The chemoattractant activity of the vitreous to human scleral fibroblasts following retinal detachment and proliferative vitreoretinopathy

N Wilson-Holt, P Khaw, F Savage, I Grierson

Abstract

The results are presented of a migration assay of scleral fibroblasts to 25% diluted vitreous samples from 27 patients with complex retinal detachments divided into three groups: group 1, no proliferative vitreoretinopathy (PVR); group 2, early PVR; and group 3, advanced PVR. There was a statistically significantly greater migration with vitreous samples from group 3 than with group 1 (p<0.0001) but no significant correlation for migration with the age of patients, duration of retinal detachment, or number of retinal procedures undertaken. Analysis of vitreous by gel electrophoresis showed that cellular migration was proportional to the number of peptide bands. Vitreous fibronectin levels were measured and there was a positive correlation between fibronectin in the vitreous samples and the migration of fibroblasts to the vitreous (r=0.68, p<0.004).

Proliferative vitreoretinopathy (PVR) is a major cause of blindness, and is a common sequel to long standing rhegmatogenous retinal detachment surgery.1,2 A critical component of the pathogenesis of PVR is the migration of cells that participate in the scarring process. Retinal pigment epithelial (RPE) cells have been implicated in this process as have macrophages and glial cells.3,4 Fibroblasts are also present in the epiretinal membranes of proliferative vitreoretinopathy and may also have an important role in the evolution of PVR.5,6

It has been shown that the various cell types can produce a variety of extracellular matrix components such as collagen and fibronectin which contribute to the structure of scar tissue.7,8 In addition epiretinal membrane cells and in particular RPE cells produce and possibly secrete into the surrounding media agents which stimulate the migration and proliferation of other cell types.9,10,11

<table>
<thead>
<tr>
<th>Table 1 Clinical data for each of groups 1, 2, and 3</th>
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<tr>
<td>Mean</td>
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<tr>
<td>Group 1 (n=12) no PVR</td>
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<tr>
<td>Age</td>
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<td>No of operations</td>
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<td>Duration of detachment</td>
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<td>Group 2 (n=3) early PVR</td>
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<td>Age</td>
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<td>No of operations</td>
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<td>Duration of detachment</td>
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<tr>
<td>Group 3 (n=12) advanced PVR</td>
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<td>Age</td>
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<tr>
<td>No of operations</td>
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<td>Duration of detachment</td>
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Figure 1 A pie chart of the aetiology of retinal detachment in group 1 patients.

The aim of the present study was to investigate the chemoattractant qualities of vitreous obtained from eyes with various types of complex retinal detachments and to determine whether this activity varied with clinical parameters. The target cells were cultured human scleral fibroblasts.

Materials and methods

Vitreous aspirates (0-5–1-0 ml) were obtained from 27 eyes in 27 patients undergoing vitrectomy for complex retinal detachments. These eyes, none of which had previously undergone vitrectomy, could be divided into three groups (Table 1): group 1 (12 eyes) no PVR (Fig 1); group 2 (three eyes) early PVR defined as vitreous haze and mild retinal wrinkling; group 3 (12 eyes) advanced PVR defined as fixed retinal folding involving one or more quadrants of the retina.

In all cases information about the age of the patient, the duration of retinal detachment, and the number of previous ‘conventional’ non-vitreoretinal detachment procedures was obtained at the time of surgery. The assays for migration and fibronectin were however performed without knowledge of this information.

PREPARATION OF VITREOUS

Aspirates of vitreous gel were obtained via the pars plana using a 1 ml syringe attached to the vitreous cutter by silicone rubber tubing. All samples of vitreous gel were obtained prior to the commencement of the pars plana infusion to prevent contamination by irrigation fluid and in no case was there contamination by admixed blood. A minimum volume of vitreous of at least 0-5 ml was obtained from all eyes.
Immediately after being obtained vitreous samples were stored at −70°C. Before use the vitreous was thawed, homogenised, and filter sterilised through a 0.22-μm filter (Millipore, USA).

**CELL CULTURE**

Human scleral fibroblasts (HSF) were grown from primary explants obtained from a single postmortem human eye (male aged 36 years) and with no known ophthalmic disease. They were grown in 25 cm² flasks (Sterilin UK) in minimal essential media (MEM) (Gibco UK) supplemented with 10% newborn calf serum (NCS, Gibco, UK) in 5% CO₂ at 37°C. They were passaged in a 1:3 ratio when they reached confluence.

**MIGRATION ASSAY**

HSF from the third to fifth passage were used in the migration assay. Forty-eight microwell chambers (Neuro Probe, Cabin John, MD, USA) were used with polycarbonate membranes coated with gelatin with pores 8 μm in diameter (Nucleopore, Pleasanton, CA, USA). Confluent HSF were detached from their culture flasks by trypsin 0.25%/EDTA 0.02%. The enzymatic treatment was terminated by the addition of MEM containing 10% NCS. The cells were washed in serum-free MEM and counted in a Coulter counter. A volume of 50 μl of serum-free MEM containing 40 000 HSF was added to the upper chambers of the wells. Vitreous samples were diluted in serum-free MEM to produce a 25% solution, the concentration found from dose-response studies to produce the optimum chemotactic effect. Twenty-five μl aliquots of the diluted vitreous were added to the lower chambers of the 48-well apparatus and the lower chambers covered with the polycarbonate membrane.

The whole apparatus was incubated in 5% CO₂ at 37°C for 5 hours following which the membrane was removed from the chamber, placed in ethanol for 15 seconds, and air dried. Membranes were stained with haematoxylin for 30 minutes, washed in cold water and mounted on a glass slide. Migrated cells on the undersurface of the membrane were counted in 20 high power fields (×1000) per well. Each assay was carried out in triplicate and a mean value for migration obtained.

**ELECTROPHORESIS**

Samples of vitreous (1 μl) were analysed by SDS-PAGE electrophoresis. Samples were run under reducing and non-reducing conditions (with or without mercaptoethanol in the sample buffer). In addition native PAGE of vitreous samples was performed. Gels were stained with Coomassie blue dye.

**FIBRONECTIN ASSAY**

Levels of fibronectin in the vitreous in 13 samples were measured using an enzyme linked immunosorbent assay (ELISA). Ninety-six well microtitre plates were coated overnight with 100 μl of rabbit anti-fibronectin antibody (Dakopatts, UK) diluted 1:1000 in phosphate buffered saline (PBS). The plates were then washed three times with PBS/Tween and coated with 200 μl of 3% bovine albumin in PBS at 37°C for 1 hour to prevent non-specific binding. The plates were then washed with PBS/Tween and 20 μl of vitreous sample were placed in three wells and made up to 100 μl with PBS/Tween. The vitreous samples were diluted 1 in 5 so that the evaluations fell within range of the exponential part of the standard curve. Standards were made up from human fibronectin (Sigma, Poole, UK), and run in the same experiment.

The samples were incubated at room temperature for 2 hours after which the plates were washed with PBS/Tween and 100 μl of rabbit antifibronectin conjugated to peroxidase (Dakopatts, UK) 1:500 with PBS/Tween was added to each well and incubated for 1 hour at room temperature. The wells were washed with PBS/Tween and a colour reagent (tetra-methyl benzidine HCl) citric acid and 5 μl of H₂O₂ were added. The colour reaction was stopped with
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GEL ELECTROPHORESIS

Analysis of vitreous samples by gel electrophoresis from eyes with no PVR showed a smaller number of peptide bands compared with eyes with advanced PVR, and the cellular migration to vitreous samples was proportional to the number of peptide bands. However no particular band distinguished the vitreous samples which stimulated more cellular migration.

FIBRONECTIN ASSAY

There was a positive correlation between the measured fibronectin levels in vitreous aspirates and the migration of fibroblasts to the vitreous (r=0.68, p<0.004) (Fig 6) with a mean of 12.96 μg/ml and standard deviation of 6.29 μg/ml (range 6.4 μg/ml to 24.75 μg/ml).

Discussion

This study demonstrated that vitreous aspirates from eyes with advanced PVR stimulate fibroblast migration to a greater extent than vitreous aspirates from eyes with no PVR. This is due to the presence of chemoattractants in the aspirates from the eyes with PVR. From the evidence that fibroblasts may play a role in PVR, the study of fibroblast migration in vitro may have clinical relevance with respect to PVR.

Clinically the development of PVR is encouraged by the presence of large areas of exposed retinal pigment epithelium, intraocular blood, or inflammatory exudate, conditions which were actually present in 11 of the 12 patients in group 1 (that is large or giant retinal tears and intraocular foreign bodies). The widespread scatter of results in group 1 may be related to the diverse nature of the retinal detachments occurring in this group. However the fact that the chemoattractant properties of the vitreous aspirates from the ‘non-PVR’ eyes were generally lower than the established PVR eyes suggests that fibroblast migration may play a role later in the pathogenesis of PVR than other cell types (for example RPE cells, macrophages) and that fibroblasts could be recruited by the presence of such cells; this is an area of future research. This relatively late recruitment of fibroblasts akin to the situation in wound healing may also explain the apparent lack of a relationship between migration and both duration of retinal detachment and the number of surgical procedures. Furthermore although the number of patients was small the relatively low level of migratory activity observed in group 2 (early PVR) might also be explained by this hypothesis.

More difficult to explain however is the absence of a relationship between patient age and the migration of fibroblasts, since there is experimental evidence from work on other tissues that healing processes occur more vigorously in youth. This may be related to the complex nature of epiretinal membrane formation compared with scar development elsewhere.

Previous studies have shown increased concentrations of fibronectin in vitreous aspirates of eyes with PVR compared with those with retinal detachments without PVR. Fibronectin is produced by fibroblasts and one would predict

STATISTICS

All data from the migration assays were expressed as the mean with the standard error of the mean. The statistical significance of the vitreous-related changes was examined by t tests and one way analysis of variance (Minitab Software, Minitab Corp, USA).

Results

MIGRATION ASSAY

There was a wide variability in the fibroblast migration to vitreous aspirates with as much as a ten-fold difference in results. However the vitreous aspirates from group 3 patients (advanced PVR) stimulated fibroblast migration to a much greater degree than vitreous aspirates from group 1 (no PVR) although wide scatter in migration results was observed in the latter group. Vitreous aspirates from group 3 stimulated a mean of 44.7 (SD 15) fibroblasts to migrate whereas group 1 stimulated 14.3 (SD 15) fibroblasts to migrate. This difference was statistically significant p<0.001 (Fig 2).

There appeared to be less migration induced by the vitreous aspirates in group 2 (early PVR) compared with group 3 (advanced PVR) but the numbers of samples in the former group were too small for statistical analysis.

We also correlated fibroblast migration against the age of the patients, the duration of retinal detachment, and the number of previous retinal detachment surgical procedures. In all three cases there appeared to be no close relationship (Figs 3, 4, 5).

Figure 5: A scattergram showing the number of cells migrated to each vitreous against the number of retinal detachment operations.

molar H2SO4 and the absorbance measured on an automated ELISA reader.

Figure 6: A scattergram showing the number of migrated cells against the fibronectin levels in the vitreous samples.
that it would be present in highest concentrations in the vitreous of eyes with advanced PVR where fibroblasts (or RPE cells with fibroblast-like characteristics) are present. Of interest are the two high results for migration to vitreous observed in group 1 which occurred in the two eyes which had sustained penetrating injuries with intraocular foreign bodies. One can speculate that fibroblasts may well have entered the vitreous at the site of perforation.

Fibronectin is also known to be chemotactic for fibroblasts as well as other cell groups, and our observation of a strong positive correlation with fibroblast migration would accord with this.19 19 20 In addition there is the evidence of inhibition of experimental PVR by a synthetic peptide derived from the cell-binding domain of fibronectin.21 This peptide appears to prevent the attachment of RPE cells to vitreous and internal limiting membrane.

However the absence of any single band on gel electrophoresis which could be related to increased migration suggests that no single chemotactic factor including fibronectin is responsible. Other putative chemotactic agents for fibroblasts include the peptide factors, platelet derived growth factor, and transforming growth factor beta22 27 but there are likely to be others.

Fibroblast chemotaxis is probably a later event in the pathogenesis of PVR, a condition which when advanced can be difficult to treat. Further understanding of the early events in the pathogenesis of PVR before fibroblast migration has occurred may provide a means of therapeutic intervention.

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