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Chapter 5

Abstract

**Purpose:** To investigate the presence of type VI collagen and glial cells in idiopathic epiretinal membrane (iERM) and the regulatory role of transforming growth factor-β (TGF-β) in the expression of collagens in retinal Müller cells.

**Methods:** Idiopathic ERM samples from vitrectomy were analyzed using flat-mount immunohistochemistry for cell specific markers and type VI collagen. To study the collagen synthesis of the myofibroblast-like phenotype of retinal Müller cells, a spontaneous immortalized human retinal Müller cells (MIO-M1) were treated with TGF-β1 for 48 hours and the expression of α-smooth muscle actin (α-SMA) and its co-localization with type I, II, IV and VI collagen were studied using immunocytology.

**Results:** The co-localization of glial acidic fibrillary protein (GFAP)/cellular retinaldehyde-binding protein (CRALBP) and GFAP/α-SMA was found in iERM indicating a dynamic process of activation and transdifferentiation of retinal Müller cells. TGF-β1 induced upregulation of α-SMA in retinal Müller cells. However, the intracellular expression of type I, II and VI was downregulated in retinal Müller cells containing α-SMA positive stress fibers.

**Conclusion:** TGF-β1 may be one of the growth factors inducing retinal Müller cells to differentiate into a myofibroblast-like phenotype that promotes the contraction of the epiretinal membrane. Unlike the fibroblasts, the expression of type I, II and VI collagens in these myofibroblast-like retinal Müller cells are not responsible for the collagen deposition in the presence of TGF-β1 during the process of iERM.
1. Introduction

Epiretinal membrane (ERM) contraction induced macular distortion is the major cause of visual disturbance in idiopathic epiretinal membrane (iERM). During the disease process, a cell-matrix mediated contraction of the ERM composes of excessive collagen deposition causes a significant macular dysfunction, which may result in symptoms like metamorphopsia, reduction in visual acuity and, on occasion, central unilateral diplopia.\textsuperscript{1, 2} Despite the advancement of vitreo-retinal surgery, iERM can result in a certain level of irreversible visual disturbance even after an uneventful iERM removal surgery due to the fact that the prior membrane contraction has resulted in permanent functional damage of the retina.\textsuperscript{3, 4} Research has been focused on the pathogenesis of iERM with the intention to develop novel non-invasive treatment strategies to prevent ERM formation and contraction.\textsuperscript{5}

The development of iERM is a fibrotic process in which myofibroblasts are responsible for the excessive production of collagenous extracellular matrix and the contractile force.\textsuperscript{6} Myofibroblasts have very heterogeneous origins in different organs.\textsuperscript{7} In fibro-contractive vitreoretinal diseases, retinal Müller cells, hyalocytes and retinal pigment epithelial cells may all differentiate into a myofibroblast-like phenotype and may thus contribute to collagen deposition and membrane contraction.\textsuperscript{8-10} Of importance in iERM are recent studies showing that retinal Müller cells can produce α-smooth muscle actin (α-SMA) and induce tissue contraction in the presence of certain pro-fibrotic cytokines.\textsuperscript{11, 12}

Transforming growth factor-beta (TGF-β) is a group of potent fibrogenic cytokines that play a crucial role in normal tissue repair and development of fibrosis by mediating inflammatory response, upregulating extracellular matrix production, decreasing protease synthesis and inducing myofibroblast differentiation.\textsuperscript{13} Two of the isoforms of TGF-β, namely TGF-β1 and β2, have been suggested to be involved in the pathogenesis of iERM based on their upregulation in the vitreous humor and
epiretinal membranes. However, the exact function of TGF-β in the pathogenesis of iERM has not yet been clarified. Several reports indicated that TGF-β regulates the proliferation, migration and transdifferentiation of retinal Müller cell. However, the role of TGF-β in collagen synthesis of retinal Müller cells has not yet been clarified. Previous study of our group showed that retinal Müller cells produce a series of collagens which could be involved in the dynamic turnover of vitreoretinal matrix in physiological situation. The aim of the current study is to explore the effect of TGF-β on the myofibroblast transdifferentiation and collagen production profile of the retinal Müller cells.

2. Materials and methods

2.1. ILM/ERM flat mount and immunofluorescence

2.1.1. General Procedures
Twenty-two patients (16 females, 6 males) who were referred to the Ophthalmology Department of the University Medical Center Groningen for idiopathic epiretinal membrane surgery were involved in the study between June 2010 and June 2012. The mean age of the patients was 69.6 years (range 53 to 84 years). Prior to their scheduled surgery, a complete ophthalmic examination was performed and written informed consent was obtained. This study was conducted in accord with the Declaration of Helsinki and was approved by the Institutional Review Board of the University Medical Center Groningen. The surgical technique involved a standard three ports trans pars plana vitrectomy, creation of a posterior vitreous detachment if necessary and ILM/ERM peeling with the aid of Membrane-blue (DORC, Zuidland, The Netherlands). Peeling was performed with an end-gripping forceps, intending to remove the ERM and ILM over an area of more than 1.5 disc diameters surrounding the macula. All vitrectomy procedures were performed by two experienced vitreo-retinal surgeons.
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(V.W.R.L. and G.P.). The ERM/ILM samples were immediately processed for flat mount immuno-histochemical analysis.

2.1.2. Flat-mount and immunohistochemistry of ILM/ERM specimens

The flat-mount and immunohistochemistry of ILM/ERM specimens was the same as we reported previously. Briefly, the ILM/ERM specimens were placed on a silicone elastomer (SYLGARD®, Dow Corning, USA) coated petri dish in 200µl of 1% phosphate buffered saline (PBS) containing 2% Tween 20 (BIO-RAD, PBST). Using a stereomicroscope, the specimens were flattened with fine glass sticks and pinned to the dish with stainless steel pins (Austerlitz Insect Pins®, Fine Science Tools Inc, California, USA). Subsequently, the flattened specimens were fixed in 2% paraformaldehyde for 30 minutes, washed three times with PBST for 15 minutes, blocked with PBST containing 5% bovine serum albumin (BSA, Sigma-Aldrich) for 60 minutes and incubated overnight at 4°C with a mixture of two primary antibodies (both diluted to 1:200). Primary antibodies used included rabbit (polyclonal, Abcam, Cambridge, UK) or mouse (monoclonal, Sigma-Aldrich,) anti-glial fibrillary acidic protein antibody (GFAP, glial cell marker, marker of Müller cell activation); rabbit anti-cellular retinaldehyde-binding protein antibody (CRALBP, Müller cell marker, UW55, a kind gift from J.C. Saari, University of Washington, Seattle, Wash), mouse anti-α-smooth muscle actin (monoclonal, Sigma-Aldrich) and rabbit anti type VI collagen antibody (polyclonal, Abcam). The combinations of the primary antibodies used in the study included: CRALBP/GFAP (2 cases), CRALBP/α-SMA (2 cases), GFAP/α-SMA (4 cases), type VI collagen/GFAP (10 cases) and type VI collagen/α-SMA (2 cases). Two samples were used as negative controls for the primary antibodies.

The samples were rinsed three times with PBST for 15 minutes and incubated for four hours at 4°C with two fluorescent-labelled secondary antibodies (diluted 1:200) combined with DAPI (diluted 1:200). Secondary antibodies used include donkey
anti-rabbit antibody conjugated with RedX, donkey anti-goat antibody with FITC and donkey anti-mouse with FITC (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA).

After incubation, the specimens were washed three times with PBST for 15 minutes and removed from the petri dish. Using a stereomicroscope, the specimens were flattened on a glass slide in a drop of antifadent (AF1, Citifluor Ltd, London, UK) and sealed with a cover-slip. Negative control samples underwent the entire procedure, except for the application of the primary antibodies.

2.2. Effect of transforming growth factor β1 (TGF-β1) on intracellular collagen expression in α-SMA positive cells.

2.2.1. Cell culture

The spontaneously immortalized human Müller cell line MIO-MI (a kind gift of G.A. Limb, UCL Institute of Ophthalmology, London, UK) has been confirmed to contain all the characteristics of human Müller cells. Dulbecco’s modification of Eagle’s medium (DMEM) with high glucose containing L-glutamax I (Life Technologies Inc., Rockville, MD) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS; Life Technologies Inc.) was the stock medium. The stock culture of MIO-MI (passage between 61 to 70) was maintained in stock medium at 37°C with 5% CO₂ for the experiments.

The fetal lung fibroblasts (FLF) were incubated in DMEM with high glucose containing L-glutamax I (Life Technologies Inc.) supplemented with gentamicin/glutamin and 10% FBS at 37°C with 5% CO₂.

The skin fibroblasts (SF, CCD-1112Sk, American Type Culture Collection, Manassas, VA) were incubated in DMEM with high glucose containing L-glutamax I (Life Technologies Inc.) supplemented with 10% FBS at 37°C with 5% CO₂.
2.2.2. The effect of TGF-β1 on retinal Müller cells

The cells were seeded on glass cover slips in 6-well plates in their stock culture medium with the addition of 0.2 mM of ascorbic acid. After 24 hours of incubation, 10 ng/ml of recombinant TGF-β1 was added to the medium for another 48 hours of culture. Subsequently, the cells were processed for immuno-double labeling to identify the co-localization of α-SMA and collagens including type I, II, IV and VI collagens. The primary and secondary antibodies used are listed in Table 1. Briefly, the cells were washed three times with phosphate buffered saline (PBS) and fixed with 1:1 acetone/methanol at -20°C for 10 minutes. Then, the fixative was discarded and the slides were air-dried and stored at -20°C until further usage. After blocking with 5% bovine serum album (BSA, Sigma-Aldrich, St. Louis, MO) in PBS, the cells were incubated with a mixture of primary antibodies including a mouse anti-α-SMA antibody and one of the anti-collagen antibodies for 2 hours at room temperature. Afterwards, they were rinsed with PBS 3 times for 5 minutes and incubated in a combination of two secondary antibodies according to the host of the primary antibodies and 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, 1:200) for 2 hours in dark room. After washing in PBS, a drop of anti-fadent (AF1, Citifluor Ltd, London, UK) was applied to each cover slip and the cover slips were placed on a slide and sealed. For negative controls, the samples underwent the entire procedure but the primary antibody was omitted.

3. Results

3.1. Immunocytochemical analysis of flat-mount surgical samples

3.1.1. General observations

Positive immuno-staining of anti-GFAP, anti-α-SMA, and anti-CRALBP was found in all tested samples (Table 1). Among the 15 samples positive to anti-GFAP antibody
staining, 10 showed a diffuse positivity throughout the entire membrane whereas 5 contained small clusters of GFAP-positive cells (Figure 1).

Table 1 Antibodies used in immuno-double labelling procedure

<table>
<thead>
<tr>
<th>Combinations of the primary antibodies</th>
<th>Combinations of the secondary antibodies</th>
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<tbody>
<tr>
<td>Mouse anti-α-SMA antibody (1:100, Sigma-Aldrich)</td>
<td>Rabbit anti type I collagen (1:100, Abcam, Cambridge, UK)</td>
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<tr>
<td></td>
<td>Rabbit anti type II collagen (1:100, Abcam, Cambridge, UK)</td>
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<td></td>
<td>Rabbit anti type VI collagen (1:100, Abcam, Cambridge, UK)</td>
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<td></td>
<td>Goat anti type IV collagen (1:100, Southern Biotechnology Associates (SBA), Birmingham, AL)</td>
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Table 2 Summary of protein expression in idiopathic epiretinal membrane samples

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Numbers of samples</th>
<th>Numbers of positive expression</th>
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<tbody>
<tr>
<td>GFAP</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>α-SMA</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>CRALBP</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Type VI collagen</td>
<td>10</td>
<td>9</td>
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GFAP: glial fibrillar acidic protein; α-SMA: α-smooth muscle actin; CRALBP: cellular retinaldehyde-binding protein.
3.1.2. Colocalization of GFAP/CRALBP and GFAP/α-SMA in iERM

In iERM samples stained for GFAP/CRALBP, a co-expression of GFAP and CRALBP was observed in some of the epiretinal cells, whereas other cells were CRALBP positive only (Fig 2). These different staining patterns were in adjacent areas. Three out of four iERM specimens stained for GFAP/α-SMA showed a co-expression of GFAP and α-SMA.

3.1.3. Presence of Type VI collagen in iERM

Nine out of ten (90%) iERM showed a positive type VI collagen staining. In six of the immuno-positive epiretinal membranes, type VI collagen formed a diffuse fine fibrillary network (Fig 4). At the edge of the ERM, a direct attachment of the network to the underlying ILM could be visualized (Fig 4B).
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Fig 2. The expression of glial fibrillary acidic protein (GFAP, green) and cellular retinaldehyde-binding protein (CRALBP, red) in iERM. Epiretinal cells are sometimes only CRALBP positive, indicating a Müller cell origin (arrow heads), whereas co-expression of GFAP and CRALBP is seen in others, indicating Müller cell activation (arrows). Bar=25µm

Fig 3. Representative epiretinal cell cluster in idiopathic epiretinal membrane that expressed both glial fibrillary acidic protein (GFAP, red) and α-smooth muscle actin (α-SMA, green). A. Variability in α-SMA positivity within one sample. Some cells have only weak and patchy α-SMA reactivity (arrow), while others are stained intensely (arrow head). Bar=100µm. B. Co-localization of GFAP (red) and α-SMA (green) positive iERM cells. Bar=10µm
Fig. 4. Expression of type VI collagen (red) in a flat-mounted idiopathic epiretinal membrane. A. Type VI collagen forms a well-arranged fibrillary network (arrow, bar=100µm). B. Diffuse attachment of a type VI collagen network to the underlying inner limiting membrane (arrow head) at the edge of the epiretinal membrane (arrow, bar=50µm).

3.2. TGF-β1 induced collagen expression in MIO-M1 cells, fetal lung fibroblasts and skin fibroblasts

TGF-β1 treatment induced significant changes in MIO-M1 cells (Fig 5. E to H) including: 1) upregulation of α-SMA expression and formation of α-SMA positive stress fibers. 2) down-regulation of type VI collagens in MIO-M1 cells which contain prominent stress fibers.

The quiescent MIO-M1 cells exhibited intracellular positivity to type I, II, IV and VI collagens (Fig 4. A to D, green). Extracellular deposition of type I, II and VI collagens could be observed as irregular fibrillar structures. Also, a faint and diffuse cytoplasmic α-SMA positivity could be observed (Fig 4. A to D, red), but the α-SMA positive stress fibers were very rare. After 48-hours of incubation in 10 ng/ml of TGF-β1, a significant upregulation of α-SMA and α-SMA positive stress fibers was observed (Fig 4. E to H, red). Furthermore, the expression of type I, II and VI
collagens was significantly decreased in MIO-M1 cells containing α-SMA positive stress fibers while the cells that did not show α-SMA positivity maintained their expression of type I, II and VI collagen. The expression of type IV collagens in α-SMA stress fiber positive cells was the least affected. The staining of type IV collagen in α-SMA stress fiber positive cells was less diffusely present than in the α-SMA negative cells and seemed to be more concentrated around the nucleus.

TGF-β1 treatment induced significant upregulation of α-SMA expression and formation of α-SMA positive stress fibers in FLF and SF as well. However, the intracellular staining of type I and VI collagens in both fibroblast cell lines containing α-SMA positive stress fibers remain to be the same with that of the α-SMA negative cells (Fig. 6).

**Discussion**

The primary goal of the current study is to provide evidences to show the direct involvement of retinal Müller cells in the formation of iERM. Of particular interest was to explore the collagens production profile in retinal Müller cells in the presence of pro-fibrotic growth factor - TGF-β1.

Consistent with previous studies of human Müller cells, MIO-M1 cells are capable of becoming an α-SMA expressing phenotype in response to TGF-β1 thus promote the contraction of iERM. The progression of iERM can be considered as a fibrotic process because the pathological finding are an increased collagen deposition and membrane contraction. Although some debates exist concerning the initial event of iERM, the general consent is that retinal Müller cells are one of the important cell types that play a central role in this process by producing various collagens and express α-SMA that involved in the membrane contraction. By being incorporated in the stress fibers, α-SMA is involved in the contractile activity of various cell types, such as fibroblasts, chondrocytes and mesenchymal stem cells,
The de novo expression of α-SMA alone has been reported to increase the contractile activity in the fibroblasts.\textsuperscript{24}
of these activated Müller cells, which indicates a dynamic activation process of Müller cells.

<table>
<thead>
<tr>
<th>MIO-M1 cell</th>
<th>Fetal lung fibroblast</th>
<th>Skin fibroblast</th>
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<tr>
<td><img src="image1" alt="" /></td>
<td><img src="image2" alt="" /></td>
<td><img src="image3" alt="" /></td>
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**Fig 6.** Expression of α-smooth muscle actin (α-SMA) and type I and VI collagens in MIO-M1 cells, fetal lung fibroblasts (FLF) and skin fibroblasts (SF) treated with transforming growth factor β1 (TGF-β1) for 48 hours. Immuno-double labelling was performed to co-localize the expression of α-SMA (red) and type I and VI collagen (green). The nuclei were counter stained with 4',6-diamidino-2-phenylindole (DAPI, blue). A to D, MIO-M1 cells; E to H, FLF; I to L, SF. A, E and I, double labelling of α-SMA and type VI collagen; each image on their right: B, F and J, is the staining of type VI collagen; C, G and K, double labelling of α-SMA and type I collagen, the image to their right: D, H and L, is the staining of type I collagen. Bar = 10 µm.

In addition, the observed variable expression of α-SMA in GFAP positive glial cells indicates that some of the retinal Müller cells can produce α-SMA and acquire the contractile activity. This is consistent with our *in vitro* results and with previous *in vitro* and animal studies which showed that Müller cells are actively involved in the formation and contraction of iERM.8, 30-32

However, the effect of TGF-β retinal Müller cells was not exactly the same as reported in fibroblasts. Our observation suggested that when the retinal Müller cells become a α-SMA expressing phenotype in response to TGF-β1, the expression
of type I, II and VI collagens in these cells will decreased. These findings indicate that the collagen synthesis of these three types of collagens are either down-regulated or they were secreted to the extracellular matrix efficiently. We favor the former hypothesis because the intracellular positivity of type I and VI collagens are not affected regardless the presence or absence of α-SMA positive stress fibers in the two types of fibroblasts we studied. In fibroblasts, it has been shown that TGF-β1 induces the concomitant upregulation of both α-SMA and type I and VI collagen production. Furthermore, the intracellular expression of type IV collagen was not affected in retinal Müller cells containing α-SMA positive fibers, which indicated that the secretion of collagens into the extracellular matrix were not enhanced. In addition, the synthesis of these collagens are completely intracellular, a significant immuno-positivity is expected if the cells are producing these collagens. Therefore, it is conceivable that the synthesis of type I, II and VI collagens in the retinal Müller cells containing α-SMA positive fibers are down-regulated. The suppression of type I, II and VI collagens may indicate a negative feedback mechanism that finely regulates the pro-fibrogenic effect of TGF-β in α-SMA positive retinal Müller cells.

High dose of TGF-β has been shown to suppress the expression of type I collagen via the induction of a negative regulator of collagen transcription in renal fibroblasts. In addition, Smad7, a key inhibitor of TGF-β/Smad signaling pathway, may also participate in the suppression of the TGF-β induced collagen production. Further research focusing on the exact mechanism of the down-regulation of collagen synthesis in retinal Müller cells that contain α-SMA positive stress fibers could provide a new approach to prevent fibrotic diseases.

Idiopathic ERM contains a complex ECM protein network containing many types of collagens, including type I, II, III, IV and VI collagens. Our observations suggested that type VI collagens form a fine fibrillar network in iERM, which is consistent with previous reports. Kritzenberger et al reported the expression of type VI collagen
predominantly in cellophane maculopathy as opposed to pre-retinal macular fibrosis. In previous research, type VI collagen expression was not detected in normal human ILM at the macular region nor in ERM from idiopathic macular hole patients with the same immunohistochemical technique. We concluded that the positive staining of type VI collagen in the iERM was specific.

In summary, our ex-vivo and in vitro studies shed some new light in the pathogenesis of iERM regarding the involvement of retinal Müller cells and the production of type I, II and VI collagens in response to TGF-β1. Although certain debate exists concerning the initial events of iERM formation, retinal Müller cells are one of the important cell types that actively are involved by migration, proliferation and transdifferentiation. During the process of iERM formation, TGF-β can be produced and activated by both retinal Müller cells and hyalocytes. Through an autocrine or paracrine fashion, TGF-β upregulates the expression of α-SMA in these epi-retinal cells resulting in the contraction of epiretinal membrane. The production of collagens in α-SMA expressing Müller cells may be down-regulated by certain negative feedback mechanisms, while the retinal Müller cells that have not become the α-SMA expressing phenotype could continue their production of collagen to form the ECM network of the iERM. Further research to understand the mechanism of down-regulation of collagen production in retinal Müller cells containing α-SMA positive stress fibers may provide novel therapeutic approach to prevent the formation of iERM.
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