Quantitative Assessment of Growth Stimulating Activity of the Vitreous During PVR

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This report postulates that the activity of cellular proliferation along the surface of the retina during proliferative vitreoretinopathy (PVR) is reflected in the aggregate effect of proliferation-inducing growth factors in the vitreous. A method for quantifying the “net” proliferation-inducing capacity of an individual vitreous sample was developed and this assay was used to evaluate the vitreous proliferative activity in a model for experimental PVR. Vitreous was aspirated sequentially after onset of fibroblast-induced PVR in a rabbit model. A simple bioassay for the “aggregate” stimulating activity was developed and each sample was assigned a quantitative value in terms of “proliferation units” (PU). Experimental eyes demonstrated a wide range of stimulating activity (0-1765 PU), but control eyes showed uniformly low levels of activity (0-337 PU). Experimental eyes that ultimately developed retinal detachment displayed higher levels of proliferative activity than did those eyes destined to remain attached. The differences were statistically different by day 3, prior to the onset of clinical retinal changes. We conclude that quantification of vitreous proliferation-stimulating activity is possible and that this method might be useful for screening eyes at high risk for the development of recurrent retinal detachment from PVR.

Proliferative vitreoretinopathy (PVR) remains the leading cause of persistent and recurrent retinal detachment, complicating as many as 10% of all retinal detachments. Although the pathogenesis of PVR is complex, at least four distinct stages appear to be important in its development, including chemotaxis and cellular migration, cellular proliferation, membrane formation, and contraction. Many studies are consistent with the notion that the vitreous fluid/gel forms a microenvironment that reflects, if not modulates, each of these phases, especially the growth and proliferation of PVR-inducing cells along the surface of the retina. The vitreous cavity, especially in a vitrectomized eye, can be thought of as a collecting pool for the aggregate growth factors produced by the proliferating cells and leaking from the blood. Not surprisingly, investigators have demonstrated that vitreous from eyes with PVR can stimulate proliferation of retinal pigment epithelium (RPE) and fibroblasts in culture. Many investigators have demonstrated that the fluid that fills the vitreous contains specific potent chemotactic factors, as well as growth-promoting cytokines.

Although specific cytokines and soluble matrix components clearly contribute to the disease process, we hypothesize that an assay that measures the aggregate proliferation-inducing activity of the vitreous aspirate might be more informative than the measurement of the concentration of any individual factor because: (1) the “proliferation-inducing microenvironment” of the vitreous is really created by the aggregate effect of the collective biological activity of multiple natural factors, including growth-stimulating and natural antiproliferative cytokines; and (2) most cytokines have pleiotropic activities that vary with biological conditions, concentration, and the target cell, so assigning a causal role to a particular factor is difficult.

If this hypothesis is correct, characterization of the “net” biological status of the vitreous microenvironment, as reflected in the total stimulatory capacity of an individual sample, might provide a useful method for quantifying the functional status of the vitreous microenvironment.

In the present study, we sought to develop a method for quantifying the capacity of a vitreous sample to stimulate RPE proliferation. We show that a simple bioassay for the “aggregate” stimulatory activity can...
be developed that assigns a relative but quantitative value in terms of arbitrary "proliferation units" to samples of human or rabbit vitreous. Using this assay to analyze an experimental rabbit model of PVR, we demonstrate that the stimulatory capacity of the vitreous varies among individual eyes according to the severity of PVR and can be used to distinguish experimental eyes that are destined to develop PVR from those that will remain quiescent.

Materials and Methods

Fibroblast Culture

Homologous dermal fibroblasts grown from explants of rabbit flank skin were cultured in Dulbecco's modified Eagle’s medium, supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified incubator with 5% CO₂ in air. Fibroblasts were carried for 3-7 passages and subculture was performed when cells reached confluence. After incubation with 0.25% trypsin for 5-10 min, cells were centrifuged, washed in phosphate-buffered saline (PBS), and counted with a hemocytometer. Greater than 90% cell viability was verified by trypan blue exclusion. The fibroblasts then were diluted to reach a final concentration of 100,000 cells per 0.1 ml of balanced salt solution (BSS) prior to intraocular injection.

Rabbit Model for PVR

All of the animal experiments in this study adhered to the ARVO Resolution on the Use of Animals in Research. A fibroblast-induced model for PVR was used for these experiments, as described previously.²⁻¹⁸ Briefly, all eyes underwent vitreous compression by injection of an expansile gas, followed two weeks later by injection with 100,000 cultivated fibroblasts in 100 μl of BSS. Control eyes received intravitreal injection of 100 μl of BSS only. At various intervals (days 1, 3, 7, 14, and 21), ophthalmoscopic examination and photography of the fundus was performed, and changes consistent with PVR, including retinal detachment, holes, or traction, were noted and photographed. Vitreous samples (100 μl) from experimental and control eyes were obtained at days 1, 3, 7, and 14 by simple vitreous aspiration. Aspirates that were insufficient in amount or contaminated with blood during the aspiration procedure were eliminated from the analysis. Previous experience with this model demonstrated that approximately 70% of eyes developed total retinal detachment with advanced PVR, whereas the remaining eyes remained relatively normal.²⁻¹⁸ Twenty one rabbits were used for this study.

Sample Preparation

Vitreous samples were spun at 250 × g to remove contaminating cells and the supernatant was transferred to a siliconized tube and stored at −70°C until analysis. For some experiments, human vitreous samples were obtained. After informed consent, 100–300 μl of undiluted human vitreous was obtained by automated vitrectomy at the time of surgery for complicated retinal detachment. All three samples were obtained from eyes with PVR resulting from recurrent tractional detachment after failed scleral buckle procedure. They all demonstrated posterior PVR (fixed folds) for at least 6 hr.

Retinal Pigment Epithelial Cell Culture

Human retinal pigment epithelium (RPE) was used as indicator cells for the proliferation bioassay. Single cell suspensions of RPE were made from freshly obtained donor eyes (gift of the Florida Lion’s Eye Bank, Miami, FL), according to the method of Flood and associates.¹⁹ The cell suspensions were plated into 25 cm² tissue culture flasks in RPE culture medium (RPMI 1640 medium supplemented with 15% fetal bovine serum, 10 mmol/l HEPES, and 35 mmol/l L-glutamine). Cultures were maintained in a humidified 37°C incubator in 5% CO₂ until the monolayers reached confluence (approximately 2 wk). Subculture was performed by brief exposure to 0.25% trypsin, washing twice in culture medium, and final resuspension at 1 × 10⁵/ml concentration in culture medium before replating.

For bioassay, subconfluent cultures of two to five passages were obtained. The RPE culture medium was replaced with basal RPMI (ie, serum-free, unadulterated media) for 16 hr to temporarily deprive the cells of basic nutrients, thus enhancing their responsiveness. One hour prior to utilization in the assay, the RPE were trypsinized, washed, and diluted in basal RPMI to reach a final concentration of 100,000 cells per ml.

Reference Standard

A reference standard was run in duplicate with each assay. Vitreous samples obtained from five rabbits with severe PVR were pooled, aliquoted into 100 μl in siliconized tubes, and stored at −70°C until they were used. This standard sample was found to produce high and reproducible proliferation (as measured by tritiated thymidine uptake) when used in the bioassay, as described below, although the maximal counts per minute (cpm) induced varied from day to day.
Proliferation Bioassay

In general, each assay contained duplicates of three types of samples: vitreous specimens, reference standard, and a background control. The bioassay was performed in 96 well, flat bottomed microtitration plates as follows. Fifty microliters of basal RPMI medium (without serum) was added to each well. Fifty microliters of the vitreous sample or reference standard was added to the top well and serially diluted in 8–12 two-fold dilutions. Finally, 50 μl of the RPE indicator cells, suspended in basal medium, was added to each well. “Background control” wells contained the indicator cells in serum free medium only. After 1 hr, 0.5 μCi of 3H-thymidine (New England Nuclear, Boston, MA) in 10 μl was added to each well. After 3 hr, no obvious differences were observed in the ability of the RPE to attach and flatten to the wells. Twenty four hours later, some wells demonstrated a significant number of detached cells, but we could not differentiate detachment because of a lack of extracellular matrix factors or because of cell death resulting from an absence of vital growth factors. The media was discarded and trypsin was added to each well. The residual cells were collected onto separate filter paper disks with a multi-channel microtitration plate harvester followed by rinsing with 95% ethanol. Proliferation was determined in cpm by measurement of the thymidine incorporation (ie, filter-adherent radioactivity) using a beta scintillation counter. Pilot studies that evaluated the role of precoating the wells with collagen I, collagen IV, fibronectin, or laminin did not improve the performance of the bioassay.

Data Analysis

We used a method for data analysis similar to that used for analysis of ELISA or cytokine bioassays. For each sample, the values for cpm versus the inverse of the dilution were plotted after log transformation. A computer program (using the least squares method) was used for analysis of ELISA or cytokine bioassays. For each sample to stimulate the proliferation of cultured RPE, the media was added and trypsin was added to each well. The residual cells were collected onto separate filter paper disks with a multi-channel microtitration plate harvester followed by rinsing with 95% ethanol. Proliferation was determined in cpm by measurement of the thymidine incorporation (ie, filter-adherent radioactivity) using a beta scintillation counter. Pilot studies that evaluated the role of precoating the wells with collagen I, collagen IV, fibronectin, or laminin did not improve the performance of the bioassay.

Activity at Various Dilutions

For each sample compared to the reference standard. Finally, 50 μl of the RPE indicator cells, suspended in basal medium, was added to each well. “Background control” wells contained the indicator cells in serum free medium only. After 1 hr, 0.5 μCi of 3H-thymidine (New England Nuclear, Boston, MA) in 10 μl was added to each well. After 3 hr, no obvious differences were observed in the ability of the RPE to attach and flatten to the wells. Twenty four hours later, some wells demonstrated a significant number of detached cells, but we could not differentiate detachment because of a lack of extracellular matrix factors or because of cell death resulting from an absence of vital growth factors. The media was discarded and trypsin was added to each well. The residual cells were collected onto separate filter paper disks with a multi-channel microtitration plate harvester followed by rinsing with 95% ethanol. Proliferation was determined in cpm by measurement of the thymidine incorporation (ie, filter-adherent radioactivity) using a beta scintillation counter. Pilot studies that evaluated the role of precoating the wells with collagen I, collagen IV, fibronectin, or laminin did not improve the performance of the bioassay.

Statistical Analysis

The Mann-Whitney test for nonparametric data was used to test for significance between groups in the bioassay. The cutoff value was determined by the following method. Using Levene’s test for heterogeneity on log transformed values for the experimental eyes on day 3, the differences between the variance of the attached and detached groups was not found to be statistically significant. This allowed a pooled estimate of variance. Using normal theory, a cutoff of net activity was generated from the sample mean net activity of the attached eyes plus a value calculated from the standard deviation multiplied by the predicted t distribution based upon the sample size (ie, SD*[(sample size)-1]). This cutoff was designed to exclude the eyes destined to remain attached with 99% specificity. The sensitivity of this cutoff would vary with the sample size according to the t distribution.

Results

Measurement of Vitreous Proliferation Stimulating Activity at Various Dilutions

First, we sought to determine whether a single measurement of the proliferation induced by a particular dilution of a vitreous sample would be adequate for comparing activity between samples. To answer this question, we chose three different human vitreous samples from eyes undergoing vitrectomy and measured the capacity of different concentrations from each sample to stimulate the proliferation of cultured RPE. As demonstrated in Figure 1, the proliferation of cultured RPE induced by the three samples varied depending on the concentration of vitreous, but important dose-dependent effects were observed. For example, at 1:8 dilution, the vitreous samples from patients 1 and 3, but not patient 2, induced significant proliferation of the cultured RPE. As the sample was diluted, vitreous from patients 1 and 2 became more stimulatory, whereas the sample obtained from patient 3 became less stimulatory. We conclude that vitreous samples

nally, to account for day-to-day variations in the maximal cpm between experiments performed on different days, the calculated dilution values were standardized according to the formula: Half max (sample)/half max (reference) X 1000. This formula generated a ratio corresponding to the relative potency of the test sample (compared to the reference standard) and the resulting value was arbitrarily called “proliferation units” (PU). Duplicate samples were run for each time point. Interplate and day-to-day variation were less than 10% with this method.
The dose response of cultured retinal pigment epithelium proliferation, as measured by tritiated thymidine uptake, is shown for three different samples of human vitreous from eyes with complicated retinal detachment. Different patterns of dose dependent inhibition and stimulation of proliferation are revealed, depending upon the sample and the concentration tested.

Could be inhibitory or stimulatory depending upon the tested concentration. Thus, comparison of proliferation stimulating activity at an arbitrarily chosen concentration could provide misleading information regarding the relative potency among samples. However, the dose-response curve formed by the analysis of serial dilutions of the specimen seemed to reflect the overall stimulating capacity of each sample.

Development of Alternative Method to Measure Net Stimulatory Activity

To avoid concentration-dependent artifacts based upon an arbitrary choice of dilution, we developed a method for the analysis of the stimulatory activity conceptually similar to the bioassay methods routinely employed to measure cytokine concentrations. Serial dilutions of each vitreous sample were performed as already described to generate a dose-response curve. Computerized curve-fitting procedures were used to generate a polynomial regression line that represented the best fit of the data. As described in the Methods, standard analytical techniques were used to assign a value to each curve, using the concept of half maximal response. A typical example is shown in Figure 2. All experimental results are reported in terms of arbitrary PU. Using this method, the three human samples were assigned values of 141 PU, 565 PU, and 600 PU (patients 1 to 3), respectively.

Change in “Net Proliferation Activity” Over Time in Experimental PVR

We tested the ability of this method to identify differences in net stimulatory activity over time in a rabbit model for PVR. One hundred microliters of the vitreous was aspirated on various days and measured for its ability to stimulate cultured human RPE. Comparison of the “net” proliferation stimulating activity (net activity) measured in vitreous aspirates from individual experimental or control eyes at various times is shown in Figure 3. As shown, the control population
showed a uniformly low level of net activity (range 0–337 PU). In contrast, experimental eyes demonstrated a wide range of net stimulatory activity (range 0–1765 PU). Many eyes exhibited high levels, first detectable by day 3 and increasing through day 14.

This PVR model results in tractional retinal detachment in approximately 70% of experimental eyes by days 14–21. In this experiment, none of the controls (0/7) but 64.2% of the fibroblast-injected eyes (9/14) developed retinal detachment. Therefore, clinical findings from day 21 were used to separate experimental eyes into two groups: those with retinal detachment and those remaining attached or demonstrating minor pre-retinal proliferation. The mean value for the net activity at each time point was plotted according to ultimate clinical outcome. As shown in Figure 4, the two experimental groups displayed a markedly different pattern. In those eyes destined to develop retinal detachment, the net activity began to rise by day 3 (563 ± 79 PU, mean ± standard error of the mean) and became maximal by day 14 (921 ± 159 PU). In contrast, those eyes destined to remain attached demonstrated low levels of net activity at day 3 (124 ± 14 PU) and remained persistently low throughout the experiment (day 14, 232 ± 114 PU). The difference in net activity between the two groups was statistically significant at day 3 (P = 0.001), day 7 (P = 0.003), and day 14 (P = 0.048).

Figure 5 demonstrates the correlation between the subsequent clinical outcome (on day 21) and net activity observed within individual eyes in the two groups at day 3. The lack of overlap between the two groups encouraged us to develop a statistically valid method to use this information as a predictive test. A "cutoff" value was calculated to separate eyes at high risk for recurrent detachment from eyes at low risk (see Methods and Materials). In the experiment shown in Figure 5, a test cutoff of 455 PU, as calculated by this method (2.08 ± 0.13 PU, log of the mean net activity of attached eyes ± SD, with log of the pooled estimate of the variance being 0.0347), was predicted to have >99% specificity but only 60% sensitivity. As shown in Figure 5, this cutoff correctly identified six of nine eyes destined to develop retinal detachment (67% sensitivity), and no attached eyes were incorrectly identified (>99% specificity).

Discussion

In this study, we developed a quantitative method for comparing the capacity of vitreous aspirates to stimulate the proliferation of cultured RPE. In aspirates obtained from rabbit eyes with experimental PVR, our results demonstrate that sequential variations in net activity can be measured in the same eye. Progressive and persistent increases in net activity were observed only in those eyes destined to develop tractional retinal detachment. Control eyes or those eyes that ultimately remained attached demonstrated very low levels of activity.

In addition, we observed that increased net activity occurred prior to the onset of retinal detachment, suggesting that proliferation-inducing changes occurred in the vitreous microenvironment early in the course of PVR. The current study suggests that high levels of growth stimulating activity were identified within the vitreous fluid of eyes destined for ultimate detachment prior to the development of clinically detectable retinal changes. The demonstration that net activity predicted the ultimate clinical outcome in this model suggests that the vitreous microenvironment reflects the "activity" of the PVR process and that quantification of the "net" proliferation stimulating soluble
growth factors within individual vitreous samples might provide information about the future clinical course of that particular eye.

The presence of soluble proliferation-inducing factors in the vitreous prior to tractional retinal detachment is consistent with the early detection of proliferating cells within the vitreous cavity in a similar model of PVR. Hatchell and coworkers found that proliferating cells could be found in the vitreous and in the retina early in the course of experimental PVR. In a previous study, we found that proliferating cells could be recovered from the vitreous cavity prior to the development of pre-retinal membrane formation, and the enumeration of these cells could be used to predict the future development of tractional detachment in individual eyes.

The present study confirms another important finding related to the identification of the physiologic role of individual factors in the production of PVR. As shown previously by Kirchhof and Sorgente, we also found that the capacity of an individual vitreous sample to stimulate PVR is dose dependent. Some samples were inhibitory at high concentrations, but stimulatory at more dilute levels. This biphasic action on cellular proliferation probably reflects the complexity of composition of vitreous from eyes with PVR. It is becoming clear that multiple matrix components and cytokines, including acidic FGF, TGF-β, interleukin-1, and others, probably are present in the vitreous. Because it is impossible to determine the precise concentration of each factor actually available to cells growing within membranes along the retinal surface, extrapolation of the physiologic role of the vitreous concentration of individual growth factors is difficult.

Furthermore, it is conceivable that the biological role of certain cytokines might be antiproliferative rather than mitogenic. For instance, although TGF-β has been shown to enhance fibroblast proliferation under certain experimental conditions, under most conditions TGF-β actually inhibits cellular proliferation (including that of RPE), although this cytokine enhances collagen synthesis and membrane formation. Thus, the ultimate action of a particular vitreous microenvironment will depend upon numerous variables, including the target cell, the presence of extracellular matrix components, the identity of individual cytokines, and their relative concentration. We hope this assay will provide a type of functional index that might allow us account for some of those variables, and in the future allow us to correlate specific factor concentrations with net proliferation-inducing activity.

The source and identity of the stimulatory factors in the vitreous is uncertain. However, other investigators have suggested at least two possibilities. Campochiaro and coworkers have demonstrated that increased vascular permeability occurs during PVR, suggesting that blood-derived factors, such as extracellular matrix proteins, might provide one source. In addition, the RPE and other cellular constituents participating in the PVR process probably also secrete factors into the vitreous cavity.

This method for quantification of “net” stimulatory activity employed analytical methods similar to those used in cytokine bioassays and ELISA. Like all bioassays, this assay requires maintenance of indicator cells in culture and is subject to biological variation. In addition, various technical aspects can be difficult to assess. The choice of reference standard was based upon an extensive series of preliminary experiments. We decided to use a “reference vitreous sample” from a rabbit model of PVR as the reference standard, because after extensive preliminary experiments, it was determined that neither serum alone nor a cocktail consisting of serum plus additional recombinant growth factors mimics the complexity of the proliferation-stimulating activity of vitreous fluid.

Although the assay described in the present study may have value as a diagnostic tool in predicting proliferative potential in vitreous samples, this assay, as currently described, has severe limitations as a tool for studying the specific physiological mechanisms of proliferation. For example, this assay cannot differentiate factors that directly influence cellular DNA synthesis from factors that indirectly impact upon proliferation via other cellular processes, such as cell attachment. This bioassay was intentionally designed to be sensitive to a spectrum of cellular processes that ultimately contribute to DNA synthesis, including cell adhesion and cell activation. Thus, enhanced DNA synthesis merely confirms that the complex sequence of events required for proliferation has successfully occurred. However, the assay is unable to determine the specific site of action in that chain of events when differences are detected. Although we suspect that mitogenic and antiproliferative cytokines are important, differences in proliferation probably also result from other cellular processes. For instance, cell adhesion to the culture plate might be suboptimal because of the absence of matrix components important for cell attachment in some vitreous samples. This occurrence also would ultimately result in diminished “proliferation units” as measured by this assay.

Nevertheless, this type of bioassay offers several advantages. Because it uses evaluation of the dose response, the effects of differing concentrations of the vitreous are partly taken into account. Also, it is conceivable that different forms of PVR will vary in the composition of the vitreous microenvironment. In this assay, the indicator cells can be varied to determine if an individual sample varies in potency at stimulating other cell types, such as glial cells, vascular...
endothelium, or fibroblasts. In addition, neutralizing antibodies to individual cytokines can be used to assess the contribution of specific growth factors to the overall stimulatory or inhibitory action of a particular sample. Also, modifications in the performance of the bioassay, such as by allowing the indicator cells to attach to the wells prior to the addition of the sample, would allow us to differentiate the effects on attachment from those on proliferation.

We intend to determine whether the measurement of "net" activity might provide a useful screening test for identifying human eyes at high risk for recurrent retinal detachment. For example, analysis of vitreous aspirates obtained at the time of post-operative fluid/gas exchange might reveal that a subset of eyes are at increased risk for retinal detachment. Timely intervention with aggressive clinical management, and possibly even antiproliferative agents, might be indicated in those high risk eyes.

Key words: proliferative vitreoretinopathy, retinal pigment epithelial cells, retinal detachment, vitreous, experimental model

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