Research Article
Evaluation of TGF-Beta 2 and VEGFα Gene Expression Levels in Epiretinal Membranes and Internal Limiting Membranes in the Course of Retinal Detachments, Proliferative Diabetic Retinopathy, Macular Holes, and Idiopathic Epiretinal Membranes

Joanna Stafiej,1 Karolina Kaźmierczak,1 Katarzyna Linkowska,2 Paweł Żuchowski,3 Tomasz Grzybowski,2 and Grażyna Malukiewicz1

1Department of Ophthalmology, Faculty of Medicine, Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz, Skłodowskiej-Curie 9, 85-094 Bydgoszcz, Poland
2Department of Molecular and Forensic Genetics, Institute of Forensic Medicine, Faculty of Medicine, Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz, Skłodowskiej-Curie 9, 85-094 Bydgoszcz, Poland
3Clinic of Rheumatology and Connective Tissue Diseases, J. Biziel University Hospital No. 2, Bydgoszcz, Poland

Correspondence should be addressed to Joanna Stafiej; joanna.stafiej@wp.pl

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Purpose. To evaluate the expression profiles of the VEGFα and TGFβ in the ERMs and ILMs in retinal disorders. Methods. In this nonrandomized prospective study, 75 patients (34 females and 41 males) referred to pars plana vitrectomy (PPV) due to different retinal diseases were enrolled to the study. The samples of ERMs and ILMs collected during PPV were immediately put in TRIzol® Reagent (Life Technologies, USA) and stored at −70°C until RNA extraction. Gene expression analysis was done with TaqMan® Gene Expression Assays (Applied Biosystems, USA) following the manufacturer’s instructions. Results. The gene expression levels of VEGFα as well as of TGFβ2 were significantly higher in ERMs than in ILMs in all studied groups. The level of TGFβ2 expression exhibits a significantly lower values in iERMs as compared with the RRD group (p=0.043). There were differences in TGFβ2 expression in ILM in groups studied: DR versus RRD, p=0.003; DR versus iERM, p=0.047; and iERM versus RRD, p=0.004. Conclusions. Our results revealed that factors associated with angiogenesis and wound healing processes in eyes with RRD, PDR, iERM, and MH were more upregulated in ERMs than in ILMs. This may indicate that ILM is not responsible for reproliferation and its peeling should be avoided in routine PPV.

1. Introduction
Proliferative vitreoretinopathy (PVR) is a severe retinal detachment (RD) and vitreoretinal surgery complication that can lead to severe vision reduction by tractional retinal detachment. Epiretinal membrane (ERM) is a semitransparent, membranous, pathologic tissue which grows on the internal surface of the retina at the vitreoretinal interface. ERMs can be either idiopathic or secondary to some pathologic conditions, including proliferative diabetic retinopathy, high myopia, uveitis, proliferative vitreoretinopathy, and other retinal degenerative diseases [1,2]. Reported frequency of ERM falls between 3% to 8.5% after scleral buckling and 6.1% to 12.8% after vitrectomy [3–8].

Due to its structure, the ILM plays a key role in the development of the vitreous–retinal boundary. Composed of a basal membrane of Müller cells, proteoglycans, and type IV collagen fibers, it constitutes the so-called framework for the development of proliferative membranes. Müller cells are responsible for homeostatic and metabolic support of
The molecular formation mechanism of both the primary and secondary ERMs in diabetic and nondiabetic patients is still poorly understood. A number of cytokines are involved in the ERM progression [21–23]. Recent technological advancements in genomics have given researchers new opportunities for identifying global gene expressions in specific tissues [24].

The aim of this prospective study was to analyse whether the gene expression profile of ERMs and ILM occurs in equal measure, which might aid in understanding ILM peeling.

2. Methods

Eighty-three patients (39 F, 44 M, age range: 26–84 years, mean age: 64.4, median: 66, standard deviation (SD): ±10.8), referred to the Department of Ophthalmology of the University Hospital in Bydgoszcz for 23-gauge pars plana vitrectomy (PPV) due to a variety of retinal diseases, were enrolled in the study. Prior to surgery, a detailed ophthalmic examination with OCT assessment was conducted, written informed consent was received from each patient before tissue sample acquisition, and approval for the study was granted by the ethics committee (KB 509/2013). Three-port PPV with ERM removal and ILM peeling was performed by two experienced vitreoretinal surgeons (JS and KK).

The patients enrolled in the study were categorized based on the disease they suffered from:

1. Idiopathic epiretinal membranes (iERMs)
2. Proliferative diabetic retinopathy (PDR)
3. Retinal detachment with epiretinal membranes (RD with ERMs)

As a control group, ILM samples collected during PPV performed due to MH from eight patients (5 females, 3 males) were used. Patient demographics are listed in Table 1. ERM and ILM samples collected during PPV were promptly put in a TRIzol Reagent (Life Technologies, Foster City, CA, USA) and stored at −70°C until RNA extraction. RNA extracts were treated with DNase I using TURBO DNA-free™ Kit (Life Technologies, Foster City, CA, USA). Total RNA (800 ng) was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA) according to the protocol. Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed by a TaqMan technology using a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Gene expression analysis was done with TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions (Table 2). Negative control consisted of a PCR mix without cDNA. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control to normalize gene expression levels for relative quantitative analysis through a comparative cycle threshold (ΔCt) method. Finally, the ΔΔCt method was used to compare gene expressions between ERM and ILM from PVR patients.

The data obtained is presented as a mean ± SD and median. For normally and equally distributed data, gene expression levels between the groups were compared using t-test. p < 0.05 was considered a statistically significant difference. Statistical analysis was performed using MS Excel 2010.

3. Results

3.1. Epiretinal Membrane. The gene expression levels of VEGFα as well as of TGFβ2 were significantly higher in ERMs than in ILMs (p = 0.009 and p = 0.015, resp.; mean and SD values are presented in Table 3).
Comparison of gene expression of VEGFα and TGFβ2 in ILM in patients who suffered from ERM to gene expression of VEGFα and TGFβ2 in ILM in patients with MH (control group) reveals no statistical differences between these two groups.

3.2. Diabetic Retinopathy. The gene expression levels of VEGFα as well as TGFβ2 were significantly higher in fibrous epiretinal membranes (FERMs) and ILMs in eyes with proliferative diabetic retinopathy (PDR).

Comparison of gene expression of VEGFα and TGFβ2 in ILM in patients who suffered from PDR to gene expression of VEGFα and TGFβ2 in ILM in the control group reveals a statistical difference between these two groups when it comes to TGFβ2 but not VEGFα (p = 0.007 and p = 0.339, resp., mean and SD values are presented in Table 5).

3.3. Rhegmatogenous Retinal Detachment. The expression levels of VEGFα and TGFβ2 were significantly higher in ERMs than in ILMs (p = 0.004 and p = 0.002, resp.; mean and SD values are presented in Table 6).

Comparison of gene expression of VEGFα and TGFβ2 in ILM in patients suffering from RD to gene expression of VEGFα and TGFβ2 in ILM in patients from the control group reveals a statistical difference between the two groups when it comes to TGFβ2 but not VEGFα (p = 0.014 and p = 0.45, resp., mean and SD values are presented in Table 7), resembling the PDR group.

### Table 3: Expression levels of VEGFα and TGFβ2 in the ERMs and ILMs in eyes with idiopathic epiretinal membranes (iERMs).

|                | ERM | ILM | p value |
|----------------|-----|-----|---------|
| VEGFα (in iERM) |     |     |         |
| Mean           | 4.43| 2.55|         |
| Median         | 4.31| 1.87|         |
| Standard deviation | 1.90| 2.34|         |
| TGFβ2 (in iERM) |     |     |         |
| Mean           | 4.16| 1.66|         |
| Median         | 4.66| 0.00|         |
| Standard deviation | 3.36| 2.84|         |

### Table 4: Expression levels of VEGFα and of TGFβ2 in the fibrous epiretinal membranes (FERMs) and ILMs in eyes with proliferative diabetic retinopathy (PDR).

|                | FERM | ILM | p value |
|----------------|------|-----|---------|
| VEGFα (in PDR) |      |     |         |
| Mean           | 4.34 | 2.96|         |
| Median         | 3.92 | 3.15|         |
| Standard deviation | 2.27| 1.71|         |
| TGFβ2 (in PDR) |      |     |         |
| Mean           | 5.53 | 3.23|         |
| Median         | 5.49 | 4.65|         |
| Standard deviation | 2.46| 2.69|         |

### Table 5: Comparison with the control group. Expression levels of VEGFα and of TGFβ2 in the ILMs in the PDR group and control group.

|                | ILM study group | ILM control group | p value |
|----------------|-----------------|-------------------|---------|
| VEGFα (in PDR) |                 |                   |         |
| Mean           | 2.96            | 2.63              |         |
| Median         | 3.15            | 2.65              | 0.339   |
| Standard deviation | 1.71| 2.46|         |
| TGFβ2 (in PDR) |                 |                   |         |
| Mean           | 3.23            | 0.64              |         |
| Median         | 4.65            | 0.00              | 0.007   |
| Standard deviation | 2.69| 1.92|         |

### Table 6: Expression levels of VEGFα and of TGFβ2 in the ERMs and ILMs in eyes with rhegmatogenous retinal detachment.

|                | ERM | ILM | p value |
|----------------|-----|-----|---------|
| VEGFα (in RRD) |     |     |         |
| Mean           | 3.71 | 2.53|         |
| Median         | 3.82 | 2.70|         |
| Standard deviation | 1.96| 1.85|         |
| TGFβ2 (in RRD) |     |     |         |
| Mean           | 5.60 | 14.92|       |
| Median         | 5.76 | 0.00 |         |
| Standard deviation | 2.46| 18.74|        |

### Table 7: Comparison with the control group. Expression levels of VEGFα and of TGFβ2 in the ILMs in the RRD group and control group.

|                | ILM study group | ILM control group | p value |
|----------------|-----------------|-------------------|---------|
| VEGFα (in RRD) |                 |                   |         |
| Mean           | 2.53            | 2.63              |         |
| Median         | 2.70            | 2.65              | 0.45    |
| Standard deviation | 1.85| 2.46|         |
| TGFβ2 (in RRD) |                 |                   |         |
| Mean           | 14.92           | 0.64              |         |
| Median         | 0.00            | 0.00              | 0.014   |
| Standard deviation | 18.74| 1.92|        |

Comparison of gene expression of VEGFα and TGFβ2 in ERMs between groups, depending on diagnosis, reveals no statistical differences between the groups when it comes to VEGFα. The level of TGFβ2 expression exhibits significantly lower values in the iERM group compared to the RRD group (p = 0.043).

Similarly, comparison of gene expression of VEGFα and TGFβ2 in ILMs between studied groups produces no
observable statistical differences in term of VEGFα; however, such differences were found between all 3 studied groups when it comes to TGFβ2 (DR versus RRD, \( p = 0.003 \); DR versus iERM, \( p = 0.047 \); and iERM versus RRD, \( p = 0.004 \)). The RRD did exhibit the highest level and the iERM the lowest level of TGFβ2.

4. Discussion

Roth and Foos postulated that an idiopathic ERM proliferates as in retinal tissue-derived glial cells escape from microde-
facts in the internal limiting membrane (ILM) that occur during posterior vitreous detachment and migrate to the sur-
face of the retina [25]. Another theory attributes the patho-
genesis of an ERM to the growth and fibrous metaplasia of
the vitreous cells that remain on the retina surface after pos-
terior vitreous detachment. On the other hand, in the ERM
that occur after rhegmatogenous RD, the retinal pigment ep-
ithelial cells are thought to migrate to the vitreous cavity
through the retinal break and settle on the retinal surface,
forming the membrane [26]. None of these theories put forth
a reason behind certain patients developing PVR rapidly
while others—not at all. The present study characterized
the expression profiles of the inflammatory cytokines VEGFα
and TGFβ2 in the ERMs and ILMs in retinal disorders such
as RRD, DR, and iERM. There might be several important
factors that determine cytokine levels either inside the oper-
ated eye or the entire body. To overcome this problem, we
collected both the ERM and ILM from the same eyes. Various
cytokines, including the vascular VEGF, have been identified
as playing a role in the pathogenesis of DR [27–29]. VEGF
that was first discovered as a vascular permeability factor is
specifically a mitogenic cytokine for vascular endothelial
cells. Retinal ischemia is the basic stimulus leading to upreg-
ulation and increase of VEGF locally and therefore plays a
major role in the progression of DR. Increased VEGF inter-
acts with its two tyrosine kinase receptors on the retinal vas-
culature, resulting in the formation of new vessels and also
disruption of the internal blood retinal barrier. The VEGF
presence has been reported in iERMs [30, 31]. In previous
studies, positive VEGF immunoreactivity of iERMs was
found, an unsurprising fact considering that retinal glia have
been known to produce VEGF. The question arose why no
blood vessels in iERM were present despite the presence of
VEGF. One possibility was the existence of other cells in
the IERM besides endothelial cells that are targeted by VEGF.
It was also plausible that the presence of endothelial growth inhibitory factors, such as TGF-β, may prevent
VEGF from exerting its angiogenic activity. Nam et al.
investigated the difference in the expression of specific
growth factors (including VEGF and TGF β1) between
diabetic and nondiabetic ERMs [32]. In our research, there
were no statistical differences in VEGFα expression between
the study groups either in ERM or ILM. There was a statistical
difference (\( p = 0.043 \)) in TGFβ2 expression only between
the RD group (mean = 5.60; SD = 2.46) and iERM group
(mean = 4.16; SD = 3.36) with regard to ERM. Selim et al.
observed that the elevation of VEGF levels was parallel to
the severity of DR and to the degree of retinal ischemia,
suggesting that the main pathogenic factor causing VEGF
elevation and responsible for DR progression in their
patients’ eyes was retinal hypoxia [33]. Retinal diseases are
closely associated with both decreased oxygenation and
increased inflammation. It is unknown whether hypoxia-
induced VEGF expression in the retina itself evokes inflam-
matation, or whether inflammation is a prerequisite for the
development of neovascularization. Interestingly, the major-
ity of the previous studies evaluating the roles of different
cytokines and growth factors in the pathogenesis of idiop-
athic epiretinal membrane were based on the vitreous
samples. Few reports are related to expression levels of these
factors between ERM and ILM and evaluated them not only
in idiopathic epiretinal membranes, but also in fibrotic ones,
usually seen in proliferative vitreoretinopathy, such as PDR
or advanced RD. To address this issue, we assessed the gene
expression levels of VEGFα and TGFβ2 in idiopathic and
fibrotic ERMs as well as in ILMs. To the best of our knowl-
edge, this is the first study that compares these parameters
in ERMs and ILMs in different diseases. Takahashi et al.
showed significant differences in VEGF levels in vitreous
body in RRD, MH, PDR, ERM, and RVO (\( p < 0.001 \)) [34].
We found no statistical differences among RRD, PDR, and
idiopathic ERM groups comparing VEGFα expression
directly in ERMs; however, in RRD, we noticed the lowest
expression. The evaluation of VEGFα expression directly in
the ILM in RRD, PDR, and idiopathic ERM groups versus
MH revealed no statistical differences contrary to Takahashi
results. TGFβ2 expressions showed statistical differences
between almost all study groups, except one (ERM group ver-
sus MH group). Myojin et al. analysed gene expression in the
irrigation solution collected during vitrectomy performed
due to ERMs and MH and found that the expression levels
of TGFβ2 and VEGFα were significantly higher in eyes with
iERM versus MH [35]. Nonetheless, we found no differences
in gene expression of VEGFα and TGFβ2 in ILM in patients
suffering from ERM versus patients with MH. Similarly,
there were no statistical differences in expression of VEGFα
in ILM between RRD versus MH and DR versus MH groups.
Interestingly, at the same time, there was a statistically higher
expression of TGFβ2 in ILM between RRD versus MH and
DR versus MH groups. As it is well known, the transforming
growth factor-β (TGF-β) and fibroblast growth factor (FGF)
play crucial cooperative roles in fibrosis. For example, in the
pathogenesis of proliferative vitreoretinopathy (PVR), TGFβ2
plays a pivotal role, promoting transition of retinal pigment
epithelial (RPE) cells into myofibroblasts. ERM is thought
to be caused by a fibrocellular proliferation of the inner lim-
iting membrane (ILM) and the subsequent vitreoretinal
adhesion and traction [36, 37]. TGFβ upregulation was
reported in eyes with iERM, PDR, and PVR, and it is associ-
ated with intraocular fibrosis [21, 23, 30, 38]. Despite
advances in surgical techniques, the percentage of unhealed
PVR remains high, producing a failure rate of up to 10% in
retinal surgical repairs [39]. In our study, TGF-β expression
responsible for fibrosis was significantly higher in ERMs than
in ILMs in all studied groups. One of the main targets of
genetic studies is to translate evidence and benefits into clin-
ical practice. ILM peeling is a subject of ongoing debate.
Rinaldi et al. found no significant differences between postoperative best-corrected visual acuity or best-corrected visual acuity change in the ILM peeling group compared with the nonpeeling group. There was no significant difference in postoperative central macular thickness and central macular thickness reduction between the two groups [40]. Similarly, Díaz-Valverde et al. noticed that internal limiting membrane peeling does not improve the functional outcome after ERM surgery. The ILM peeling reduces ERM recurrences, but few recurrences are clinically significant [41]. Moreover, a number of researchers noticed that ILM peeling had been considered to cause mechanical retinal damage, including physiological alterations in Müller cells, irregularities of the nerve fiber layer, small paracentral scotomas, loss of Müller cells end-feet within the peeling area, and weakening of the macular glial structure [17, 42–44]. Müller cells react to mechanical and hypoxic stimuli by hypertrophy to resist and protect the neuroretinal layers from traction and to protect photoreceptors from apoptosis [45]. Gao et al. suggested that in some cases of myopic foveoschisis ILM removal resulted in the development of postoperative full-thickness macular holes [46]. Sakamoto et al. found by an image of en face OCT a retinal dimple signs after ILM peeling [47]. There are also results suggesting that ILM peeling may reduce retinal sensitivity and significantly increase the incidence of microscotomas [48]. A meta-analysis of vitrectomy with or without internal limiting membrane peeling for macular hole coexisting with retinal detachment in the highly myopic eyes done by Gao et al. revealed no definite benefit of postoperative visual improvement [49].

5. Conclusions

Our results reveal that VEGFα and TGFβ2 associated with angiogenesis and wound healing processes in eyes with RRD, PDR, and iERM were more upregulated in ERMs than in ILMs. This may indicate that ILM is not responsible for reproliferation and its peeling should be avoided in routine PPV. Further studies are needed to better understand the ILM role in reproliferation and the need of ILM peeling and its consequences.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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