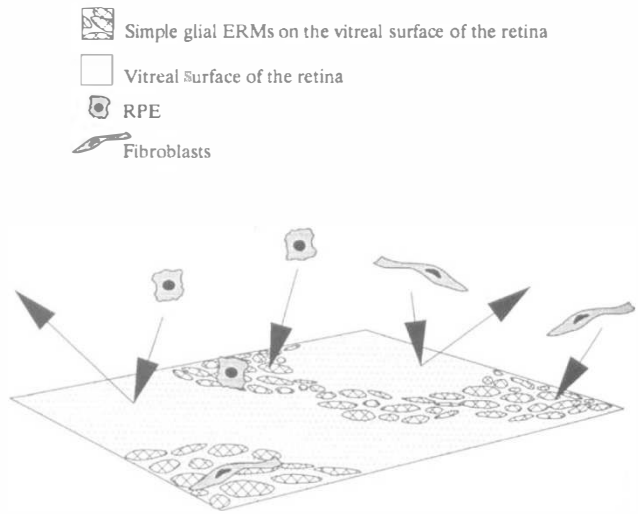

Fig. 5. *Velcro hypothesis, which proposes that RPE and fibroblasts adhere to and thrive better on simple glial ERMs than on the surface of the retina.*

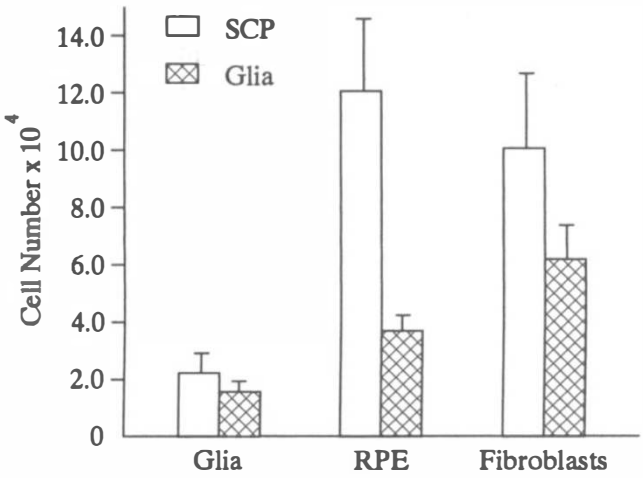

Fig. 6. *Histogram of settlement for 30 minutes, followed by washing, of [^{14}C]adenine-labelled glia, RPE and fibroblasts onto serum-coated plastic (SCP) or a glial monolayer. Scintillation counting gave an indirect measure of settlement and adhesion which could then be read off as numbers from an appropriate calibration curve. The glial monolayer can be seen to be a poor substrate* (n = *8; bar is SEM).*

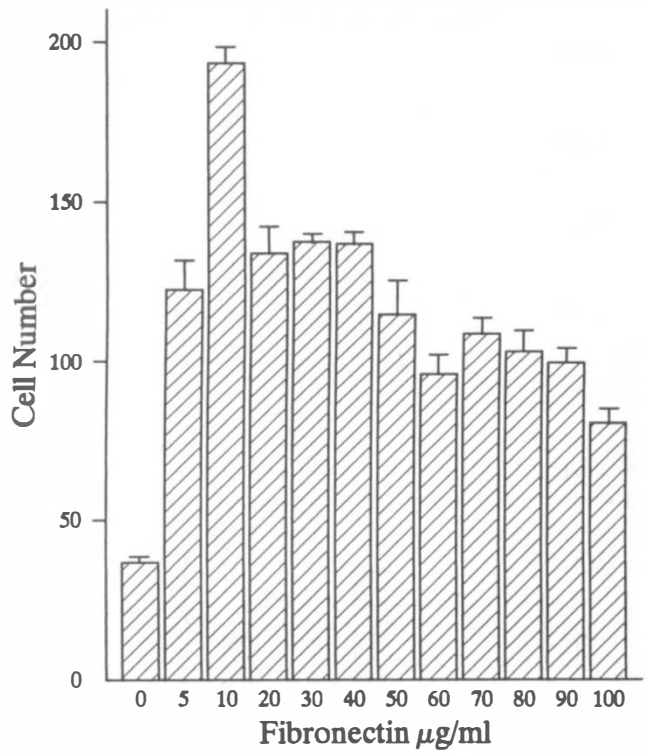

Fig. 7. *Cells grown from a segment of complex ERM (removed at surgery) were exposed to fibronectin in a 48-well microchemoattraction chamber. Fibronectin can be seen to be an effective migratory stimulant for these cells, with an optimum dose response at 10 μg/ml* (n = *4; bar is SEM).*

and 4 times the number of RPE cells settled on the plastic surface compared with the glial sheet. The Velcro idea did not seem to be holding up, and when the adhesiveness of a glial sheet was compared with that of ocular fibroblasts and RPE and found to be no different, we abandoned that line of thinking.

There may not be something physically special about the glial surface but our assay was weak in that it did not take into account the complex environment in which ERMs form. As we have said earlier, growth factors,[4,15] cytokines[14] and bioactive glycoproteins such as fibronectin[16] are crucial behavioural modulators which influence not only cell division but also other important events such as migration, synthesis and substrate adhesion. Fibronectin, for example, at concentrations as low as 5 μg/ml, doubles the settlement of RPE cells on plastic; fibroblasts are not so affected by 5 μg/ml but 10 μg/ml does increase their settlement by about 40%.

Migration *in vitro* can be measured in a variety of ways, but one quantitative procedure that we use involves the microchemoattraction chamber.[53,54] The chamber consists of two segments, each with the same number of microwells which come into register when the two segments are fitted together. Between the two segments is a gasket and a cell-permeable polycarbonate membrane. The substance to be tested fills the lower wells and a cell suspension is placed in the upper wells. The cells settle on the membrane and if they respond to the attractant they migrate through pores in the polycarbonate membrane to the lower side where they can be counted. Fibronectin is an effective attractant for RPE, fibroblasts, retinal glia and, for that matter, cells grown from segments of ERMs put up in culture (Fig. 7). For each cell type the optimum migratory response was evoked by between 10 and 15 μg/ml of fibronectin.

It is possible that the release of bioactive substances, such as fibronectin, during the genesis and development of ERMs attracts cells to the site of the scar. To test whether the cells involved in ERM formation do release substances which influence adhesion and migration we collected conditioned medium. For the conditioning process the cells were kept in serum-free medium for 48 hours,[55] checked for viability, and then the medium collected and stored at −70 °C until required. All the bioactive substances released by the cells are then, it is hoped, contained within the harvested medium. Initial tests have shown that our glial cultures release soluble factors that promote their own settlement and the settlement of RPE cells but have no effect on fibroblasts. In addition, migration of both glial cells and fibroblasts was enhanced by glial conditioned medium, but this time the RPE cells were only poorly responsive. Thus it would seem that glial cells release soluble factors that have the potential of acting remotely or locally to, on the one hand, enhance RPE adhesion and settlement and, on the other, provoke fibroblast migration. In addition to this we have been able to show that conditioned medium

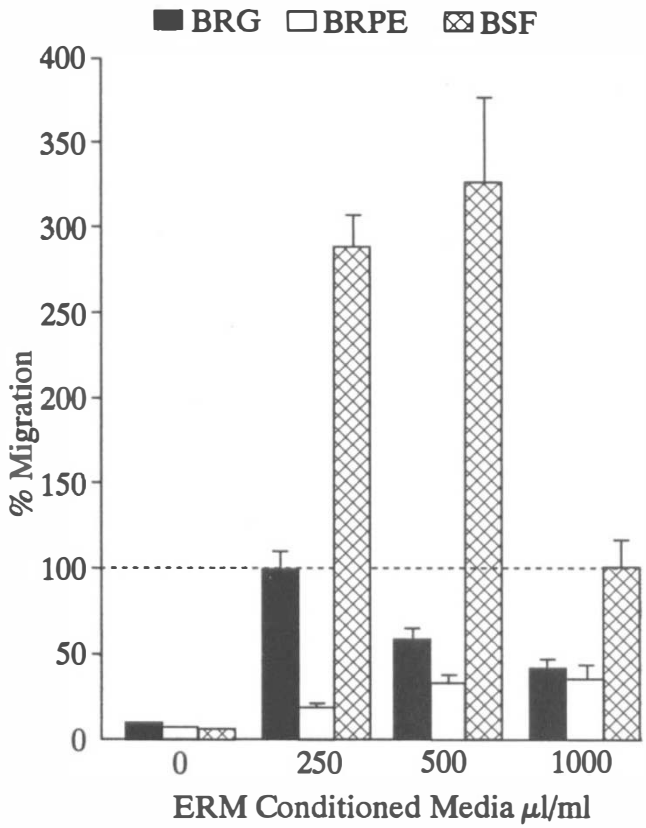

Fig. 8. *The response of bovine retinal glia (BRG), bovine retinal pigment epithelium (BRPE) and bovine scleral fibroblasts (BSF) in a microchemoattraction assay, to medium conditioned by cells cultured from ERM tissue. The migration of fibroblasts to ERM-conditioned medium is much greater than that of the other two cell types and is also greater than the response to an optimum dose of fibronectin (Fn; dashed line) (*n = *3; bar is SEM).*

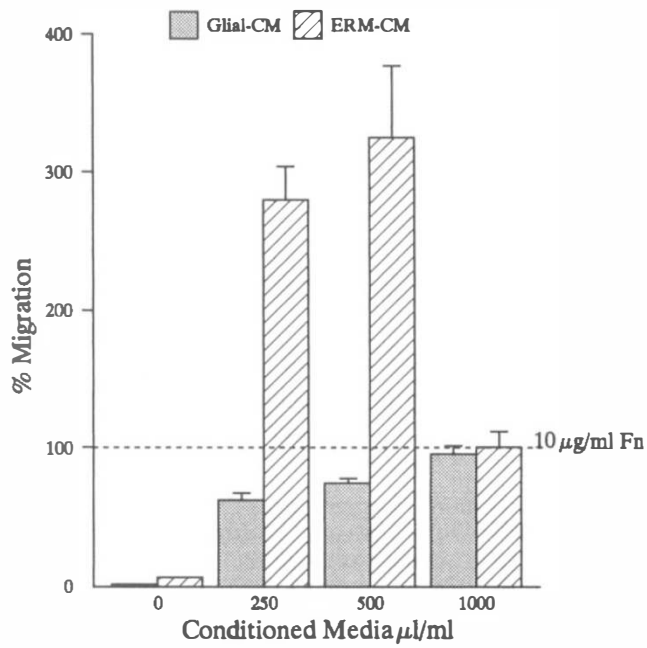

Fig. 9. *The response of bovine scleral fibroblasts to medium conditioned by retinal glia (glial-CM) and by cultured ERM cells (ERM-CM). The fibroblast's migratory response to glial-CM is comparable to optimum fibronectin (10 μg/ml Fn) (*n = *3; bar is SEM).*

from ERM cells grown up in culture contains stimulants that have little effect on RPE, some effect on glia, but have a massive effect on fibroblast migration (Fig. 8). Due to the difficulties of growing ERM cells in culture and further difficulties in obtaining suitable conditioned medium, we have not as yet looked for settlement effects, but this would be an interesting next step.

We do not know what the bioactive factors are in these various conditioned fluids and this work is currently in progress. Fibronectin was an obvious starting point for us, given its action on the target cells (see above) and on cells in other tissues.[56] We found by ELISA that the amount of fibronectin produced by the glial cells and ERM cells during the conditioning process was around 10–15 μg/ml. From our previous data this is a concentration which we know aids RPE settlement and fibroblast migration, but if fibronectin was the only factor involved then why were fibroblast settlement and RPE migration not stimulated? In addition, when we tested the optimum glial-conditioned medium settlement response against the optimal fibronectin response for RPE, the former was significantly higher ($p<0.01$, Student's *t*-test). This is also the case when fibroblast migration to ERM-conditioned medium is compared with the response to optimal fibronectin ($p<0.001$, Student's *t*-test). However, glial-conditioned medium only provokes a migratory stimulus similar to that produced by fibronectin (Fig. 9). Clearly fibronectin may well be a key player, but its interaction with other bioactive stimulators and inhibitors requires consideration and further evaluation.

CONTRACTION

To answer the question why complex ERMs contract and simple ones do not, we have to examine the whole basis of scar contraction and wound closure. Contraction is now known to be a cell-based, rather than a collagen-based activity as was thought.[5] How precisely the contraction is brought about remains controversial, but two theories hold sway. The first was introduced by Gabbiani *et al.*[5] and, in its most developed form, suggests that a subset of fibroblasts come together in the form of a sheet of attached cells and that this stationary aggregate acts like a muscle to bring about scar contraction and wound closure. These contractile fibroblasts were called myofibroblasts[5,57-59] and were distinguished by their elongated spindle shape, invaginated nucleus, the presence of gap junctions between adjacent cells, the formation of extracellular material called MAS (myofibroblast associated substance) and, most of all, by the presence of aggregations of actin microfilaments in their cytoplasm to form stress bundles.[57,58] The

myofibroblasts were thought of as a half-way house between true fibroblasts and smooth muscle cells and they were identified not only in granulation tissue[5] but also in tumours and even in normal tissue of various types.[59]

Cells with the characteristics of myofibroblasts have been identified in contractile ERMs removed at surgery for treatment of human disease[9,16,60,61] or produced experimentally in animal models.[62] However, it is our experience that their numbers are never abundant. ERMs have been removed and investigated for their overall contractile potential from both experimental[63] and pathological eyes.[60] In agreement with similar studies conducted by others on granulation tissue[64] the ERMs were shown to have intrinsic contractility. Some movement was produced in ERM tissue that was placed in an organ culture bath, fitted to a transducer and then exposed to agents which contract smooth muscle.[63] We were never able, in the conditions of our own laboratory, to reproduce this experimental finding (C. A. Hitchins and W. Unger, unpublished), but we did show that cells grown out of ERMs in culture contracted when they were exposed to MgATP.[65] Cytoskeletal staining for keratins demonstrated that a proportion of the contracting cells were RPE, but the others remained unclassified. In a separate study, cultured RPE contraction induced by MgATP was compared with that of fibroblasts and smooth muscle cells. All three cell types responded but the response of RPE and fibroblasts was less than that of aortic smooth muscle.[66] It is not surprising that cells from ERMs are actin rich[9,16,60] and can contract in the presence of ATP,[65] because this would seem to be a property of most cells – even those such as macrophages[67] and glia[27] that are tractionally incompetent *in vivo*. The actin in fibroblasts and RPE is non-muscle actin, similar to that in glia, but these cells do not express desmin and the analogy of their muscle-like properties does not bear too close a scrutiny.

The alternative proposal is that contraction has little to do with stationary fibroblasts (or RPE) with muscle-like characteristics forming a cellular unit which undergoes synchronised contraction. Instead contraction is the consequence of isolated migratory cells moving through a scaffold or on top of a substrate applying traction through adhesion between the cells and the extracellular matrix. The important factors, therefore, are cell movement on a scaffold, adhesion to the scaffold and pliability of the scaffold. The last simply means that wound closure requires a non-rigid substrate to move (clot or collagen); the more unyielding the scaffold the more cells are needed to produce a contraction. That is probably why no contraction takes place around subretinal membranes (rich in RPE) where attached to Bruch's membrane whereas the relatively few cells that form a preretinal ERM (also a site of RPE accumulation) have a devastating effect on the delicate neural retina.

The work of Harris and his group[68] in the early 1980s first illustrated how locomotion and adhesion worked together to bring about contraction through surface wrinkling. They put a viscous drop of high-centistoke silicone oil on a slide and polymerised the surface of the oil by rapidly passing it through a Bunsen flame. This created a skin over the liquid silicone (like the skin on a rice pudding) onto which cells could be seeded. Macrophages moved about the silicone without leaving a trace, but fibroblasts produced initially deep lateral folds in the silicone sheet.[68,69] Similar findings have been reported by Khaw *et al.*[70] in their studies of Tenon's capsule fibroblasts.

We repeated the Harris experiments using scleral fibroblasts, RPE and retinal glia and filmed the process by time-lapse photography. The retinal glia moved on the surface of the silicone but did not mark the surface in any way we could recognise. The fibroblasts and RPE settled on silicone skins and in less than an hour had spread out and had developed a distinctive frontal fan of cytoplasm with a ruffling membrane. The two cell types looked very similar as they moved but the RPE cells tended to have a larger fan than the fibroblasts. Movement was not very efficient on this delicate surface and lateral folds in the substrate were seen extending from each of the mobile cells. After 4 hours the cells had come together to form islands, between each of which were deep contraction groves in the silicone sheet. Sometimes the contraction was so strong that the polymerised silicone split and was dragged in towards the cellular foci, exposing the underlying liquid (Fig. 10). A very similar picture can be seen on the surface of the retina when ERMs begin to form. Under the electron microscope retinal folds can be seen between the foci of cells which make up the developing ERMs, that appear identical to the folds which were so distinctive in the silicone rubber. To explain this contraction process we like to use the analogy of a baby on a blanket. Imagine a baby on a blanket on a polished floor. As the baby starts to crawl, the blanket begins to wrinkle laterally (Fig. 11); the more the baby moves the more wrinkled the blanket becomes (migration); the more purchase the baby exerts the more the blanket will wrinkle (adhesion); and the more pliable the blanket the more it will wrinkle (substrate).[6] Thus our experiments indicate that the reason retinal glial cells are clinically innocuous has to do with their adhesion to the substrate: they are as mobile as fibroblasts and RPE cells but do not have the strong surface purchase to distort the surface they move over.

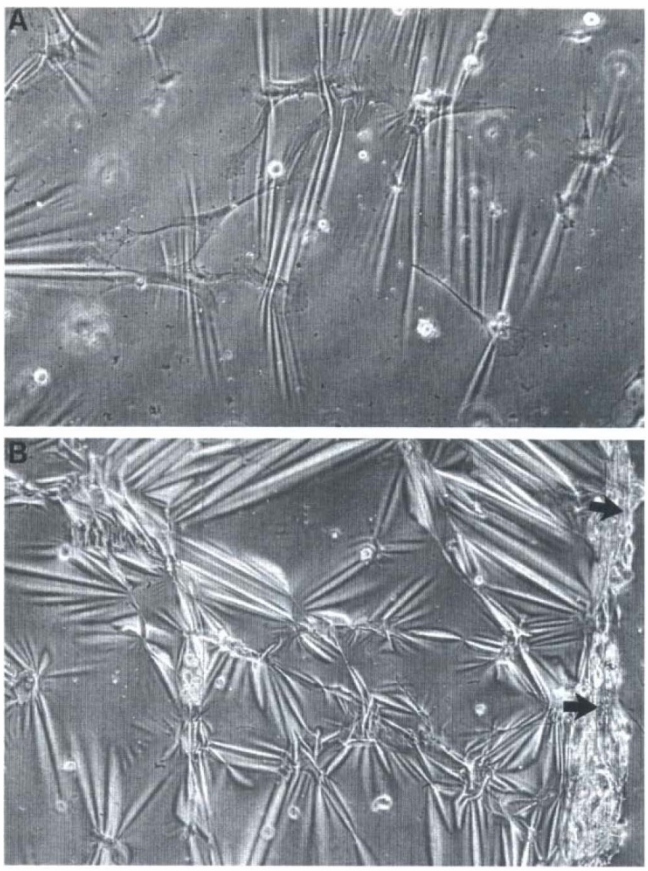

Fig. 10. *Phase-contrast micrographs of RPE cells on a thin silicone sheet 1 hour* (A) *and 6 hours* (B) *after settlement. (A) Mobile cells are spread out and have produced wrinkles in the sheet. (B) By 6 hours the wrinkles have become deep grooves connecting islands of cells. The traction is so fierce that the thin sheet has been split and is rolling like a blind (arrows). (×160)*

A test system used to investigate the strengths and weaknesses of the myofibroblast and migratory theories of scar contraction is the three-dimensional collagen matrix model. This simple model is established by taking collagen in solution (usually type I), seeding it with fibroblasts or another cell type, and then polymerising the collagen into a matrix by a change in pH. The matrix is then overlaid with medium and released from the sides of its dish. Over a period of a few days the matrix contracts down to a small fraction of its original size (Fig. 12). The rate of contraction is dependent on the collagen concentration and the number of cells seeded into the matrix.[71,72] The process is highly dependent on there being serum in the medium,[72] it does not seem to involve the action of enzymes on the collagen at a gross level[73] nor does it depend on cell proliferation.[71,74] Rather, what appears to happen is that the fibroblasts, which are initially rounded, spread out and then migrate to the surface of the collagen where they form a layer.[69,75,76] During their migration the collagen matrix becomes reorganised into bundles and previously trapped water is released into the medium.[69,76–78] This mechanism is similar to the rearrangement of extracellular matrix materials which can occur in the early stages of wound healing.[71,79]

Ehrlich[74,80] has examined the collagen matrix model in some detail to see whether the contraction is brought about by myofibroblasts acting in a coordinated manner as a multicellular unit (Gabbiani

Fig. 11. *A baby on a blanket on a slippery floor. The more the child struggles to move the more wrinkles are produced in the blanket.*

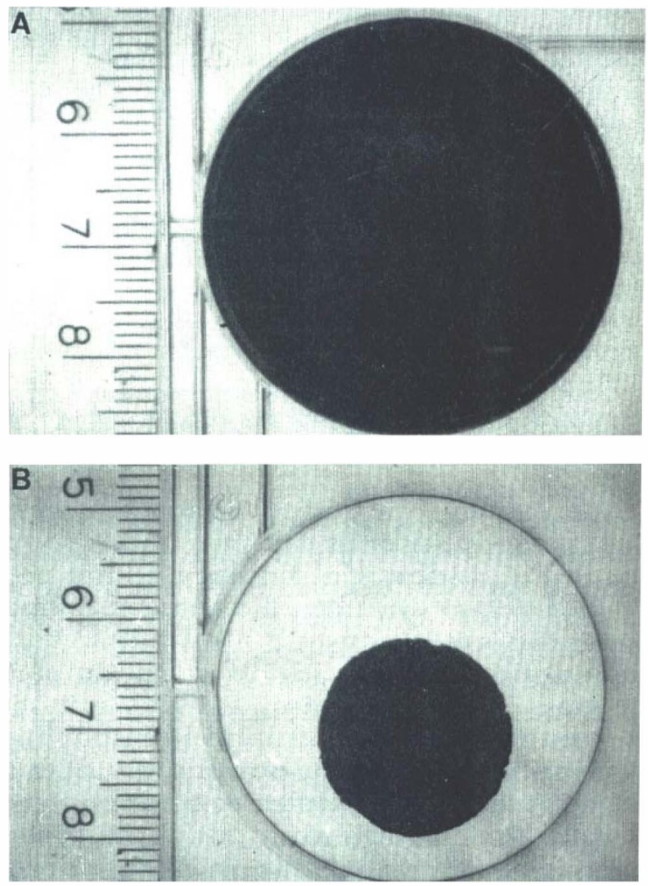

Fig. 12. *Macrophotographs of wells containing collagen seeded with fibroblasts. In (A) the collagen fills the well, but by 4 days (B) the collagen has contracted to a fraction of its original size.*

model[5]) or whether it is produced by isolated migratory cells exerting tractional forces on their immediate extracellular environment (the Harris model[68,69]). His experiments and observations have shown that myofibroblasts could be found in these matrices but that it is the migratory fibroblast which produces the contraction. Our own findings showed that there was a physical rearrangement of the collagen in association with migrating ocular fibroblasts and RPE which was not so evident around migrating glia. When 250 000 cells were seeded into each matrix then the fibroblasts reduced the collagen down to less than 20% of its original size in 7 days; the RPE's result of 50% was less dramatic and glia had no effect at all (Fig. 13). This was not because the glia did not spread and migrate; initially they did so as well as the other two cell types. It was our consideration that they failed to reorganise the collagen into bundles because they exerted less force on their surroundings as they moved.[76,79] This was not an all-or-nothing event because glial cells were capable of producing some contraction when their numbers in the collagen matrix were increased to 750 000, although the response was nothing like as good as that produced by the other two cell types. Surprisingly one other report[81] in the literature comparing glial contraction of collagen matrices with that of fibroblasts and RPE contradicts our findings by showing that glia are more effective than fibroblasts and RPE! We can only say that our experimental runs were repeated many times and, at low cell numbers, glia were never able to contract a collagen matrix while at high numbers their contraction was feeble compared with the other two cell types.

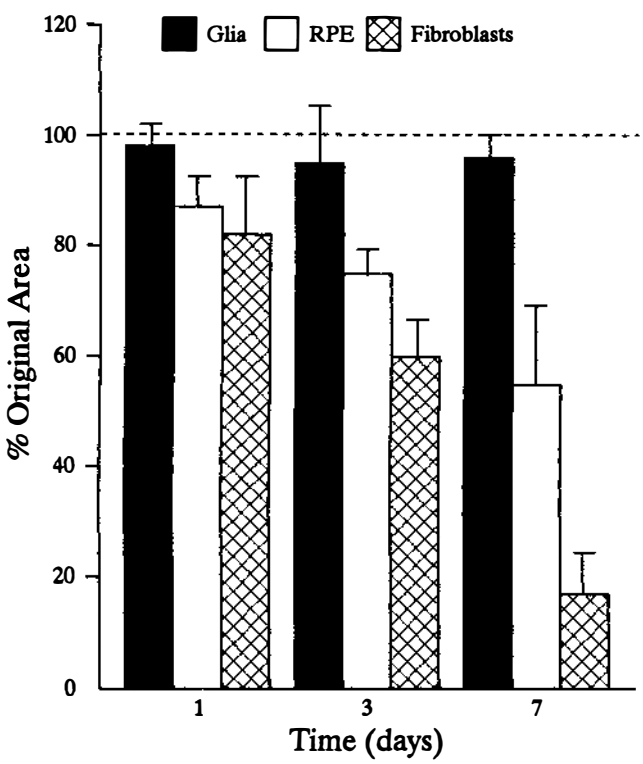

Fig. 13. *The effect of 250 000 glia, RPE and fibroblasts on collagen matrices. The fibroblasts contract the matrix down to less than 20% of its original size in 7 days, RPE cells reduce it to little more than 50% and glia have virtually no effect* (n = *4–15; bar is SD).*

Our experiments support the idea that isolated migratory cells produce most of the contraction of collagen matrices. A multicellular unit was evident only at the latter stages of contraction when the cells (fibroblasts and RPE) had migrated out of the contracted collagen matrix and formed a cellular skin around it. Electron microscopy showed that a myofibroblast phenotype was rare among the migrating cells within the matrix but that cells with the distinctive invaginated nucleus and aggregated bundles of actin microfilaments were more common in the cellular skin which formed around the reorganised collagen in the later stages of the assay.[79] Similar to the findings of others,[80] one of us showed that exposing the cell-populated matrices to ATP had no effect on contraction rate although it did cause the cells to be more rounded.[79] Immunohistochemical and time-lapse studies conducted by us on fibroblast injection into the rabbit vitreous *in vivo* showed that migratory fibroblasts were dominant when scar membranes were forming and retinal detachments were beginning to occur whereas myofibroblasts were latecomers on the scene.

It is becoming accepted by some[6,68,69,74,79,80,82] that the myofibroblast exists but is not central to the contraction process. If that is so, what role does it have? We have suggested[6] that myofibroblasts act as cellular 'staples' to hold the contracted tissue in place long enough for collagen to be synthesised in sufficient quantities to secure the scar. This would explain why myofibroblasts are seen late when contraction is peaking. Many cell types, when spread on the tissue culture dish or another firm substrate, express an elaborate arrangement of aggregated actin in their cytoplasm. These stress fibres are associated with stationary cells exerting isometric force on an immobile surface.[6] Stress fibres are a characteristic feature of myofibroblasts,[57–59,82] and we know that myofibroblasts are immobile cells. It is conceivable that they form not to produce isotonic contraction but to hold things steady by exerting isometric force at the end or near the end of the contraction process. In support of this idea Farsi and Aubin[83] found that if a collagen matrix was secured so it could not contract then the cells in it adopted a myofibroblast form with distinctive bundles of actin microfilaments in their cytoplasm, but that if the matrix was then released then the myofibroblast phenotype was lost.

Fig. 14. *Weaver ants making their leaf nest. The ants which scurry about on the surface of the leaf (mobile fibroblasts; arrows) bind the leaf over (isotonic force), whereas the ants which line up grasping the leaf with their mandibles and legs (myofibroblasts; arrowheads) hold the leaf in place (isometric force).*

We like to compare the role of mobile fibroblasts and myofibroblasts to the activities of weaver ants when they make a leaf nest (Fig. 14). These ants scurry around the chosen leaf and start to bend it over (the isotonic action of the migratory fibroblasts); when it is folded over sufficiently other ants hold the strain by grasping one edge of the leaf with their back feet and the other edge with their mandibles (the isometric force generated by myofibroblasts). The ants holding the leaf in position are gradually incarcerated into a silk mesh which acts as the final seal for the nest. The temporary seal provided by myofibroblasts is also replaced by a more permanent collagenous mesh when a scar becomes fixed. With time the fixed scar has more and more collagen and fewer fibroblasts of either type.

The term 'tractional structuring' was introduced by Stopak and Harris[84] to explain the process by which scar cells *in vivo* remodel extracellular fibres to form connective tissue bundles. It also helps to explain what is happening to flat surfaces when they go into folds and pucker (silicone sheet or neural retina). In general, a migrating cell adheres to its substrate at various points on the surface and exerts a contractile force or traction on each of these adhesions. The cell initially pulls in all directions, but it is the area of strongest adhesion that resolves the direction of locomotion.[85] If the substrate is weak it will be drawn in; if it is rigid the cell will move forward easily.

In the collagen matrix the cells which contracted the collagen (fibroblasts and RPE) exerted a pulling force on the surrounding tissue, dragging in the thin collagen fibrils which were unable to resist this force. As the collagen fibrils were reeled in (Glaser *et al.*[86] have calculated that collagen strands can be dragged at a rate of 2.5 μm/min by RPE cells) they clumped together to form bundles. The bundles enlarged by this tug-of-war process until the tensile strength of the aggregated collagen was sufficient to allow the cell to move forward.[87] Cells behind would tend to follow the same route as the lead cell because of contact guidance (a directed response to axial cues) and in response to chemoattractants synthesised by the lead cell(s), so reinforcing collagen bundle formation. Our glial cells could not produce the same effect despite having a well-developed cytoplasmic actin microfilament system and, in the early stages, being able to move through the matrix as quickly and as well as the other two cell types. It would seem that within the hypothesis of tractional structuring that they can move forward on collagen matrices without much bundle production and on flat surfaces without need for much wrinkling.

FINAL COMMENTS

The ERM is a fascinating scar system which combines two types of repair: gliosis and fibrosis. Gliosis seems to dominate early membrane development and is associated with minimal contraction, whereas fibrosis brings with it strong traction on the retina. Proliferation of the various cell types involved is taken as the main characteristic of the condition and, therefore, it is not surprising that research to find adjuncts or alternatives to PVR surgery has been dominated by antiproliferative agents.[6,88] Division of cells that would otherwise be non-dividing is an important feature of PVR, and justifies the name, but it should not be forgotten that the key pathological event is cell-mediated contraction. There is *not* a major increase in tissue bulk during ERM growth and because of the delicate nature of the neural retina relatively small foci of scar tissue development can have dramatic distortional effects. Thus it is quite possible to stop proliferation but not inhibit contraction and the subsequent loss of vision.

Migration of the key cells and their aggregation at the site of ERM formation are fundamental events which also need to be modulated if medical treatment of PVR is ever to be effective. This is all the more important now that we know migration and adhesion to a distortable substrate are how fibroblasts and their like bring about contraction and wound closure.[6,69,74.] Future research may concentrate on the adhesion between fibroblast/RPE and substrate to find out what is crucial to the 'reeling in'

Table I. Collagen matrix contraction (250 000 cells per gel)

	Fibroblasts	RPE	Glia
Contraction	Yes	Yes	No
Fibronectin	Yes	Yes	Yes
Thrombospondin	Yes	Yes	No
Vitronectin	Yes	Yes	No

The cells which contract collagen matrices (fibroblasts and RPE) are positive for fibronectin, thrombospondin and vitronectin, whereas glia, which do not contract matrices at this concentration of cells, are positive for fibronectin (immunohistochemistry).

process. Which glycoproteins are needed and which are incidental? Of the integrin receptors involved in cell attachment and migration, which are bystanders and which, if blocked, would impede migration and contraction? To date fibronectin, thrombospondin and vitronectin seem to be elaborated around the mobile cells in collagen matrices and in some cases their migratory pathways (i.e. the forming collagen bundles)[79] (Table I). Whether or not fibronectin is essential for contraction is controversial, with some evidence for[79,89,90] and some against.[91,92] This problem remains to be resolved and information on the effect of other glycoproteins is entirely lacking.

It is now known from studies of chick[93] and human[94] RPE that these cells express β_1 integrins in tissue culture. Despite some evidence to the contrary[95] integrins also seem to be present *in vivo*.[96] Antibody blockade of β_1 integrin affects cell-substrate adhesion[94] in RPE cells. Studies of ERMs show the presence of both the β_1 family and $\alpha_v\beta_3$ integrins, and the authors suggest that fibronectin–cell interactions (β_1) are important in the early stages while laminin–cell interactions (β_1 and β_3) modulate adhesion later in the life of the scar.[97] Immunocytochemical staining shows the β_1 family of integrins but not β_3 are associated with human RPE cells after they are seeded into collagen matrices.[79] Much work still needs to be done and little is yet known about integrin expression in three-dimensional matrices of the other cell types involved in ERM formation, although non-ocular fibroblasts at least, like RPE, also express β_1.[98]

Work on medical or biological agents directed against adhesion and migration of cells is lacking in PVR research. However, it is encouraging that monoclonal antibodies raised against the classical collagen type I receptor, $\alpha_2\beta_1$ integrin, seem able to inhibit collagen matrix contraction,[99] presumably by an effect on adhesion that in turn causes failure of tractional structuring. However, this remains to be determined. More wide-ranging antibodies raised against the β_1 family also do the same thing,[98] and a synthetic peptide (RGDS) derived from the fibronectin binding domain inhibits the attachment of RPE to collagen types I and II.[100] Treatments directed against adhesion and migration offer important research avenues for the future treatment of PVR.

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Key words: PVR, ERMs, glia, retinal pigment epithelium, wound healing, retinal detachment.

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