Differential Effects of TGFβ and Vitreous on the Transformation of Retinal Pigment Epithelial Cells

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PURPOSE. In proliferative vitreoretinopathy retinal pigment epithelial (RPE) cells undergo epithelial-mesenchymal transformation (EMT). Vitreous and transforming growth factor-β (TGFβ) have been implicated in this EMT. The role of TGFβ in the vitreous-mediated transformation of low-passage human RPE cells was investigated.

METHODS. Cells were treated with vitreous or TGFβ2. SB431542 was used to inhibit TGFβ signaling. Morphology was investigated using phase-contrast or confocal microscopy. Motility was measured using a monolayer-wounding assay. Invasion was determined using basement membrane matrix-based assays. Gene expression was measured by quantitative PCR, immunohistochemistry, or immunoblotting.

RESULTS. Changes in phosphorylation or cellular localization of Smad -2, -3, or -4 indicated a TGFβ-like activity in vitreous. Cortical actin filaments in untreated cells were replaced by stress fibers after TGFβ treatment, but peripheral actin aggregates were seen in vitreous-treated cells. SB431542 did not block the morphologic change induced by vitreous. Vitreous-treated cells exhibited increased motility and invasion, whereas TGFβ-treated cells did not. However, SB431542 decreased vitreous-mediated changes in motility and invasion. The levels of mRNA for genes indicative of myofibroblast differentiation (α-SMA and CTGF) were increased by treatment with TGFβ but suppressed by vitreous. TGFβ or vitreous caused increased expression of Snail1.

CONCLUSIONS. Vitreous or TGFβ caused a fibroblast-like morphology and induced Snail1, a marker of EMT. TGFβ activity in vitreous was necessary but not sufficient for the vitreous-induced motile, invasive phenotype. However, differences in the cytoskeletal organization and in the expression of CTGF and α-SMA suggested that TGFβ-treatment caused differentiation along a myofibroblast pathway, whereas vitreous treatment suppressed myofibroblast formation. (Invest Ophthalmol Vis Sci. 2009;50:5965–5974) DOI:10.1167/iovs.09-3621

Proliferative vitreoretinopathy (PVR) is a major cause of retinal detachment and vision loss in developed countries and results from the formation of fibrotic epiretinal membranes either on the surface of the retina or within the vitreous. Retinal pigment epithelial (RPE) cells are almost always present in epiretinal membranes and are believed to have a profibrotic role in the development of PVR. RPE cells in the epiretinal membranes, which have a fibroblast-like morphology, undergo epithelial-mesenchymal transformation (EMT) and are considered to be contractile, leading to the contraction of epiretinal membranes and to retinal detachment.

Important risk factors for PVR include inflammation and the breach of the blood-retinal barrier, allowing leakage of serum or blood into the eye and breaks in the neural retina. Retinal tears allow vitreous to come in contact with the apical surface of the retinal pigment epithelium. A role for vitreous in the transformation of RPE cells is supported by the observation that RPE cells cultured in vitreous-containing medium undergo a change in morphology that is similar to the EMT seen in RPE cells in epiretinal membranes. In this process, quiescent, columnar RPE cells change shape to a fibroblast-like appearance and become motile.

Identification of components of vitreous that cause RPE cell transformation could provide an avenue to treat PVR or inhibit its onset. Increased concentrations of TGFβ in the vitreous of patients with PVR have been especially implicated in the progress of PVR because of the strong profibrotic effects of TGFβ. Exogenous TGFβ can replicate certain aspects of RPE cell transformation, and mice lacking Smad3, a transcription factor involved in TGFβ signaling, are resistant to retinal fibrosis. However, it is not resolved whether TGFβ in the vitreous is vital for the transformation of RPE cells. Conversely, it is also not clear whether this growth factor is sufficient for transformation or whether other factors act in concert with TGFβ to elicit transformation of RPE cells when they come in contact with vitreous.

To determine whether TGFβ is involved in the transformation of vitreous-treated cultured RPE cells, we have examined whether TGFβ alone can replicate some of the effects of vitreous and whether inhibition of TGFβ signaling induced by vitreous can inhibit the transformation process. EMT is frequently defined as a morphologic change, but we have investigated several other properties that are typically associated with EMT and fibrosis: changes in cytoskeletal organization, motility, and the ability to invade extracellular matrix. We have also examined the expression of certain genes that typically exhibit altered expression during EMT. We find that though TGFβ is necessary for some aspects of RPE cell EMT, it alone cannot explain all the changes. In the case of TGFβ, RPE cells appear to differentiate along a myofibroblast pathway, but this is not the case in the presence of vitreous, which appears to suppress myofibroblast formation.
METHODS

RPE Cells

Human donor eyes were obtained from Lifepoint (Lexington, SC) or the Oregon Lions' Eye Bank (Portland, OR). The protocol adhered to the tenets of the Declaration of Helsinki for research involving human tissue. Eyes were not used if there was a known history of retinal disease or diabetes. RPE cells were grown as previously described in heat-inactivated serum-containing medium or the same medium supplemented with 25% human vitreous. Recombinant human TGFβ2 (R&D Systems, Minneapolis, MN), various inhibitors, or all of these. The TGFβ reporter assay had not progressed beyond the very early stages of this process. The cells used in the scratch assay had not progressed beyond the very early stages of this process.

Immunofluorescence Microscopy

Cells growing on glass coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. They were then incubated with fluorescence-labeled phallloidin to stain actin. Vinculin was detected using a mouse anti-vinculin antibody (Clone V284; Upstate Biotechnology, Waltham, MA) and Alexa 594-conjugated rabbit anti-mouse IgG as a secondary antibody. Smads were detected using a goat antibody to Smad4 (sc-1909; Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit antibody that binds to both Smad2 and Smad3 (Smad2/3, sc-8332; Santa Cruz Biotechnology). Primary Smad antibodies were detected using Cy3- or Cy2-labeled donkey IgG (Jackson ImmunoResearch, West Grove, PA). A rabbit antibody against a synthetic peptide sequence was used to detect Snail1 (Ab177732; Abcam). Immunofluorescence was detected with CDP-Star chemiluminescence reagent (NEL 602; Perkin Elmer). Luminescence was observed using imaging technology (2000MM Image Station; Eastman Kodak, Rochester, NY). For Snail1 detection, cell extracts were analyzed in a similar fashion using the rabbit anti-Snail1 antibody (Ab177732; Abcam).

Quantitative Real-Time PCR

Details of the primers used for quantitative real-time PCR (qPCR) analysis of ribosomal protein large P0 (RPLP0), α-smooth muscle actin (α-SMA), Snail1, or connective tissue growth factor (CTGF) mRNA expression are shown in Table 1. The specificity of all primer pairs was checked by direct sequencing of the PCR product. qPCR was performed using RPLP0 as an internal standard, as described previously. Results were calculated using the Pfaffl method, as described previously.

Motility

Cells were seeded on 12-well culture plates (BD Biosciences) and allowed to grow to for approximately 2 days after confluence in normal serum-containing medium. A scratch was made in the monolayer using a sterile pipette. Detached cells were removed by rinsing, and the medium was replaced with normal serum-containing medium (with various additives) with or without 5 μM aphidicolin (Sigma-Aldrich, St Louis, MO) to inhibit cell division. The migration of cells into the scratched area was recorded using phase-contrast microscopy. The micrographs were analyzed with a graphics editing program (Photoshop 6; Adobe, San Jose, CA). Two straight lines were drawn at the migration front edges, and the distance between the two lines was measured in arbitrary units. The distances in the control wells at 24 hours were set to be 100, and all other distances were expressed relative to the control well value. Hence, values less than 100% of control indicated increased migration. Adult human RPE cells in culture require a very long postconfluent period (weeks or months) to develop the mature, highly differentiated RPE phenotype, which may include reexpression of pigmentation, the cells used in the scratch assay had not progressed beyond the very early stages of this process.

Invasion

Approximately 30,000 cells were seeded onto the upper surface of basement membrane matrix (Matrigel; BD Biosciences, San Jose, CA)-coated culture well inserts in a 24-well plate serum-containing medium. After a 3-hour incubation to allow cell attachment, the medium was removed and replaced with serum-containing medium with various additives. After 20 hours, the cells on the upper surface were removed with a cotton swab, and the cells that had migrated to the lower surface of the insert (though the basement membrane matrix)
**Inhibition of TGFβ Signaling**

SB431542 (Tocris Bioscience, Ellisville, MO), a potent and selective inhibitor of the TGFβ type 1 receptor (activin receptor-like kinase [ALK5]; IC50 = 94 nM) and of ALK4 and ALK7, was added to serum-containing medium at a concentration of 20 μM.

**RESULTS**

**Effects of Vitreous and TGFβ on the Morphology of Human RPE Cells**

In PVR, RPE cells detach from their basement membrane, divide, migrate toward and into the vitreous, and undergo EMT-like changes. Our in vitro system examines the initial events when dividing RPE cells are treated with vitreous and, hence, is a model for some of the early stages of PVR as the cells encounter vitreous components, a stage at which the cells are presumed to be subconfluent. EMT results in a morphologic change in which cuboidal cells become fibroblast-like. TGFβ signaling has frequently been implicated in this process, both in vivo and in vitro. Several studies have shown that vitreous can induce an EMT-like change in the morphology of human RPE cells similar to that seen with TGFβ in RPE cells and other epithelial cell types. To compare the effects of vitreous and TGFβ2 on RPE cell morphology, cells were grown in serum-containing medium for 48 hours in the presence or absence of vitreous or TGFβ2. Subconfluent cultured RPE cells, growing in normal medium, grew as clumps with a flattened cuboidal morphology (Fig. 1A). In the presence of vitreous, the cells became elongated (spindle-shaped) and moved apart so that there were few regions of intercellular contact (Fig. 1D), suggesting increased motility. They also appeared less flat, suggesting less extensive adhesion to the substrate. In the presence of TGFβ, the cells also became spindle-shaped but their edges were more flattened and had prominent finger-like projections; they retained more intercellular contacts, suggesting that there may be less of a motility increase compared with vitreous (Fig. 1B).

**Differential Effects of Vitreous and TGFβ on the RPE Cell Cytoskeleton**

The actin cytoskeleton of RPE cells that were grown to near confluence was arranged as a circumferential belt of microfilaments (Fig. 2A), an appearance similar to that observed in vivo. Vitreous treatment resulted in the loss of the cortical filaments and their redistribution as peripheral aggregates (Fig. 2B). It has been shown previously that this actin accumulation is at the leading edge of the motile cell at which prominent vinculin-containing focal adhesions are formed. In TGFβ-treated cells, the cortical filaments were also lost, but they were replaced by prominent stress fibers extending throughout the length of the cell (Fig. 2C). Thus, the distinct morphologic changes induced by vitreous and TGFβ are associated with different changes in the cytoskeleton.

**TGFβ-Like Activity in Vitreous**

Vitreous contains many TGFβ-binding and -inactivating proteins. In addition, vitreous treatment of cultured RPE cells modulates the expression of some of these genes. Thus, levels of total TGFβ, as measured by such techniques as ELISA, may not reflect activation or signaling. To determine whether vitreous contains active TGFβ, the activation of specific Smad transcription factors was investigated because this would indicate TGFβ-like activity as opposed to total levels, inactive and active, of this cytokine.

When TGFβ binds to its receptors, the latter are phosphorylated and, in turn, phosphorylate Smad2 and Smad3 that then combine with the co-Smad (Smad4) and enter the nucleus. To investigate Smad phosphorylation, cells were serum-starved for 16 hours and then incubated in vitreous-containing medium (also without serum) for up to 90 minutes. Without vitreous, the cells contained little detectable phospho-Smad2 (Fig. 3A, lane 1), whereas, when serum was present, phospho-Smad2 was readily detectable (data not shown). Addition of vitreous in the absence of serum resulted in Smad2 phosphorylation within 15 minutes (Fig. 3A, lane 2), after which the level of Smad2 phosphorylation diminished (Fig. 3A, lanes 3–5). In contrast, there was little change in the level of total Smad2 (Fig. 3B). This indicates that vitreous contains TGFβ-like activity because Smad2 activation is specific to TGFβ signaling.

The possibility that vitreous in the presence of serum could activate Smad4 or Smad2/3 was investigated by observing the translocation of these transcription factors into the nucleus. When RPE cells growing in normal serum-containing medium were stained with hematoxylin and eosin and examined by microscopy, the actin cytoskeleton of RPE cells that were grown to near confluence was arranged as a circumferential belt of microfilaments, an appearance similar to that observed in vivo. Vitreous treatment resulted in the loss of the cortical filaments and their redistribution as peripheral aggregates. It has been shown previously that this actin accumulation is at the leading edge of the motile cell at which prominent vinculin-containing focal adhesions are formed. In TGFβ-treated cells, the cortical filaments were also lost, but they were replaced by prominent stress fibers extending throughout the length of the cell. Thus, the distinct morphologic changes induced by vitreous and TGFβ are associated with different changes in the cytoskeleton.

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**FIGURE 1.** Morphology of cultured human RPE cells after incubation for 48 hours in serum-containing medium under various conditions. (A) Control cells form an epithelial monolayer. (B) TGFβ2 (5 ng/mL) causes the cells to become more fibroblast-like. (C) The effect of TGFβ2 (5 ng/mL) is reduced by SB431542 (20 μM), an inhibitor of TGFβ signaling. (D) Vitreous (25%) causes a morphologic transformation and the cells appear to move apart. (E) The effect of vitreous is not prevented by SB431542 (20 μM). (F) SB431542 (20 μM) alone does not affect the appearance of cells.
Effect of Inhibition of TGFβ Signaling on Vitreous-Induced Changes in RPE
Cell Morphology

SB431542 is a TGFβ type 1 receptor antagonist.34 This agent did not affect the cuboidal, closely packed morphology of RPE cells growing in normal serum-containing medium (Fig. 1F) but, as expected, abolished the morphology change that resulted from TGFβ (Fig. 1C). However, when vitreous-treated RPE cells were coincubated with SB431542, the morphology change was not abolished; indeed, it was exacerbated (Fig. 1E). This indicates that other factors in vitreous can influence the morphology of RPE cells in the absence of TGFβ signaling.

Effect of Inhibition of TGFβ Signaling on Vitreous-Induced Changes in the Cytoskeleton of RPE Cells

Treatment of cells in normal medium with SB431542 did not alter the circumferential distribution of actin (Fig. 2D) and did not alter actin distribution in vitreous-treated cells (Fig. 2E). However, SB431542, as expected, restored the circumferential distribution of actin fibers in TGFβ-treated cells (Fig. 2F). Thus, it appears that changes in actin organization as a result of vitreous treatment are not the result of TGFβ in the vitreous.
The shape and motility of epithelial cells is influenced by their ability to form focal contacts (adhesions) with their underlying extracellular matrix. We therefore determined the distribution of focal adhesions using antibodies against vinculin. In epithelial RPE cells, vinculin was found at the edges of the cell (Fig. 2G) and underneath as “spot welds” to the extracellular matrix. There was also considerable reticular cytoplasmic staining (Fig. 2G). TGFβ/H9252 caused a redistribution of vinculin from the cytoplasm to focal adhesions that terminate the long stress fibers (Fig. 2I), and this was, as expected, reversed by SB431542 (Fig. 2L). In vitreous-treated cells, far fewer focal adhesions were seen (Fig. 2H), and much of the vinculin was reticular in the cytoplasm; those focal adhesions that were formed in these cells were at the edges of the cell, and this was not affected by SB431542 (Fig. 2K).

These observations suggest that in the presence of vitreous, the cells move away from one another but form prominent stress fibers indicative of contractile cells.

**Vitreous but Not TGFβ Increases RPE Cell Motility, but Increased Vitreous-Mediated Motility Is TGFβ Dependent**

Incubation of human RPE cells in the presence of vitreous leads to increased migration,13 and, given that TGFβ has been shown to enhance human RPE cell motility under some circumstances,21,22 it may be the component responsible. However, bovine44 and chick45 RPE cell motility is not enhanced by TGFβ.

The motility of RPE cells was determined using an assay in which a confluent monolayer was scratched, and the rate of migration of the cells into the scratch was assessed. To ensure that cell motility rather than cell division was being measured, the experiments were carried out with or without aphidicolin, a DNA synthesis inhibitor that does not affect migration,31 and...
the results were similar (data not shown). Vitreous-containing medium, in the presence of aphidicolin, caused the cells to fill in the scratch more rapidly than normal medium (compare Figs. 5A and C). TGFβ did not replicate the effects of vitreous; rather, its effects were small and inconsistent (Fig. 5B). SB431542 had no effect when added alone to the cells (Fig. 5E); however, it slowed vitreous-induced migration (Fig. 5D). The results of three such experiments are summarized in Figure 5G. Thus, although TGFβ did not cause increased mobility by itself, it was an essential factor in the vitreous-mediated increase in RPE cell motility because the presence of SB431542 abolished the vitreous-mediated increase in motility.

**Effect of Vitreous on the Invasion of Basement Membrane Matrix by RPE Cells**

In PVR, RPE cell migration into the vitreous not only involves increased mobility, it involves invasion of the extracellular matrix of the vitreous. From the effects of vitreous and TGFβ on motility, it would be predicted that only the former would greatly alter the invasive potential of the cells. This was the case. RPE cells were seeded in culture well inserts coated with basement membrane matrix (Matrigel; BD Biosciences), a natural extracellular matrix produced by mouse EHS sarcoma cells, and the number of cells that penetrated the basement membrane matrix to appear on the lower surface of the culture well insert was determined. Vitreous promoted the invasion of basement membrane matrix, but the effect of TGFβ was significantly less (Fig. 6). However, the effect of vitreous was inhibited by SB431542 (Fig. 6). Thus, although TGFβ alone did not cause major changes in invasiveness or motility, it did play an important role in the ability of vitreous to increase both.

**Vitreous and TGFβ Induce the Expression of Genes Associated with EMT, but Only TGFβ Induces the Expression of Genes Typical of Myofibroblast Formation**

TGFβ has been reported to induce many epithelial cells to transform into myofibroblast-like cells, and this is supported by gene array studies of changes in RPE cell gene transcription (our unpublished data, 2006). Our previous report showed that, after 12 to 48 hours of vitreous treatment, the gene expression profile is consistent with the downregulation of the TGFβ pathway and of genes associated with myofibroblasts and the upregulation of BMP signaling. The present study shows that vitreous-induced changes observed in RPE cell morphology and cytoskeletal organization are independent of TGFβ, but TGFβ is necessary for the vitreous-mediated increase in RPE cell motility and invasion. We therefore investigated whether changes in gene expression in TGFβ-treated RPE cells reflect a myofibroblast differentiation pathway and whether the more complex vitreous led to another pathway of differentiation that nevertheless contained a TGFβ-mediated component.

Snail1 (SNAI1) is a transcription repressor that is often used as a marker for EMT. Its expression is typically upregulated in EMT, often by TGFβ. After 24 hours treatment of RPE cells with either vitreous or TGFβ, Snail1 mRNA expression was elevated approximately threefold (Fig. 7). Similarly, immunofluorescence microscopy and immunoblotting showed an increase in Snail1 protein (Fig. 8) with both vitreous and TGFβ. TGFβ in vitreous was necessary for the elevation of Snail1 mRNA expression because the latter was completely abolished by SB431542 (Fig. 7).

To determine whether treatment with either TGFβ or vitreous leads to differentiation of human RPE cells into myofibroblasts, two markers of myofibroblast formation were investigated. CTGF expression is controlled by TGFβ and, in turn, controls the expression of αSMA, the most widely observed marker of myofibroblast formation. TGFβ caused RPE cells to express increased levels of both CTGF mRNA (2-fold to 8-fold, depending on the primary cell donor) and αSMA mRNA.

![Figure 5](https://iovs.arvojournals.org/)

**Figure 5.** Changes in the motility of RPE cells. Cells were grown to confluent monolayers, and a scratch was made in the monolayer with a pipette tip. Cells were then grown for another 24 hours in various media supplemented with aphidicolin to inhibit proliferation. The rate at which the cells repopulated the denuded area was observed. Lines show the approximate edge of the scratch. (A) Control cells in serum-containing medium. (B) Cells in serum-containing medium plus TGFβ2 (5 ng/mL). (C) Cells in serum-containing medium plus 25% vitreous. (D) Cells in serum-containing medium plus 25% vitreous plus SB431542 (20 μM). (E) Control cells in serum-containing medium plus SB431542. (F) Cells in serum-containing medium plus TGFβ2 plus SB431542. (G) Summary of three experiments in which migration was measured in duplicate using scratch assays with three different vitreous-cell donor pairs.
TGFβ is frequently present in biological tissues in a latent, inactive form. Our results show that TGFβ is, indeed, present in human vitreous in the active form because vitreous causes changes in Smad distribution and phosphorylation in RPE cells. TGFβ is, however, not required for the morphologic or cytoskeletal changes that are observed in the presence of vitreous because an inhibitor of TGFβ receptor 1 (ALK5) signaling, SB431542, does not reverse the vitreous-induced changes, indicating the role of other signaling molecules. In contrast, TGFβ is necessary for the effect of vitreous in increasing motility and invasion given that there are reductions in both when SB431542 is added to the vitreous.

Unlike epithelial cells, many mesenchymal cells can invade and migrate through the extracellular matrix. Increased motility and increased invasiveness are often part of EMT and characteristic of RPE cells in PVR. Vitreous contains many chemotactic factors that may cause the transformed RPE cells to migrate to the vitreous cavity. Based on the results reported here, it is apparent that TGFβ is necessary for vitreous-induced migration but that alone it is not sufficient. This is in contrast to the results of others who showed that TGFβ1 or TGFβ2 alone could stimulate RPE cell migration. Although we observed that a few cultures of low-passage RPE cells showed increased migration in response to TGFβ alone, the magnitude of the response was small compared with vitreous, and the results were inconsistent.

Increased motility requires alteration of the cytoskeleton and interactions with the extracellular matrix, and our results show that TGFβ and vitreous have very different effects. When subconfluent, low-passage RPE cells are transformed by TGFβ, they reorganize their cytoplasmic and circumferential actin filaments into prominent stress fibers terminating in focal adhesions; as a result, they appear similar to myofibroblasts. This is supported by the observation of increased expression of such myofibroblast-specific genes as αSMA and CTGF. Interestingly, in a mouse model of PVR, TGFβ signaling has been shown to play a critical role in myofibroblast differentiation of RPE cells. Unlike epithelial cells, many mesenchymal cells can invade and migrate through the extracellular matrix. Increased motility and increased invasiveness are often part of EMT and characteristic of RPE cells in PVR. Vitreous contains many chemotactic factors that may cause the transformed RPE cells to migrate to the vitreous cavity. Based on the results reported here, it is apparent that TGFβ is necessary for vitreous-induced migration but that alone it is not sufficient. This is in contrast to the results of others who showed that TGFβ1 or TGFβ2 alone could stimulate RPE cell migration. Although we observed that a few cultures of low-passage RPE cells showed increased migration in response to TGFβ alone, the magnitude of the response was small compared with vitreous, and the results were inconsistent.

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Increased motility requires alteration of the cytoskeleton and interactions with the extracellular matrix, and our results show that TGFβ and vitreous have very different effects. When subconfluent, low-passage RPE cells are transformed by TGFβ, they reorganize their cytoplasmic and circumferential actin filaments into prominent stress fibers terminating in focal adhesions; as a result, they appear similar to myofibroblasts. This is supported by the observation of increased expression of such myofibroblast-specific genes as αSMA and CTGF. Interestingly, in a mouse model of PVR, TGFβ signaling has been shown to play a critical role in myofibroblast differentiation of RPE cells. Unlike epithelial cells, many mesenchymal cells can invade and migrate through the extracellular matrix. Increased motility and increased invasiveness are often part of EMT and characteristic of RPE cells in PVR. Vitreous contains many chemotactic factors that may cause the transformed RPE cells to migrate to the vitreous cavity. Based on the results reported here, it is apparent that TGFβ is necessary for vitreous-induced migration but that alone it is not sufficient. This is in contrast to the results of others who showed that TGFβ1 or TGFβ2 alone could stimulate RPE cell migration. Although we observed that a few cultures of low-passage RPE cells showed increased migration in response to TGFβ alone, the magnitude of the response was small compared with vitreous, and the results were inconsistent.
ganization of the cytoskeleton in vitreous-treated cells may be the basis of the cells’ increased motility.

Our results imply that in the presence of TGFβ/H9252, but not in the presence of normal vitreous, RPE cells adopt a myofibroblast differentiation pathway. This raises the question of the nature of the RPE cells formed in the presence of vitreous. Are they indeed proceeding along an EMT pathway, as would be suggested by their increased motility and invasion? When epithelial cells undergo EMT, they may first form a multipotential fibroblast-like cell as a result of the loss of intercellular adhesion proteins such as cadherins, claudins, and occludins and focal adhesion proteins such as α5 integrin; vitreous has been found to induce such changes.13,28 The EMT process is under the control of a number of transcription repressors, the best studied of which are Snail1 and Slug (Snail2), which directly repress mRNA transcription of genes associated with the epithelial phenotype.59 We have observed increased levels of Slug mRNA28 and Snail mRNA and protein (this article) in vitreous-treated cells. Increased expression of Snail in particular has been regarded as a marker of EMT.60,61 Thus, it would be expected that Snail1 would be expressed in the first phase of all EMT processes, and this was the case whether RPE cells were exposed to TGFβ or to vitreous. In the presence of TGFβ, the cells differentiated in the direction of myofibroblastogenesis. However, in the presence of vitreous, the myofibroblast differentiation pathway was suppressed, as shown by decreased αSMA and CTGF mRNA expression. This suppression may be associated with the downregulation of the TGFβ pathway by 6 hours of vitreous treatment observed in our microarray studies.28 This raises the question of what might be the component of vitreous that suppresses differentiation along the myofibroblast pathway. BMP-2 is a member of the TGFβ superfamily and can promote EMT transformation62,63 and is also known to suppress genes associated with myofibroblastogenesis and myogenesis.64–66 Interestingly, vitreous-treatment of RPE cells results in the upregulation of BMP-2 mRNA and protein, decreased expression of inhibitors of the BMP-2 pathway, and increased expression of BMP-2 target genes.28 Thus, vitreous treatment may result in an EMT change in which TGFβ plays an important initial role, followed by downregulation of the TGFβ pathway and upregulation of the BMP-2 pathway, which protects the RPE cells from becoming myofibroblasts, a potentially dangerous cell type because of the ability to promote the formation and contraction of epiretinal membranes. Thus, this and other protective mechanisms in vitreous may prevent myofibroblast formation and PVR in many persons, but in the unfortunate minority of cases, additional factors may overcome these protective mechanisms and promote myofibroblast formation.

Our data show that TGFβ in the vitreous plays a role in some of the transforming effects of the latter but clearly demonstrate that vitreous does not cause a myofibroblastic change in RPE cells. This was observed in subconfluent, proliferating cells that might be expected to be more responsive to morphogenetic influences (including TGFβ) than to mature, highly differentiated, nondividing barrier epithelial RPE cells. This indicates a complex balance and a probable role of other factors present in vitreous. Our data challenge the long-held notion that vitreous can induce myofibroblastic changes in RPE. However, it cannot be excluded that increased levels of
TGFβ could induce myofibroblastic changes in RPE cells at later stages of PVR given that TGFβ signaling is known to be disregulated during many instances of EMT. However, a recent study has shown that the presence of higher levels of TGFβ in subretinal fluids of patients with retinal detachment might be protective against the progress of PVR and has implications for the role of TGFβ in PVR. Manipulation of TGFβ signaling thus is a valuable therapeutic target to prevent PVR; however, further studies have to be carried out on the dynamics of TGFβ signaling, including the amounts of TGFβ receptors at various stages of PVR and the influence of other factors in the vitreous.

References


