Cellular Proliferation in the Vitreous: The Use of Vitreous Explants as a Model System

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An in vitro model of cellular proliferation in the vitreous has been developed using explants of bovine vitreous gel. Various cell types, including chick embryo pigmented retinal epithelium, choroidal fibroblasts, retinal glial cells, bovine retinal capillary endothelial cells, and peritoneal mouse macrophages, were cultured at 37°C on vitreous gels for periods up to 8 days and compared for their effects on the structure of the gel. Choroidal fibroblasts caused a very marked reduction in the size of the gel and produced the strongest traction on the gel fibril structure; in contrast, peritoneal macrophages caused virtually no reduction in vitreous volume and little or no visible traction on the gel as detected by phase-contrast microscopy. The remaining cell types usually formed sheets on the surface of the gel; this was associated with an initial reduction in vitreous volume of approximately 50%, but little change thereafter. The cell mediated traction events on the structure of the vitreous gel were followed by time lapse video microscopy and by electron microscopy at various times after seeding of cells on the gels. The appearances corresponded closely with reported clinico-pathological studies of cellular proliferation in the vitreous. We believe that this in vitro model has several advantages over in vivo models of cellular proliferation in the vitreous, in that it permits analysis of individual cell behaviour and it is eminently suitable for pharmacologic manipulation.


Cellular proliferation with retino-vitreal traction is a major complication of retinal reattachment surgery, occurring in 5–10% of cases.1 The cause of the traction effects on the vitreous is unknown, but there is strong evidence that it is a cell-mediated event.2 Several cell types have been observed in cellular proliferation in the vitreous, including glial cells, pigmented retinal epithelial cells, macrophages, and fibroblasts. In human pathological specimens, all of these cells have been isolated.3 It has been suggested that the vitreous gel provides a scaffold for cell movement and traction in this condition.4 Intraocular proliferations occur in other disease states, such as diabetic retinopathy, and following penetrating injury of the posterior segment, especially if this is associated with vitreous haemorrhage.5

Several in vivo models of cellular proliferation in the vitreous have been developed (see Discussion), which involve either the injection of cultured cells into the vitreous gel, or the infliction of a standardised penetrating injury with vitreous bleeding. The disadvantages of these methods are that they rely on clinical scoring methods, which are subject to considerable error and are barely quantitative; that as in vivo models, they involve several other variables which cannot be controlled easily (e.g., secondary inflammation); and direct observation of cell behaviour is difficult.

An alternative approach to the investigation of cellular proliferation in the vitreous is the use of an in vitro model, such as vitreous explants; under these conditions, cells can be directly visualised, and their effects on the gel compared. In addition, the system can be manipulated with the addition of pharmacological agents in an attempt to modify the behaviour of the cells on the gel.

Materials and Methods

Fresh bovine eyes were transported from the slaughterhouse to the laboratory on ice and used within 4 hr of death. Under sterile conditions, a circumferential incision was made in the eye so that the anterior segment (cornea and lens) with attached vitreous gel could be removed. Intact vitreous gel was either dissected from, or was allowed to detach under gravity from, the posterior lens surface. The gels were dialysed for at least 24 hr against culture medium (usually Minimum Essential Medium containing penicillin and streptomycin). The gel retained its mechanical integrity during this stage. In initial experiments, vitreous explants were sterilised by irradiation with UV light, but this was

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Submitted for publication: October 5, 1984.

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found to be unnecessary and was omitted in most experiments.

Cells

Five cell types were used in this study: retinal pigment epithelium, choroidal fibroblasts, and retinal glial cells were from chick embryos; bovine retinal capillary endothelium were from adult bovine eyes; and peritoneal macrophages were from mice. These cell types were chosen to provide a wide range of cell types which were known to grow easily in standard tissue culture systems. Retinal pigment epithelium, choroidal fibroblasts, and retinal glial cells were isolated from 11-day-old White Leghorn chick eyes by the methods of Bultjens and Edwards, and grown to confluence in plastic tissue culture flasks in MEM + 10% FCS. Previous studies have shown that these methods yield pure culture of each cell type.

Bovine retinal capillary endothelium were isolated by the method of Bowman et al. and briefly was as follows: retinas were dissected from eye-cups from which the vitreous gels had been removed, gently homogenised in MEM, and passed through a 75 μm nylon mesh. Capillary networks were recovered from the filter and incubated for 4 hr at 37°C in MEM + collagenase/dispase (1.0 mg ml⁻¹: Worthington Diagnostics; Bedford, U.K.). Proteolysis was stopped with the addition of FCS and the sample layered onto a preformed gradient of 50% Percoll in MEM + 10% FCS (Pharmacia, London, UK) and centrifuged for 10 min at 1000 g. A single band of cells above the midpoint of the gradient was found to contain small clumps of cells which were cultured in fibronectin-coated plastic bottles in MEM + 10% platelet deprived serum (PDS). Confluent cultures of cells with “cobblestone” morphology were established by 2 weeks. A more detailed account of this procedure is the subject of a report in preparation.

Mouse (peritoneal) macrophages (MM) were obtained by lavage of the peritoneal cavity with 1.0 ml of HBSS containing 0.1 μ ml⁻¹ heparin. Activated macrophages were similarly obtained from mice which had been injected intraperitoneally with 1 ml horse serum, 4 days prior to cell collection. Macrophages were cultured directly on vitreous explants (see below).

Cell Culture on Vitreous Explants

Pieces of dialysed vitreous were placed in 35 mm diameter plastic petri dishes or 16 mm diameter wells (Costar Ltd.; Northumberland, U.K.) plates, and single-cell suspensions at various concentrations were seeded onto the surface of the gels. Cell suspensions were prepared from confluent cultures by treatment with calcium and magnesium-free Eagles' medium containing EDTA, followed by brief trypsinisation, as described previously. Mouse macrophages, however, were used without prior culture. Cells were placed on the slightly convex surface of the vitreous gel in a small volume (100 μl) of medium and allowed to adhere for 15 min before the addition of further medium. Under these conditions, cell adhesion occurred rapidly, and it was difficult to dislodge the cells from the vitreous gel. The cells were incubated at 37°C in a humidified atmosphere and photographed at intervals using an inverted microscope with phase contrast optics (Leitz Ltd.; Luton, U.K.).

Macroscopic effects of cell growth on vitreous gels were observed by seeding cells onto whole intact vitreous gels (volume approx. 6 ml) in plastic petri dishes: after various times of incubation at 37°C, the gels were fixed in 3% glutaraldehyde in phosphate buffered saline, stained with Coomassie blue for 1 hr, and washed with distilled water. Control vitreous gels without cells were incubated and treated identically.

An estimate of the changes induced by cell growth on the vitreous gels was made by measuring the volume of the vitreous gel before and after cell culture using a volumetric cylinder. Gels were placed in a known volume of fluid in the cylinder, and the volume of the gel was calculated from readings of the displacement in the fluid level (Archimedes, circa 80 B.C.).

Time Lapse Video Microscopy

The behaviour of single cells and small groups of cells on vitreous gels was observed using a time lapse video microscopy system (National NV 8030). Cultures were placed on the warm stage of an inverted microscope and viewed through a television camera attached to a video monitor. Cells in a single field were observed for periods up to 8 hr, and photographs at different times were taken directly from the monitor. Pieces of vitreous were immobilised using heavy stainless steel rings.

Electron Microscopy

Cultures of cells on vitreous gels were fixed in 3% phosphate-buffered glutaraldehyde for 2–4 hr, and small pieces of gel were processed for scanning transmission electron microscopy as described previously.

Results

Macroscopic Changes in Vitreous Gels

Choroidal fibroblasts grown on whole pieces of intact vitreous gel (1 × 10⁶ cells per gel, volume approximately 4 ml) caused shrinkage and contraction of the gel (Fig. 1). In addition; contracted gels stained more...
intensely with Coomassie blue than identically treated control gels (i.e., gels without cells; see Materials and Methods) suggesting that there was no loss of protein (collagen) from the shrunken gel. The degree of gel contraction varied with the numbers of cells, and often resulted in a very small, compacted pellet of tissue. Gel contraction also varied with cell type. Choroidal fibroblasts caused a progressive and considerable shrinkage of the gel over a period of 6 days, by which time the gel was reduced to about 5% of its original volume (Fig. 2A). Retinal pigment epithelium, however, caused an initial rapid shrinkage (to approximately half of its original volume after 24 hr) but little further change occurred over the next 2–7 days (Fig. 2B).

Other cell types, such as glial cells, also produced visible reduction in the size of the vitreous gel (Fig. 2C), although the effect was less marked, and this was reflected in the different behaviour of each cell type seen microscopically. On a semi-quantitative basis, the order of gel shrinkage was choroidal fibroblasts, retinal pigment epithelium, retinal glial cells, and bovine capillary retinal epithelium. In contrast, cultures of MM on vitreous explants caused virtually no shrinkage of the gel and minimal reduction in its volume (Fig. 2D).

Contraction of the gel induced by cells appeared to occur around a central focal point, from which visible traction bands radiated. This effect was most marked with choroidal fibroblasts, and less so with other cell types.

Microscopic Changes in Vitreous Gels

Cells seeded onto the surface of vitreous gels adhered rapidly to the gel and grew well. Choroidal fibroblasts invaded the gel as single cells and, to a lesser extent, retinal glial cells also were invasive. Retinal pigment epithelium and bovine retinal capillary endothelium proliferated on the surface of the gel and formed confluent sheets of cells before invasion of the gel occurred.

Pigment Epithelium

Fifteen min after seeding onto vitreous, retinal pigment epithelium were adherent to the surface of the gel but were still rounded (Fig. 3A). Single cells then tended to aggregate and spread. After 2 hr, cells had become aligned in rows; larger groups of cells had formed linear aggregates, while single cells had adopted a very elongated "spindle" morphology (Fig. 3B) along visible fibers in the gel substrate. After 24 hr, these effects were more marked. Some larger aggregates of retinal pigment epithelium had formed sheets of cells (Fig. 3C), and most of the other cells were spindle-shaped and aligned along the now prominent parallel bundles of fibrils in the gel. After prolonged culture (5–7 days), the cells had aggregated, usually to one or two large dense sheets, with extensive traction lines evident in the vitreous gel (Fig. 3D).

Choroidal Fibroblasts

Choroidal fibroblasts rapidly adhered to the gels (within 1–2 min) and almost all the cells had a spindle-shaped morphology and were aligned in parallel 2 hr after seeding (Fig. 4A). At this stage, traction lines were barely visible. Choroidal fibroblasts did not form sheets of cells, but generally clustered together in a large cell mass (Fig. 4B). Traction lines in the gel were very marked at this stage, and the direction of cell movement was toward the largest cell aggregate.
Fig. 3. Phase contrast microscopy of pigmented retinal epithelial cells seeded onto bovine vitreous explants A, 15 min, B, 2 hr, C 24 hr, and D, 5 days incubation at 37°C. Note progressive aggregation of cell monolayer to form a large sheet. Note also development of traction lines in vitreous gel (arrows, B, C) with linearly arranged PRE cells.
Other Cells

Retinal glial cells also produced traction lines in vitreous gels, but their effect was less dramatic than choroidal fibroblasts (Fig. 5). In addition, although long parallel bundles of gel fibrils were observed as with retinal pigment epithelium and choroidal fibroblasts, they were fewer, and other arrangements of the gel surface were seen, such as surface wrinkling around a small cell aggregate (Fig. 5). Of the cells studied, bovine retinal capillary endothelium adhered least well to the gel surface, and were generally less invasive (Fig. 6A). Twenty-four hr after seeding, islands of bovine retinal capillary endothelium had formed monolayer colonies of cells growing on the surface of the gel. Faint traction lines radiating from these cell sheets were visible, but gel destruction was not prominent (Fig. 6B). Even after several days growth, groups of cells were seen arranged...
in linear aggregates, but dense traction lines were much less frequent than with choroidal fibroblasts or even retinal pigment epithelium (Fig. 6C).

Mouse macrophages adhered very rapidly to the surface of the gel (Fig. 7A–C), and within 1–2 hr had spread widely, particularly activated mouse macrophages. Mouse macrophages also adhered to collagen fibrils; but, although they formed cell aggregates, they

Fig. 5. Phase contrast microscopy of retinal glial cells 24 hr after seeding on bovine vitreous explant. Note fine wrinkling of surface of gel (small arrows), plus long single traction lines (large arrows).

Fig. 6. Phase contrast microscopy of bovine retinal capillary endothelium on bovine vitreous explants A, 2 hr, B, 24 hr, and C, 7 days incubation at 37°C. Traction lines are faint (arrows) and cells tend to form aggregates.
Fig. 7. Phase contrast microscopy of mouse macrophages on bovine vitreous explants A, 15 min, B, 2 hr, and C 24 hr incubation at 37°C. Note that cells adhere to and spread well on vitreous surface (arrows, B), form larger linear cell aggregates but do not produce traction lines.

did not cause significant disruption of the gel or form obvious traction lines. However, mouse macrophages, particularly activated mouse macrophages, invaded the gel, generally as single cells.

**Time Lapse Studies**

Time lapse video recordings of cell behaviour on vitreous gels provided a permanent record of the above changes within a single group of cells. Cells were seen to form adhesions to collagen fibrils, and appeared to migrate (along the length of the fibril), at the same time exerting traction on increasing numbers of fibrils until the fibrils formed parallel bundles beneath the cells. In the case of retinal pigment epithelium, the cells then approached neighbouring cells, often by extending very long processes. Single cells and small groups of cells appeared to be drawn towards larger clusters of cells. Traction between groups of cells was transmitted through the fibrils, and increased to a critical point when the fibril appeared to break and the two groups of cells rounded up and separated. In this manner, the gel structure was progressively disrupted. Similar observations were made with other cell types used in this study.

**Ultrastructure**

Scanning and transmission electron microscopy (SEM, TEM) confirmed the findings of phase contrast light microscopy. Fifteen min after seeding, choroidal fibroblasts on the surface of the vitreous were mostly rounded, but were showing pseudopodial attachment. After 2 hr, many cells were extremely flattened and adherent mainly through their lamellipodia. After 24 hr, linear aggregates of cells were seen within condensed layers of vitreous fibrils which could be visualised in relief aligned along the surface of the cells. Such changes correlated with invasion of the cells into the vitreous gel. Cross-sections through the gel showed parallel bundles of collagen fibrils in numerous planes. The cells were elongated with very extensive lamellipodia, but few cytoplasmic microfilament bundles or adhesion plaques were seen; however, areas of close contact with the collagen fibrils were seen, especially around the edges of lamellipodia where points of attachment ap-
Fig. 8. Ultrastructure of pigmented retinal epithelial cells on surface of bovine vitreous explants A 15 min, B 15 min, and C, 2 hr after seeding. Note early formation of close inter-cellular contacts between still rounded PRE (A, B); after 2 hr the cells have adopted a more spread morphology and have aggregated together.

TEM and SEM of retinal pigment epithelium 15 min after seeding on a vitreous gel showed that these cells had formed close intercellular contacts at an early stage when vitreous was still a very loose, fine collagenous structure (Fig. 8A, B). After 2 hr, the cells had adopted a more spread morphology (Fig. 8C), and a fine network of fibrils was visible on the surface of the vitreous, interlacing larger cellular aggregates. After 24 hr, sheets of cells forming discrete islands and showing a degree of differentiation, such as close contacts and rudimentary apical microvilli, were observed. By 4 days, PRE cells had adopted a typical monolayer appearance with obvious cell polarity (Fig. 9A). Junctional complex formation was a prominent feature at the base of the apical microvilli, as was basement membrane formation on the surface of the now condensed vitreous collagenous matrix (Fig. 9B).

Macrophage morphology changed little after cells became spread on the gel surface (1–2 hr, see above). Close association with the collagenous matrix was seen, but the vitreous did not become condensed and microfilament bundles were not prominent within the cell.

Discussion

We believe that this study has shown that vitreous explants can be used as a suitable in vitro model for cellular proliferation in the vitreous, and that it provides direct evidence for the cell-mediated nature of this phenomenon. The in vitro system has a number of...
advantages over in vivo models, not the least of which are that it permits analysis of the behaviour of defined cell populations and is readily amenable to experimental manipulation. For instance, several studies have suggested that modulation of in vivo models of cellular proliferation in the vitreous, with drugs such as inhibitors of collagen cross-linking penicillamine\(^1\) or antimitotic agents (triacsinolone, dexamethasone),\(^12,13\) reduce the severity of the cellular proliferation. Such studies could be more easily and reliably performed using the simple system described here. Furthermore, this system avoids the complications of uncontrolled variables (e.g., secondary inflammation). It has been suggested on the basis of preliminary evidence using OH-proline analogues that cell proliferation in collagen gels is under negative feedback control via extracellular matrix components\(^14\) (Docherty and Forrester, unpublished data), which emphasises the prominent role of the extracellular matrix in these conditions.

Clinical\(^15-21\) and experimental\(^22-27\) studies of cellular proliferation in the vitreous cavity have implicated a wide variety of cell types, including retinal pigment epithelium, macrophages, fibroblasts, glial cells, and myofibroblasts, a term used to describe fibroblasts (in healing wounds) which contain large numbers of actin microfilaments. Similar studies of penetrating eye injuries have identified the (myo-) fibroblast, usually from the choroid, as the major cell type responsible for vitreous contraction and traction retinal detachment. In both situations, it was suggested that the cells used the vitreous as a scaffold for cell migration.\(^4\) The results of the present study using vitreous explants support this notion, since cells were seen to adhere to and spread out on the fibrils of the vitreous gel. In so doing, they caused disruption of the gel by creating numerous traction or “stress” lines in the gel. The degree of gel disruption varied with the ratio of the gel volume to the cell concentration, which corroborates indirect evidence from in vivo experiments.\(^28\) However, not all cells were equally effective: choroidal fibroblasts were the most invasive and gel-disruptive cell type, while bovine retinal capillary endothelium were less effective. Retinal pigment epithelium caused severe destruction of the vitreous gels, but tended to do so as sheets of cells, while glial cells caused less disturbance to the structure of the gel, but produced other effects, such as surface wrinkling, similar to surface wrinkling (cellophane) retinopathy, in the human eye.\(^29\) In contrast, macrophages caused no disruption of the gel, although they spread on its surface and invaded the gel with ease. In some respects, this result is rather surprising, since macrophages, especially activated macrophages, are known to secrete large amounts of collagenase, an enzyme which had been considered important in matrix modification by invasive cells.\(^30\) Other cells, however, can invade collagen gels in vitro in the absence of collagenase.\(^31\) It is possible that some of these differences in cell behaviour relate to the different origins of the cell; i.e., that embryonic cells are more likely to cause gel destruction than adult cells. However, the fact that different cells produce different effects on the gel indicates the importance of identifying precisely which cells are involved in the various cellular proliferations in clinical disease states. It must also be noted that proliferations in vivo frequently involve interactions between different cells, and the present model has so far only approached the study of individual cell types and their effects on the gel. However, the model is eminently suitable for the study of cellular interaction in matrices.

Apart from its clinical relevance, this model has applicability to more fundamental cell behavioural studies. Interestingly, the hierarchy of cell types, in terms of the damage they cause to the vitreous gel, parallels the stress which each exerts on the substratum. Thus Harris\(^32\) showed that fibroblasts would only spread on a very viscous silicone oil, whereas carcinoma cells, macrophages, and leukocytes would, respectively, spread on oils of lower and lower viscosity. Harris\(^33\) has recently suggested that traction events produced by cells in collagen gels may have a role in collagen morphogenesis, both in the developmental context and
in pathological processes, such as capsule formation during wound healing and around benign tumors. For instance, the ability of a tumor cell to induce traction and coagmentation of fibrils in a collagen gel (i.e., to form a capsule) may be inversely related to its invasive and metastatic potential. Such a concept would be partly supported by the results of this study, in which the most rapidly invasive cell (the macrophage) caused virtually no traction on vitreous collagen fibres, although choroidal fibroblasts, which had greatest traction effects and caused the most damage to the gel structure, were also invasive. Clearly, the relationship between invasiveness and traction cannot be described in such simple terms. Previous studies of this nature have been performed using artificial, reconstituted gels, and the use of a native gel (vitreous) for such studies may be more realistic. In addition, the vitreous is reported to have growth-promoting and growth-inhibiting factors.34-36 The methods described here should provide a system for analysing these factors.

Key words: cellular proliferation, vitreous, vitreous gels, cell culture

References


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