Vitreous Treatment of Retinal Pigment Epithelial Cells Results in Decreased Expression of FGF-2

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Purpose. Changes in gene expression were investigated after treatment of cultured human retinal pigment epithelial (RPE) cells with vitreous. This may have implications for proliferative diseases such as proliferative vitreo-retinopathy.

Methods. Cells were cultured in the presence or absence of human vitreous, and gene expression was examined using the differential display polymerase chain reaction technique. Differentially expressed RNAs were cloned, screened for differential expression, and sequenced. The expression of one of these RNAs (that for fibroblast growth factor [FGF]-2/basic FGF) was examined by in situ hybridization and ribonuclease protection assays. The level of FGF-2 protein was examined by immunoblot analysis. The effects of adding FGF-2 to cells cultured in the presence of vitreous were examined.

Results. Treatment of low passage human RPE cells with 25% vitreous resulted in the epithelial-to-fibroblast-like morphologic changes reported by others and in the decreased expression of FGF-2 mRNA and FGF-2 protein. Addition of FGF-2 to cultures at the same time as addition of vitreous prevented some of the effects of vitreous on these cells.

Conclusions. Vitreous treatment of RPE cells in culture results in decreased expression of FGF-2 mRNA and protein. Because supplementation of FGF-2 prevents some of the vitreous-mediated effects, this may indicate that modulation of FGF-2 levels by the vitreous may play an important role in the phenotypic changes seen in RPE cells exposed to vitreous. (Invest Ophthalmol Vis Sci. 1998; 39:2111-2120)

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tinal pigment epithelial (RPE) cells form a columnar, barrier epithelium between the neural retina and the choriocapillaris at the back of the eye and do not normally divide in the adult human. In some disease states, such as proliferative vitreo-retinopathy (PVR), cells gain access to and proliferate in the vitreous humor and elaborate extracellular epiretinal membranes, the contraction of which may result in detachment of the retina and blindness. RPE cells are thought to play a major role in PVR and may be important in the initiation of the processes that lead to this disease. In addition, damage to the neural retina resulting in exposure of RPE cells to the vitreous is an important risk factor in PVR.

When RPE cells proliferate in the vitreous in vivo, as occurs in PVR, they usually lose their typical columnar epithelial morphology and appear more fibroblast-like; similar morphologic changes have been observed on exposure of cultured RPE cells to vitreous humor. Identification of the proteins downregulated by the addition of vitreous humor could provide information about genes that play an important role in maintenance and function of the more epithelial-like state of RPE cells and may increase our understanding of the processes involved in PVR. The polymerase chain reaction (PCR)-based differential display technique, which can be used to identify such genes, allows multiple RNA samples to be screened rapidly and simultaneously for induction or suppression of gene expression. With this technique, it was found that expression of the basic fibroblast growth factor (FGF-2) gene is downregulated on treatment of cultured human RPE cells with vitreous humor.

Methods

Cells

Donated human eyes (no more than 10 hours after death) were obtained from the South Carolina Lions’ Eye Bank. RPE cells were isolated and cultured as previously described except that retinal extracts were not used to supplement the culture medium. The epithelioid nature of the cells was confirmed by staining cells for cytokeratin-18. The spontaneously transformed human D407 RPE cell line was grown in Dulbecco’s modified Eagle’s medium (high glucose) with 3% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml. Human vitreous humor was dissected from donor eyes, diluted with medium to a final concentration of 25% vitreous, and filtered through a 0.45 μm filter. Subconfluent cells were treated with fresh medium with or without 25% vitreous for 48 hours, and total cellular RNA was extracted. In the case of cultures grown on ProNectin F, which contains repeats of the RGD-containing site of fibronectin (Protein Polymer Technologies, San Diego, CA), tissue cul-
Differential display

Differential display was performed as detailed in the RNAmap (GenHunter, Nashville, TN) kit with some modifications. RNA was extracted using RNASTAT-60 (Tel-Test B, Friends-wood, TX) according to the manufacturer’s directions. Before use in differential display reactions, total cellular RNA was treated with RNase-free DNase (GIBCO-BRL, Gaithersburg, MD) in the presence of RNasin (Promega, Madison, WI), extracted with phenol-chloroform (1:1), ethanol-precipitated, and dissolved in glass-distilled water.

Cloning of Differentially Expressed Bands

Bands were excised from the gel and amplified by PCR as described by Liang et al., except that the dNTP concentrations were increased to 50 mM. A single band was seen when the reaction mix was diluted to 50 µl with distilled water and heated in a boiling water bath for 5 minutes immediately before using 5 µl in the PCR. The PCR was performed essentially as described by Liang and coworkers, using the nucleotide concentrations described by Aiello et al., using the same T12VN primer that was used in the reverse transcription reaction, and one of the arbitrary primers (AP) supplied with the Genhunter kit. The products of the PCRs were subjected to polyacrylamide gel electrophoresis and stained with ethidium bromide. The PCR products were purified by ultrafiltration using Microcon-100 filters (Amicon, Beverly, MA), using four washes with distilled water.

Determination of FGF-2 Protein Levels (Immunoblot Analysis)

Cells were incubated at 37°C in control medium for 48 hours; control medium for 24 hours and then medium containing 25% vitreous for 24 hours; or medium containing 25% vitreous for 48 hours. Protein extracts were prepared by a method similar to that of Dono and Zeller. Cells were washed twice with phosphate-buffered saline (pH 7.0) and extracted with 0.1 M extraction buffer (0.3 M NaCl, 20 mM Tris-Cl [pH 7.2], 0.5% Triton X-100, 0.5 µg aprotinin/ml, 0.5 µg phenylmethyl-sulfonyl fluoride/ml) per 25-cm² flask. After homogenization, the suspensions were centrifuged at 10,000g for 10 minutes, and the protein concentration in the supernatant fraction was determined by the method of Bradford using bovine gamma globulin as a standard (Bio-Rad Laboratories, Hercules, CA). Heparin binding proteins in each sample were enriched by incubating 0.6 ml of the supernatant fraction overnight with}

Cloning of Part of the Protein Coding Region of FGF-2

To obtain a clone corresponding to the protein coding region of FGF-2, a 398-bp region corresponding to nucleotides 468 to 865 of Genbank Accession Number J04513 was amplified by PCR (sense primer 5’TGGCAGCCGGGAGCATCAG; anti-sense primer 5’ATACTGGCCAGTTGTTTCA) and then was cloned into the pCR-Script vector as above. Only one positive colony was obtained, and it was used for the generation of probes for in situ hybridization. Because sequencing indicated that this clone originated from a rare trimming event 31 nucleotides downstream from the correct site, we re-cloned FGF-2 for the RNase protection assay experiments using PCR (sense primer 5’ACCTCAAGGACCCCAAGC; anti-sense primer 5’TTCGGCCAGGTCCGTG) to amplify a 358-bp region corresponding to nucleotides 540 to 897 of Genbank Accession Number J04513. This fragment was cloned as above, and multiple positive colonies in both orientations were isolated. All the FGF-2 PCR primer pairs used were designed such that there were two exon/exon boundaries between them to exclude interference from any contaminating DNA.

In Situ Hybridization

Digoxigenin-labeled anti-sense or sense riboprobes were made using a Maxiscript Transcription system (Ambion, Austin, TX) with digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN) and T7 or T3 polymerase. In situ hybridization was performed using a modification of the methods of Schulte-Merker and Panoskaltsis-Mortari and Bucy. Hybridized riboprobe was detected using alkaline phosphatase-coupled digoxigenin-anti-sense or sense riboprobes. Radioactive hybridization probes were made using PCR amplification with the same primers used in the differential display reaction in the presence of [α-32P] dCTP (M. Liu and F. Berger, unpublished data). To ascertain whether the insert was indeed present in the colonies, one filter was hybridized with a probe made by amplification of either cDNA derived from control cells or cDNA derived from vitreous-treated cells (M. Liu and F. Berger, unpublished). The insert in colonies that appeared to correspond to differentially expressed genes was amplified by colony PCR, followed by asymmetrical PCR and then was sequenced as previously described. The BLAST program was used to screen Genbank for similar sequences.

Analysis of FGF-2 Protein Levels (Immunoblot Analysis)

To determine whether the insert corresponded to a differentially expressed sequence, the other two filters were hybridized with a probe made by amplification of either cDNA derived from control cells or cDNA derived from vitreous-treated cells (M. Liu and F. Berger, unpublished). The insert in colonies that appeared to correspond to differentially expressed genes was amplified by colony PCR, followed by asymmetrical PCR and then was sequenced as previously described. The BLAST program was used to screen Genbank for similar sequences.
FIGURE 1. Effect of 25% or 50% vitreous on human retinal pigment epithelial (RPE) cells in tissue culture. Subconfluent cultures of human RPE cells were treated with medium containing 0%, 25%, or 50% vitreous for 48 hours and examined by light microscopy. (A) 0% vitreous; (B) 25% vitreous; (C) 50% vitreous. The upper and lower parts of the figure show independent experiments.

100 μl preswollen heparin-Sepharose (Sigma Chemical, St. Louis, MO) under slow rotation. The beads were washed with 0.6 M NaCl, 10 mM Tris-HCl (pH 7.0) and then with 10 mM Tris-HCl (pH 7.0). All procedures above were performed at 4°C. Proteins were eluted from the beads by boiling for 5 minutes in gel sample buffer (5% sodium dodecyl sulfate [SDS], 50% glycerol, 60 mM Tris-HCl [pH 6.8], 10 μg dithionitrobenzoic acid/ml, 0.005% bromphenol blue). Samples were adjusted to correct for any differences in the amount of protein in the original extract and analyzed in duplicate, along with prestained molecular weight standards and recombinant human FGF-2 (R&D Systems, Minneapolis, MN), by SDS-polyacrylamide gel electrophoresis using 17.5% gels. The proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-ECL; Amersham, Arlington, IL). After preincubation with 3% bovine serum albumin in TTBS (0.3 M NaCl, 20 mM Tris-HCl [pH 7.0], 0.1% Tween-20), the membranes were incubated with goat anti-human FGF-2 (R&D Systems; diluted 1:2000 with TTBS) for 1 hour at room temperature on a rotator. The membranes were washed three times (10 minutes per wash) with TTBS before incubation with peroxidase-conjugated rabbit anti-goat IgG antibody (Sigma Chemical; 1:2000 dilution in TTBS) for 30 minutes. After washing, the blots were developed using the ECL western blotting detection system (Amersham) according to the manufacturer’s instructions. Densitometry was performed using an Alphalmager 2000 (Alpha Innotech, San Leandro, CA).

Ribonuclease Protection Assays
Radioactive anti-sense riboprobes were synthesized using the T7 promoter in the pCR Script plasmid (FGF-2) or the pTRI-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plasmid (Ambion) using a Maxiscript system (Ambion) and [α-32P] UTP (NEN; 800 Ci/Mol) in the presence of equimolar unlabeled UTP (FGF-2) or a 1:1 ratio of nonradioactive UTP to radioactive UTP (GADPH). The probes were purified on microspin columns (Pharmacia, Piscataway, NJ). RNA (2 μg total RNA adjusted to a total of 10 μg with yeast tRNA) was mixed with excess probe, freeze-dried, and dissolved in hybridization buffer (Pharmingen, San Diego, CA). Ribonuclease protection assays were performed essentially as recommended by Pharmingen using their reagents, except that the temperature for the overnight hybridization was 47°C. The size and amount of protected probe were examined by electrophoresis on 6% acrylamide-7 M urea sequencing gels with Century Plus Markers (Ambion) followed by autoradiography and gel scanning using an Alphalmager 2000 (Alpha Innotech).

RESULTS
Treatment with Vitreous
As reported by others, treatment of cultured human RPE cells with vitreous resulted in marked morphologic changes, with the cells becoming more mesenchymal in appearance (compare panels B and C in Fig. 1 with panels A). This morphologic change occurred reproducibly in the presence of 25% vitreous, but did not occur if medium diluted with 25% serum-free medium was used (data not shown). Thus, it appears that the effects of vitreous are due to the vitreous itself and not merely due to dilution of serum factors. Morphologic changes were often but not always visible after 24 hours, but were
FIGURE 2. Differential mRNA display. Total RNA from control (C) or 25% vitreous-treated (V) cells was subjected to differential mRNA display using T\(\beta\)VG as the anchor primer and AP6 (A) or AP2 (B). The PCR products were analyzed as duplicate lanes on a denaturing sequencing gel with a single lane of a sequencing reaction as a marker (M). Only the part of the gel corresponding to the region between ~530 and ~330 nucleotides is shown (top and bottom small arrowheads, respectively, in each panel). Group 1 corresponds to donor A cells at passage 4 incubated for 48 hours in the absence (C) or presence (V) of homologous vitreous. Group 2 corresponds to donor B cells at passage 6 incubated for 48 hours in the absence (C) or presence (V) of donor A vitreous. Group 3 corresponds to donor B cells at passage 7 incubated for 48 hours in the absence (C) or presence (V) of homologous vitreous, and group 4 corresponds to the same RNA as used in group 3 but with reverse transcriptase omitted from the cDNA reaction. The large arrowhead indicates where a 395-nucleotide DNA would be expected to migrate.

usually more marked after 48 hours. Total RNA for differential display was extracted after 48 hours incubation in the presence of 25% vitreous because this gave reproducible results.

Differential Display

To ensure that bands identified as differentially expressed were reproducible, differential displays were generated with RNA from RPE cells of donor A (fourth passage) treated with or without homologous vitreous, donor B (seventh passage) treated with or without homologous vitreous, and donor B (sixth passage) treated with or without vitreous from donor A. A section of the display generated using T\(\beta\)VG and AP6 (GenHunter) is shown in Figure 2A. A differentially displayed band that was downregulated in the presence of vitreous and that migrated at a similar position to a 395-nucleotide fragment in the marker lane was observed (compare control [C] lanes and vitreous-treated [V] lanes in Fig. 2A, groups 1, 2, and 3). This band was excised from a control lane, amplified, and cloned. The resulting “mini-library” was screened by the method of M. Liu and F. Berger (unpublished data) (Fig. 3). Of 13 clones that contained an insert (Fig. 3A), 6 appeared to correspond to a differentially expressed insert in that they hybridized to a probe made by PCR of control cell cDNA (Fig. 3B), but not to a probe made by PCR of vitreous-treated cell cDNA (Fig. 3C). Four of the clones that appeared to be differentially expressed were sequenced. All four clones contained the identical 397-bp insert, which had the T\(\beta\)VG anchor primer at each end, and thus represented 369 nucleotides of the putatively differentially expressed mRNA. The sequence of the insert corresponded to the extreme 3'-end of the 6757-nucleotide mRNA for human basic FGF14 (FGF-2) (GenBank J04513). The fragment had been amplified using the T\(\beta\)VG primer at the junction with the poly(A) tail on the sense strand and using the T\(\beta\)VG primer hybridized to an A-rich region on the anti-sense strand (with mismatches at ~8 and ~11 nucleotides from the 3'-end of the primer). This would imply that if the differentially displayed fragment were indeed from FGF-2 mRNA, it should be seen on all displays using the T\(\beta\)VG primer, unless the additional AP interfered. Indeed, as expected, a differentially displayed fragment of the same size was seen when differential displays with T\(\beta\)VG and AP6 (Fig. 2A), AP2 (Fig. 2B), or API (data not shown) were examined. This gives additional support to the reliability of the differential display process.

In Situ Hybridization

The human FGF-2 cDNA sequence codes for a 6757-nucleotide mRNA, the protein coding sequence of which corresponds to nucleotides 302 to 934. The cloned sequence from the differential display reactions corresponded to nucleotides 6389 to 6757. Because RPE cells contain 7 Kb, 3.7 Kb, and other smaller species of FGF-2 mRNA, it is possible that differential expression was associated with differential processing of the 3'-end rather than modulation of the levels of coding sequence. Therefore, part of the coding region of the mRNA was cloned into pCR-Script and used to make anti-sense and sense digoxigenin-labeled riboprobes that were used for in situ hybridization. The results (Fig. 4) indicated that FGF-2 was expressed in virtually all the untreated cells (Fig. 4B) and that it was markedly downregulated in cells treated for 48 hours with 25% vitreous from donors B (Fig. 4D) or C (data not shown). Actin was used as a control probe to show that this
FGF-2 and RPE Cells

Ribonuclease Protection Assays

To examine further the modulation of FGF-2 mRNA in control versus vitreous-treated cells, the relative amounts of this mRNA were determined using ribonuclease protection assays. Results were normalized using the expression of GAPDH mRNA as an internal control and indicated (Fig. 5) that there was a decrease in the expression of FGF-2 mRNA, although the extent varied (compare control [C] and vitreous-treated [V] lanes for the different donors). The nature of the substrate (tissue culture plastic or ProNectin P) did not seem to affect the degree of downregulation in the case of donor D.

FGF-2 Expression in Cultured Human RPE Cells

Because the differential display results and the ribonuclease protection assays suggested that the mRNA for FGF-2 was decreased in vitreous-treated cells, the levels of FGF-2 protein were measured. Cells and medium were examined after concentration of FGF-2 by binding to heparin-Sepharose beads, elution of bound proteins, gel electrophoresis, and immunoblot analysis to detect the presence of FGF-2 protein. No FGF-2 was detected in the medium (data not shown), but three putative FGF-2 bands were observed that were associated with the cellular fraction (Fig. 6A). From their apparent molecular weight on the gel, these were assumed to correspond to the 18-kDa form (band L) of FGF-2 and the higher molecular weight forms translated from the same mRNA but from alternate start codons (band H and the band indicated by arrowhead in Fig. 6A). The lower two bands, which had apparent molecular weights of 18 kDa (Fig. 6A, band L) and 24 kDa (Fig. 6A, band H) in this system, were quantified by densitometry. There was a decrease of more than 50% in the amount of both forms of FGF-2 after a 48-hour incubation in the presence of 25% vitreous compared with control cells incubated for 48 hours in the absence of vitreous (Fig. 6B). Cells incubated for 24 hours in control medium and then for 24 hours in the presence of 25% vitreous showed intermediate values (Fig. 6B).

Effects of FGF-2 on Properties of D407 RPE Cells

From the experiments outlined above, it appears that down-modulation of FGF-2 mRNA and protein may occur in response to vitreous treatment of human RPE cells in culture. To determine whether decreased FGF-2 expression might play any role in the effect of vitreous on RPE cells in culture, the effect of FGF-2 on a line of human RPE cells was examined (Fig. 7). Cells grown in the presence of medium alone had an epithelial morphology (Fig. 7A, bottom). If cells were grown in the presence of 25% vitreous, they assumed a more elongated morphology initially (not shown), continued to proliferate, and eventually formed multilayered sheets (Fig. 7B, bottom). The addition of human recombinant FGF-2 (50 ng/ml) to the vitreous-containing medium resulted in cells that appeared very similar to those incubated in medium alone (Fig. 7C, bottom). Thus, added FGF-2 appeared to be able to counteract this aspect of vitreous-induced phenotypic changes. Although it is difficult to quantify morphologic data in this kind of experiment, independent observers who were unaware of cell treatment examined the cultures and scored the cells for their epithelial/fibroblastic appearance on a scale of 1 to 4. A typical experiment in which D407 cells were treated with vitreous for 24 hours gave scores of 1 for control, 4 for vitreous, and 1.5 for vitreous plus FGF-2 (50 ng/ml). A typical experiment with low

was not due to generalized loss of mRNA (Fig. 4A, 4C). The sense-probe results (Fig. 4E, 4F) indicated that the signal was specific.

FIGURE 3. Screening of bacterial colonies for the presence of a differentially expressed insert. The band at -395 nucleotides on the differential display was excised from a control lane (Fig. 2A), reamplified with the same primers (F12VG and AP6), polished with Pfu polymerase, ligated into pCR-Script, transformed into Escherichia coli, and the bacteria plated on ampicillin/X-gal plates. White or pale blue colonies were selected and grown on replicate nitrocellulose filters. One filter (A) was hybridized with a radioactive probe made by amplification of the excised fragment in the presence of [α-32P]dCTP to ascertain if the insert was indeed present in the colonies. A second filter (B) was hybridized with a radioactive probe made by PCR amplification, in the presence of [α-32P]dCTP, of cDNA derived from vitreous-treated cells. The colony marked (*) represents a colony transformed with pUC 18 (negative control).
Figure 4. In situ hybridization of control or vitreous-treated cells with actin or fibroblast growth factor 2 (FGF-2) probes. Subconfluent human retinal pigment epithelial (RPE) (donor B) cells growing on multichambered slides were treated with medium (A, B, E, F) or with medium containing 25% vitreous (C, D) and incubated for 48 hours. The cells were then processed for in situ hybridization and hybridized with a digoxigenin-labeled antisense RNA probe for actin mRNA (A, C) or for the protein-coding region of FGF-2 mRNA (B, D). Panels labeled (A) and (C) correspond to different chambers of the same slide, as do (B) and (D). The
FIGURE 5. Estimation of the amount of fibroblast growth factor 2 (FGF-2) mRNA by ribonuclease protection assays. Cells were treated with vitreous (V) or without vitreous (C) for 48 hours, and RNA extracted. RNA (2 μg) was hybridized with radioactively labeled antisense RNA corresponding to the coding region of FGF-2 and antisense RNA corresponding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal loading control). After ribonuclease digestion, the amount of protected probe was determined by electrophoresis on 6% acrylamide-7M urea gels and autoradiography (A). Lanes 1 and 2: RNA from donor B cells ± vitreous (corresponds to group 2 in Fig. 2); lanes 3 and 4: RNA from donor A cells ± vitreous (corresponds to group 1 in Fig. 2); lanes 8 and 9: RNA from donor D cells ± vitreous; lanes 10 and 11: as in lanes 8 and 9, but the cells were grown on ProNectin F- coated plastic; position of band corresponding to GAPDH RNA (*), position of band corresponding to FGF-2 RNA (arrowhead). Lane 5: No donor RNA added (r), negative control. Lanes 6 and 12: undigested control (u), ribonuclease protection assay performed with no donor RNA and with ribonuclease omitted from the ribonuclease digestion buffer, only 10% of product loaded compared with other lanes. Lane 7: RNA marker (m), bottom band is 300 nucleotides, middle band is 400 nucleotides, upper band is 500 nucleotides. Size of probes before digestion—FGF-2, 440 nucleotides; GAPDH, 383 nucleotides. Expected size of probes after digestion—FGF-2, 358 nucleotides; GAPDH, 316 nucleotides (others have reported a doublet for this probe [Ambion]). The results were quantified by densitometry. The ratio of FGF-2 RNA to GAPDH RNA, in arbitrary units, is shown graphically in (B). The lane numbers on the x-axis correspond to those in (A).

passage donor cells treated with vitreous for 48 hours gave scores of 1 for control, 3.5 for vitreous, and 1.5 for vitreous plus FGF-2 (50 ng/ml). Scores cannot be validly averaged between experiments because the lack of stringent criteria for a score of 4 and the variability in the degree of response according to the donor of the cells and of the vitreous meant that, although scores were consistent within an experiment, they could not be compared between experiments. It thus appears that FGF-2 might also be able to prevent the effects of vitreous on low passage donor cells.

DISCUSSION

It is known from in vivo and in vitro evidence that exposure of RPE cells to vitreous, particularly in the presence of serum or serum-derived factors, results in changes in the morphologic and biochemical properties of RPE cells.1 The differential display technique was used in an in vitro system to investigate changes in steady state levels of mRNA that might occur during vitreous treatment. FGF-2 (basic FGF) was observed to undergo downregulation at the mRNA level as determined by differential display, differential colony hybridization, in situ hybridization, and ribonuclease protection assays and at the protein level as determined by immunoblot analysis. In situ hybridization suggested that the decrease in mRNA expression occurred in most cells of the culture and that it was not due to a subpopulation of cells. Because increasing the amount of FGF-2 by the addition of FGF-2 to the medium prevented vitreous-induced phenotypic changes in a line of human RPE cells, a decrease in FGF-2 may indeed play an important role in the vitreous modulation of these cells. Moreover, because the addition of exogenous FGF-2 can prevent at least some of the effects of vitreous, it appears that the downregulation of the FGF-2 gene in the presence of vitreous is not merely the result of feedback inhibition due to FGF-2 present in vitreous.

These findings raise the question as to whether FGF-2 is present in human vitreous and if so at what concentration. Baird et al.32 examined ocular fluid from previously "vitrectomized" eyes and found detectable levels of FGF-2 (from 0.6 to 8.9 pmoles/ml, or approximately 10 to 140 ng/ml). The only patient with a value of more than 26 ng/ml was the only one with advanced proliferative diabetic retinopathy. Sivalingam et al.33 examined vitreous from patients with various forms of retinal disease and found that FGF-2 was present in D3.

To estimate background levels of signal, control cells were hybridized with digoxigenin-labeled sense riboprobes corresponding to the same region of actin mRNA (E) or FGF-2 mRNA (F). (Vitreous-treated cells hybridized with sense probes gave similar results to E and F; data not shown). All pictures were taken at the same time after addition of the alkaline-phosphatase substrate.
FIGURE 6. Estimation of the relative amounts of fibroblast growth factor 2 (FGF-2) protein in control or vitreous-treated cells by immunoblot analysis. Subconfluent, low passage, human donor cells were treated with medium for 48 hours (48 hr control) (lanes 2 and 3), medium for 24 hours and then medium containing 25% vitreous for 24 hours (24 hr control + 24 hr vitreous) (lanes 4 and 5), or medium containing 25% vitreous for 48 hours (48 hr vitreous) (lanes 6 and 7). The cells were lysed, and the FGF-2 from equivalent amounts of cell protein was concentrated by adsorption to heparin-Sepharose, followed by elution with 6 M NaCl. The eluted material and human recombinant FGF-2 (slightly shorter than the natural form) (lane 7) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the relative amount of FGF-2 present was determined by immunoblot analysis using the ECL system (Amersham) (A). Bands, identified as L (low-molecular-weight form of FGF-2) or H (higher-molecular-weight forms of FGF-2) in lanes 2 to 7, were quantified by densitometry, and the average for each time point is shown in (B). The band indicated by an arrowhead is probably another higher-molecular-weight form of FGF-2, but was too faint to quantify reliably.

undetectable (<2.4 ng/ml) in 25 of 36 patients and that its presence (at concentrations >30 ng/ml) in the remaining 11 patients appeared to correlate with active vasoproliferative disease. Boulton et al. 34 also examined vitreous from patients with retinal disease, but used a more sensitive assay and found FGF-2 in all vitreous samples tested in the range of 0.3 to 6.3 ng/ml, with the higher levels (>3 ng/ml) found in non-insulin treated diabetic patients with active neovascularization. It thus appears that FGF-2 may be present in normal human vitreous, but the normal levels are not clear in view of the disparity between the above reports and the fact that samples were from patients with retinal disease. Although it would not be surprising if FGF-2 were present at low concentrations in normal vitreous, considering that the

FIGURE 7. Comparison of control, vitreous-treated, or vitreous + fibroblast growth factor 2 (FGF-2)-treated cells. A human retinal pigment epithelial cell line (D407)7 was treated when subconfluent with fresh medium (A, a), fresh medium containing 25% vitreous (B, b), or fresh medium containing 25% vitreous + 50 ng human recombinant FGF-2/ml (C, c). After 70 hours, the cells were examined directly by light microscopy using Hoffman Contrast Modulation optics (A, B, C) or fixed, sectioned, stained with toluidine blue, and then examined by light microscopy (a, b, c).
retina contains FGF-2, this FGF-2 may not be biologically available, because the vitreous also contains FGF-2 binding proteins. The latter include soluble forms of at least one of the high affinity FGF receptors (FGFR), probably FGFR-1, and thus any FGF-2 present in the vitreous of normal individuals may not be available for cell surface-receptor binding. Thus, not only may vitreous treatment result in a decreased level of FGF-2 mRNA and protein in human RPE cells, but it may also sequester extracellular FGF-2.

Although FGF-2 was originally named for its growth-promoting potential, it can have many different activities on a variety of cells, including effects on differentiation, migration, and survival. The possibility that the effect of vitreous on the properties of RPE cells may be mediated in part via decreased levels of FGF-2 is intriguing, because FGF-2 has been reported to play an important role in differentiation in various tissues of the eye such as the lens, neural retina, and RPE. Camposchiro and Hackett found that FGF-2 was active in promoting expression of markers of differentiated RPE cells in culture. It is thus possible that downregulation of this gene on exposure to the vitreous might be involved not only in the dedifferentiation of RPE cells but also in the dedifferentiation observed when differentiated cells of other types gain access to the vitreous.

Multiple forms of FGF-2 can be translated from a single species of mRNA by use of alternate start codons, giving rise to a lower-molecular-weight form (~18 kDa), which can be exported from the cell, and to three higher-molecular-weight forms that possess nuclear localization signals and may have different biological effects. Vitreous caused a decrease in the total amount of both the lower-molecular-weight and at least one of the higher-molecular-weight forms (the 22 kDa and 22.5 kDa forms cannot be distinguished on our gel system). Thus, the effect of vitreous may be associated with a decrease in either form. However, the prevention of the vitreous-induced changes in the D407 RPE cell system on the addition of recombinant FGF-2 (146 amino acids, a slightly truncated form of the 18-kDa FGF-2) suggests that some effects can be mediated by an extracellular form that lacks the nuclear localization signal. The importance of binding of extracellular FGF-2 to cell-surface high affinity receptors versus that of intracellular FGF-2 is currently being investigated.

The cultured RPE cell system is useful in searching for alterations in the expression of genes that may play a role in the phenotypic changes observed in the presence of vitreous, because such genes are likely to be particularly important in the initial events in the development of proliferative retinopathy. However, in vivo experiments will be needed to confirm any hypotheses as to their role in pathogenesis. Screening for other effects exerted by vitreous on cultured RPE cells and determination of whether FGF-2 might play a role in modulating these is currently under way by our group. Once RPE cells have gained access to the vitreous and altered their phenotypic properties, synthesis of growth factors and responses to growth factors will not be necessarily the same as in earlier stages of the disease. Thus, reports of increased levels of FGF-2 in vitreous from patients with proliferative vitreoretinopathy, particularly in association with neovascularization, do not contradict our results.

References


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