High TGF-β2 Levels during Primary Retinal Detachment May Protect against Proliferative Vitreoretinopathy

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Purpose. Transforming growth factor (TGF)-β2 and hepatocyte growth factor (HGF) have been implicated in the pathogenesis of proliferative vitreoretinopathy (PVR) after retinal detachment surgery. The exact role of these factors in the early events, immediately after primary retinal detachment, is not yet known, and determining their roles was therefore the purpose of this study.

Methods. Subretinal fluids were collected prospectively from 144 patients during surgery for scleral buckling. TGF-β2 and HGF were measured with commercially available ELISA kits. Thirty patients in whom a redetachment caused by postoperative PVR developed, were compared with 114 patients with an uncomplicated retinal detachment. The controls included 18 vitreous samples from patients with macular hole or pucker. Multivariate regression analysis was used to compare the relative roles of growth factors and clinical factors in the development of PVR.

Results. The median amount of subretinal TGF-β2 was approximately two times lower in patients with postoperative PVR (1.9 ng/mL) than in the uncomplicated detachment group (3.3 ng/mL; $P = 0.002$). TGF-β2 levels in the PVR-positive group were similar to control vitreous levels (1.8 ng/mL). Subretinal HGF concentrations were not significantly different between the two groups of patients (PVR positive: 8.8 ng/mL; PVR negative: 8.9 ng/mL), but were higher than control vitreous levels (4.6 ng/mL; $P = 0.01$). Stepwise multivariate logistic regression analysis revealed that of all factors under study, decreased TGF-β2 content was the exclusive predictor of postoperative PVR ($P = 0.01$).

Conclusions. High TGF-β2 levels in subretinal fluid at the time of primary retinal detachment may protect a patient against subsequent development of PVR. (*Invest Ophthalmol Vis Sci.* 2004;45:4113–4118) DOI:10.1167/iovs.04-0643

In approximately 10% of eyes that are treated for rhegmatogenous retinal detachments, fibrotic membranes develop, resulting in tractional retinal detachment. This complication is called proliferative vitreoretinopathy (PVR) and often leads to blindness. It usually ensues within 3 months after primary surgery. The RPE cell is considered to be the major player. After trauma it transforms into an activated mesenchymal cell type that migrates out of its own monolayer and starts dividing. Together with glial cells, fibroblasts, and macrophages, the dedifferentiated RPE cells move into the epiretinal and subretinal spaces and produce extracellular matrix resulting in the fibrotic membranes.[1] The mechanism of uncontrolled fibrosis is unclear, but it has been hypothesized that growth factors such as transforming growth factor (TGF)-β2[2,3] and hepatocyte growth factor (HGF) are involved.[4–7] An interesting model has been postulated by Hinton et al.,[7] who suggested an early role of HGF in PVR pathogenesis. In their model, an inflammatory response occurs after injury of the retina, leading to the activation of RPE cells and stimulation of HGF expression. In an autocrine loop, HGF causes the RPE cell to mobilize and leave the monolayer.

After RPE cells release from their monolayer, HGF may continue PVR progression by acting as a mitogen, as has been shown for hepatocytes and several other cell types.[8] At this stage, the process becomes more complex, involving other factors that contribute to the progression of PVR. For example, it has been suggested that latent TGF-β2, present in high levels in the vitreous, becomes activated when it is exposed to the RPE cell layer and that it exacerbates the deposition of collagen.[9] Furthermore, the detection of three TGF-β isoforms and TGF-β receptors I and II in epiretinal membranes is indicative of their involvement in PVR.[10,11]

Several studies have further supported a role for TGF-β2 as shown by the elevated TGF-β2 levels in vitreous aspirates of patients with PVR.[2,12,13] These studies can indeed give clues about growth factor involvement. Vitreous, however, is not the fluid that surrounds the RPE cell layer after initial retinal detachment, and, in that situation, analysis of subretinal fluid would be more appropriate. Furthermore, the time point at which subretinal fluid is obtained (i.e., at the time of primary retinal detachment surgery) can give clues to the initial local factors that may trigger the RPE cells. To investigate, we analyzed TGF-β2 and HGF levels in subretinal fluids aspirated during primary detachment surgery. Data from patients who had postsurgical development of PVR were compared with those of patients in who did not. We found decreased TGF-β2 levels in patients with PVR, whereas HGF levels were similar. The higher TGF-β2 levels observed in uncomplicated retinal detachments suggest a local suppressive role in the early stages of PVR development.

Methods

Subjects

Subretinal fluids were routinely collected in our department during conventional scleral buckling surgery for rhegmatogenous retinal detachment. Patients with a local detachment of 1 clock hour or less were excluded, because retinal surgeons generally do not perform a scleral puncture in these cases. Patients in whom the sampled subretinal fluid showed macroscopic hemorrhage were also excluded. From samples collected between November 1999 and April 2003 we se-

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TABLE 1. Potential Clinical Risk Factors for PVR

Risk Factor	Postoperative PVR Negative (n = 114)	Postoperative PVR Positive (n = 30)	Univariate Testing
Age (y)	62 (27–80)	63 (18–81)	NS
Sex (%)			
Female	34	27	NS
Male	66	73	NS
Size of retinal detachment (quadrants)	2 (1–4)	2 (1–4)	NS
Total size of retinal defects (optic disc diameters)	2 (0–11)	1 (0–3)	NS
Macular detachment (%)	60.2	69.0	NS
Detachment duration (interval)	2 (1–5)	2 (1–5)	NS
Preoperative PVR grade	1 (0–3)	1 (0–3)	NS
Diabetes mellitus (%)	8.8	3.4	NS
Preoperative myopia > -0.5 D (%)	22.1	20.7	NS
Preoperative uveitis (%)	0	3.4	NS
Preoperative vitreous hemorrhage (%)	2.7	3.4	NS
Preoperative cryotherapy (%)	0.9	0	NS
Preoperative lens status (%)			
Pseudophakia	23	20.7	NS
Aphakia	0	0	NS
Laser treatments after primary surgery (n)	0 (0–5)	0 (0–6)	NS
Intraoperative cryotherapy (%)	74.3	72.4	NS
Intraoperative gas (%)	82.3	75.9	NS
Intraoperative vitreous hemorrhage (%)	0	6.9	$P = 0.04$

Data are the Median (range) and frequency (%).

lected 30 consecutive patients who had postsurgical PVR. During that time, we selected three to four separate age- and sex-matched control patients who did not have a redetachment caused by PVR. This resulted in 114 patients with uncomplicated retinal detachment. As a further control, we used vitreous samples from 15 patients with idiopathic pucker and 3 with macular hole. The study was performed with the agreement of the University Hospital ethics committee. All patients gave informed consent before inclusion in the study and after the nature of the study was explained. The study adhered to the tenets of the Declaration of Helsinki.

Sample Collection

Undiluted subretinal fluid samples were obtained after scleral puncture with a 25-gauge bent needle and before possible cryotherapy, as described elsewhere.[14,15] Undiluted vitrectomy samples were obtained by conventional three-port, closed vitrectomy by manual suction at the start of vitrectomy before opening the infusion line. All samples were collected in sterile tubes (Eppendorf, Fremont, CA) and stored at −80°C. Sample volumes ranged from 100 μL to 1 mL.

Protein and Growth Factor Assays

Total protein concentration was measured in triplicate by the Lowry method, with bovine serum albumin as a standard. Singular growth factor measurements were performed with HGF and TGF-β2 ELISA kits (Quantikine; R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions. The assay standards ranged from 31.2 to 2000 pg/mL for TGF-β2, and 125 to 8000 pg/mL for HGF. Subretinal fluids and vitreous were diluted 7.8 times for TGF-β2 and 3 times for HGF. These dilution factors were calculated from data of other investigators.[6,13] The lowest vitreous concentrations these studies report—that is, 2.4 ng/mL for TGF-β2 and 2 ng/mL for HGF[6,13]—would, after dilution, result in concentrations that are in the logistic middle of the detection range of the assay, 300 and 700 pg/mL, respectively. To measure total TGF-β2 activity, samples were acid activated before the assay, according to the manufacturer's protocol. In brief, 4 μL of 1 N HCl was added to a 20-μL ocular fluid sample. After 10 minutes, the sample was neutralized with 4 μL 1.2 N NaOH/0.5 M HEPES, and 128 μL of calibrator diluent from the kit was added, resulting in a 7.8× dilution. If measurements exceeded the standard range, samples were diluted accordingly, and the assay was repeated. In some cases, we could not test all biochemical variables in the immunoassays due to a limited sample volume. The following sample sizes for each variable remained: TGF-β2 with $n = 122$, HGF with $n = 119$, total protein with $n = 128$.

Clinical Variables

For all patients, we collected potential clinical risk factors (Table 1)[16] and the following clinical variables: sex, follow-up time, occurrence of a redetachment, postoperative PVR grade, and preoperative and final postoperative best corrected Snellen visual acuity (also with pinhole correction). When present, PVR was graded according to the Classification of Retinal Detachment with PVR.[17] Data were collected as 0 (no PVR), 1 (grade A), 2 (grade B), 3 (grade C), and 4 (grade D). In some patients in the PVR-negative group, preoperative grades up to level 3 were observed. This did not lead to a redetachment after surgery, and therefore these patients were categorized in the patient group that did not have a redetachment caused by PVR. Moreover, after primary surgery, PVR was absent in these patients, according to the aforementioned classification system,[17] although it cannot exclude the microscopic presence of membranes.

By carefully interviewing the patient, we determined the approximate time of onset of the detachment. Some patients were not aware, however, that they had a visual field defect caused by the retinal detachment and were not able to give the exact date. Therefore, the duration of detachment before surgery was categorized into the following five intervals: 1 (<3 days), 2 (4–7 days), 3 (8–14 days), 4 (2–4 weeks), and 5 (>1 month), as described elsewhere.[18]

For statistical analysis, Snellen visual acuity was transformed into logMAR (logarithm of minimal angle of resolution) acuity, as described earlier.[19] Net visual outcome was calculated by subtracting logMAR visual acuity at final follow-up from logMAR visual acuity at primary detachment surgery. All possible causes of poor postoperative visual acuity were noted—for example, macular degeneration, macular hole, and glaucoma.

Statistical Analysis

Data were not normally distributed and therefore are presented as the median with minimum and maximum values in box-and-whisker plots.

TABLE 2. TGF-β2, HGF, and Total Protein Levels in Subretinal Fluid of Patients with Retinal Detachment and in Vitreous of Patients with Macular Hole or Pucker

	Subretinal Fluid, Postoperative PVR Negative	Subretinal Fluid, Postoperative PVR Positive	Vitreous, Macular Hole or Pucker
TGF-β2 (ng/mL)			
Median (range)	3.3 (0.4–30.5)	1.9 (0.4–8.8)*	1.8 (0.6–2.6)‡
Mean (SD)	5.6 (5.4)	2.8 (2.1)	1.7 (0.6)
Patients (*n*)	92	30	17
HGF (ng/mL)			
Median (range)	8.9 (1.1–64.7)	8.8 (1.7–21.8)	4.6 (2.7–10.1)§
Mean (SD)	10.8 (9.5)	10.0 (5.9)	5.1 (2.0)
Patients (*n*)	96	23	11
Total protein (mg/mL)			
Median (range)	3.9 (0.4–85.9)	22.1 (0.3–21.8)†	0.7 (0.1–2.6)‡
Mean (SD)	9.4 (14.1)	4.1 (5.5)	0.8 (0.6)
Patients (*n*)	100	28	16

*† Comparison between subretinal fluid groups: * $P = 0.002$; † $P = 0.02$.
‡§ Comparison between vitreous and combined subretinal fluid groups: ‡ $P < 0.001$; § $P = 0.01$.

Patients who had a redetachment due to postoperative PVR were compared with patients who did not. The nonparametric Mann-Whitney test was used for ordinal variables such as growth factor level and duration of detachment. The χ^2 test was used to compare nominal variables such as diabetes mellitus status and preoperative myopia. The Spearman rank correlation test was used to test the correlation between the levels of total protein, TGF-β2, and HGF, and to test their association with ordinal clinical variables. Differences were considered significant at $P < 0.05$, with two-tailed testing. The biochemical and clinical variables that scored as significant or close to significance ($P < 0.15$) in the univariate tests were further analyzed with forward stepwise multivariate regression analysis. The biochemical data were categorized into tertiles, and missing values were assigned to the fourth category. Logistic regression analysis was used to assess the data's predictive ability in determining the occurrence of a redetachment caused by postoperative PVR. Linear regression analysis was used to assess the data's predictive value in determining postoperative visual acuity. Using the analysis, we present a mathematical model to quantify the risk of development of PVR-associated redetachment in relation to growth factor levels, as previously described.[13]

RESULTS

Subretinal fluids from 144 patients with a rhegmatogenous retinal detachment were analyzed for growth factor and protein content. Thirty samples were obtained from patients with postoperative PVR. This group included 8 women (27%) and 22 men (73%) with a median age of 63 years (range, 18–81). Ten patients were classified with PVR grade B, 18 with PVR grade C, and 2 with PVR grade D. The other group of patients with uncomplicated retinal detachment consisted of 39 women (34%) and 75 men (66%) with a median age of 62 years (range, 27–80). The mean follow-up time was 14 months. To compare our results with control vitreous levels, we also analyzed vitreous from 18 patients with macular hole or pucker. They consisted of 11 women (61%) and 7 men (39%) with a median age of 68 years (range, 55–78).

Median subretinal TGF-β2 levels were approximately two times lower in patients with postoperative PVR, compared with those without. The TGF-β2 levels of the PVR-positive group were close to the vitreous levels observed in patients with macular hole and pucker (Table 2, Fig. 1). The levels represent the sum of both biologically active and latent TGF-β2, because samples were acidified before the assay, a treatment that activates latent TGF-β. To determine the proportion of latent TGF-β2, two samples were run with and without prior acidification. One sample displayed undetectable levels of active TGF-β2 (after 3.5 dilution). The other sample measured 0.4 ng/mL active TGF-β2, whereas acid activation revealed 9.6 ng/mL of total TGF-β activity. Thus, 96% of TGF-β2 in that sample was latent.

Subretinal HGF levels were comparable between the two retinal detachment groups, whereas they were almost two times higher than vitreous levels in the control group with macular hole and pucker (Table 2, Fig. 2). Similar to TGF-β2, median protein levels were almost twice as low in the PVR-positive group as in the PVR-negative group (Table 2, Fig. 3). Overall, subretinal protein levels were substantially (almost five times) higher than vitreous levels. There was a strong correlation between levels of TGF-β2 and total protein ($r = 0.8$, $P < 0.001$), whereas HGF content correlated only moderately with these biochemical variables ($r = 0.5$, $P < 0.001$).

Complete data were available on clinical variables of the 144 patients (Table 1). There were no significant differences between the two groups, except for hemorrhage during pri-

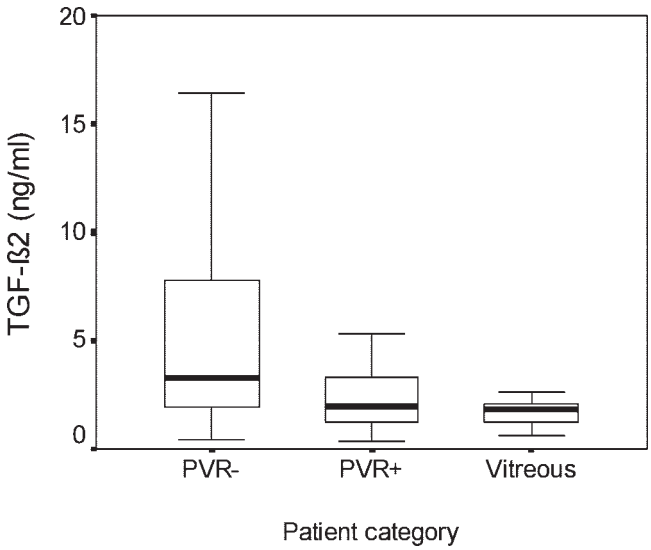

FIGURE 1. Box-and-whisker plot of TGF-β2 levels in subretinal fluids of patients with (PVR+) or without postoperative PVR (PVR−) and in vitreous of patients with macular hole or pucker. *Box*: lower and upper quartiles; *horizontal line*: the median. The points at the ends of the whiskers are the 2.5 and 97.5 percentiles.

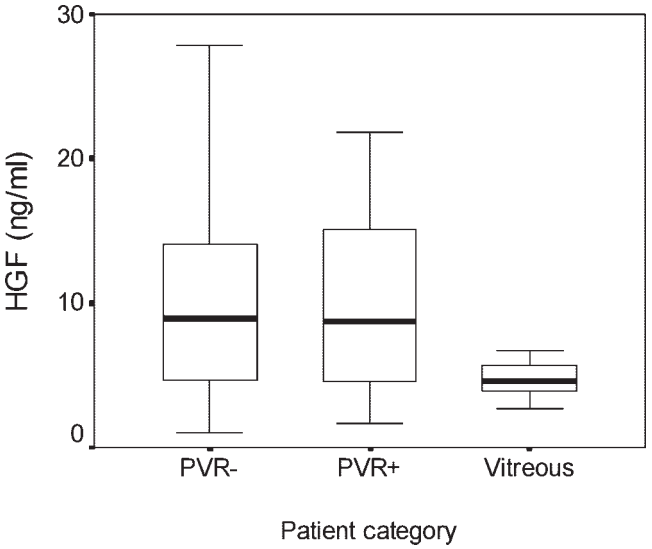

FIGURE 2. Box-and-whisker plot of HGF levels in subretinal fluids of patients with (PVR+) or without postoperative PVR (PVR−) and in vitreous of patients with macular hole or pucker. The plots are as described in Figure 1.

mary detachment surgery, which occurred more often in the PVR-positive group than in the PVR-negative group ($P = 0.04$). We did not include this outcome, however, because only two in the entire patient group had this complication. When comparing the three biochemical factors with the clinical variables, we found a significant positive correlation with duration and size of detachment and preoperative stage of PVR, but correlation coefficients were low ($r < 0.5$). For example, the correlation coefficients for detachment duration were 0.3, 0.45, and 0.3 for TGF-β2, HGF, and protein, respectively ($P < 0.001$). We did not find any correlation between TGF-β2 levels and age within both patient groups nor within the PVR-positive group alone. Similarly, there was no correlation with inflammation, because only one patient in the entire population had preoperative uveitis. The TGF-β2 level of this PVR-positive patient was 3.7 ng/mL, which was within the range in patients without uveitis. Any postoperative signs of uveitis were absent, probably because our patients received local steroids as a standard treatment after surgery, which may suppress postoperative inflammation.

As expected, the postoperative and net visual acuities of the PVR-negative group was significantly better than those of the PVR-positive group at final follow-up ($P < 0.002$). The logMAR acuity before operation was not significantly different between the two patient groups ($P = 0.095$). However, throughout the groups, it correlated moderately but significantly with final and net visual outcome ($P < 0.001$; $r = 0.5$ and $r = 0.7$, respectively). Of all other clinical variables, only the size of detachment correlated with final and net visual outcome but with a low correlation ($r < 0.3$, $P = 0.002$). There was no correlation between any of the biochemical factors and the preoperative, postoperative, and net logMAR acuities.

Based on our inclusion criteria, TGF-β2, total protein level, preoperative logMAR visual acuity, and size of detachment were selected for multivariate regression analysis. With logistic regression analysis we found that low TGF-β2 content was the exclusive predictor of the occurrence of PVR ($P = 0.01$), whereas linear regression showed that only the preoperative logMAR acuity, not growth factor or protein concentration, was indicative of final visual outcome ($P < 0.001$). Based on estimated coefficients, the following logistic regression equation was determined to calculate the changes in risk of a redetachment caused by PVR in relation to quantitative changes in TGF-β2 level. The estimated probability of development of PVR $= 1/(1 + e^{0.246+0.233(\text{TGF-}\beta 2)})$.

In the equation, TGF-β2 is measured in units of 1 ng/mL. We found that for each nanogram per milliliter increase in TGF-β2 content the probability of development of PVR decreased, with an odds ratio of 0.8 ($P = 0.01$).

DISCUSSION

In the present study, TGF-β2 levels were lower in subretinal fluid of patients who had a redetachment caused by PVR than in patients with uncomplicated retinal detachment. Statistical analysis of biochemical and clinical variables revealed that TGF-β2 was the exclusive predictor of development of PVR. Our findings were different, even opposite to those of previous investigations in which raised levels of TGF-β2 were found in association with PVR,[2,12,13] although others could not confirm these findings.[20,21] The main difference in the present study was that these earlier studies involved analysis of vitreous instead of subretinal fluid. Another dissimilarity was the PVR state of the eyes, because most investigations were performed in eyes already affected by PVR. The study by Kon et al.[13] was most comparable to ours, analyzing eyes prospectively in a large sample. It appears, however, that the Moorfields study also included eyes already affected by postoperative PVR at the start of the study, because 46% of the patients had already undergone a scleral buckling operation and/or cryolaser therapy before the analysis. Also, the PVR-negative group included lower PVR grades—that is, 1 clock hour of grade C or less, and the patients included were only those in whom vitrectomy was considered necessary.[13] Another important difference is the time interval between detachment and surgery, which was 51 days in Kon et al., whereas most of our patients underwent surgery within a week. Taken together, this implies that the conflicting findings in TGF-β2 levels are probably due to a difference in patient population and sampling time. Our study primarily dealt with the early events after retinal detachment.

The present study represents levels of total TGF-β2 activity (active plus latent). In one exemplary sample, the majority (96%) was latent. Similar results have been found by Connor et

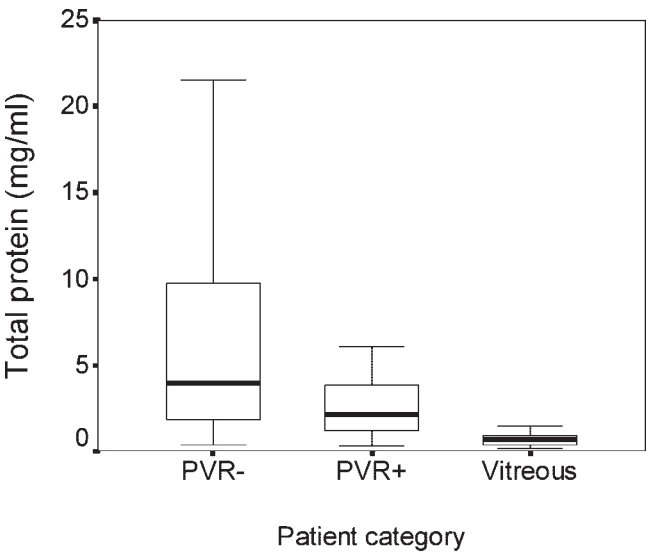

FIGURE 3. Box-and-whisker plot of protein levels in subretinal fluids of patients with (PVR+) or without postoperative PVR (PVR−) and in vitreous of patients with macular hole or pucker. The plots are as described in Figure 1.

al.,[2] in which the majority (87%) of vitreous TGF-β was latent. Of importance, the authors did not find any association between latency and PVR state, and later studies report only on TGF-β2 activity after acid activation.[13,20,21] Therefore, in our studies, we also focused on total TGF-β2 levels.

TGF-β1 has been established as an important mitogen, and it has been shown to be profibrotic, in that it stimulates matrix synthesis in many cell types.[22] These characteristics and its expression in epiretinal membranes[10,11] make the protein a candidate player in PVR pathogenesis. Next to TGF-β1, TGF-β2 has been detected in epiretinal membranes,[10,12] indicating that eventual PVR may depend on the balance between the two isoforms. TGF-β2 accounts for most TGF-β activity in vitreous (84%–100%), whereas only 10% to 21% is from TGF-β1.[2] In addition, in a study on retinal detachments in cat eyes, TGF-β2 levels increased in the vitreous, whereas TGF-β1 levels remained undetectable.[23] These studies indicate that TGF-β2, rather than TGF-β1, plays a central regulatory role at the vitreoretinal interface.

In primary RPE cultures, TGF-β2 can act as a negative regulator of cell proliferation,[12,24,25] whereas in passaged cells it was a more potent stimulator of matrix synthesis.[25] The negative effect on RPE cell proliferation coincided with cell death and DNA fragmentation, suggesting a mediating role in apoptosis.[12] The TGF-β2 levels causing apoptosis were within the range of the subretinal levels of our study. In a recent investigation, apoptosis markers were increased in the vitreous of eyes with a retinal detachment, with or without PVR, compared with the control.[21] Of note, one of the markers correlated with TGF-β2 levels. Taking these and our data into consideration, we propose that the PVR-affected patients may lack a form of TGF-β2–mediated protection that controls RPE cell proliferation. We further suggest that in the early events after retinal detachment, high TGF-β2 levels may suppress PVR pathogenesis by stimulating apoptosis and/or inhibiting proliferation of uncontrolled RPE cells. Further investigations are necessary to unravel the exact mechanism and to establish the source of TGF-β2 at the vitreoretinal interface. For that matter, RPE cells, glial cells, microglial cells, fibroblasts, and macrophages[26–28] have been shown to express both TGF-β and TGF-β receptors.[10,11,23,29] Moreover, an autocrine suicidal feedback mechanism of the RPE cell has been suggested.[12]

With regard to total protein levels, the results from subretinal fluids also deviate from earlier vitreous studies. For instance, high total protein levels in vitreous have been associated with PVR,[13,30,31] whereas in our study decreased levels were found. That and the strong correlation with TGF-β2 levels indicate that other proteins may be involved in RPE suppression as well. In any case, in the early events, HGF may not be among the candidates, because its levels were PVR independent. In contrast, overall subretinal HGF levels in both patient groups were higher than in control vitreous of eyes without detachment, consistent with earlier findings.[6] Also, in a recent paper, Jin et al.[32] reported that HGF overexpression induced retinal detachment in rabbit eyes followed by subretinal proliferation of RPE cells. These findings and our data indicate that HGF elevation is primarily associated with retinal detachment and, to a lesser extent, with PVR.

Our study did not show that potential risk factors such as age and level of inflammation are involved in the development of PVR or that they correlate with TGF-β2 concentrations, except for duration and size of detachment and preoperative PVR stage, although these variables correlated only moderately with TGF-β2 concentration. Another determinant may be the degree of retinal hypoxia that is probably present because of the detachment. This, however, cannot be measured clinically, and therefore we do not know whether retinal hypoxia is a contributing risk factor.

In summary, our findings imply that TGF-β2 is an important suppressor of the events that lead to PVR. Furthermore, low subretinal TGF-β2 levels were predictive of development of PVR and may therefore be used as a clinical biomarker for this complication. Our analysis showed that for each nanogram increase in subretinal TGF-β2 level, the odds of development of a redetachment caused by postoperative PVR were decreased 0.8 times. We suggest that in PVR-affected eyes, TGF-β2 suppression of the formation of fibrotic membranes is absent. This implies that recent views on therapeutic strategies need major adjustment. Rather than scavenging the factor from the injured site, improved outcome of retinal detachment surgery may be achieved by application of TGF-β2 in those patients at risk. Pharmacologic treatment at the time of surgery still presents a practical problem, because patient classification based on immunoassay measurements takes longer than the time needed for surgery.

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