Localization of TGF-β type II receptor and ED-A fibronectin in normal conjunctiva and failed filtering blebs

Tobias Meyer-ter-Vehn, Franz Grehn, Günther Schlunck

Department of Ophthalmology, University of Würzburg, D 97080 Würzburg, Germany

Purpose: The cytokine transforming growth factor-β (TGF-β), and the ED-A splice variant of the extracellular matrix protein fibronectin modulate wound healing and scar formation. To further elucidate their possible role in filtering bleb scarring after glaucoma surgery in human eyes in vivo, we studied the cell type specific localization of TGF-β receptors and the presence of ED-A fibronectin in sections of normal conjunctiva and scarred filtering blebs.

Methods: Cryosections of normal conjunctiva (four patients) and scarred filtering blebs (seven patients) were studied by double-label immunofluorescence. Antibodies against PECAM-1 and prolyl-4-hydroxylase allowed for specification of vascular endothelial cells and activated fibroblasts, respectively. TGF-β receptor type II (TGF-β-RII), α-smooth muscle actin, O-linked sialoglycoprotein, fibronectin and the ED-A fibronectin splice-variant were also detected using specific antibodies. Labeled sections were viewed with a confocal laser scanning microscope.

Results: Vascular endothelial cells expressed TGF-β-RII in both normal and scarred tissue. TGF-β-RII was sparsely detected in the fibroblasts of normal conjunctiva while it was strongly expressed in most fibroblasts of the scarred filtering blebs. Similarly, ED-A fibronectin was not detected in the extracellular matrix of normal conjunctiva but abundantly present in scarred filtering blebs.

Conclusions: Filtering bleb scarring is associated with an abundant expression of TGF-β receptors in activated fibroblasts and the deposition of the fibrogenic ED-A fibronectin splice-variant. These data support the concept of targeting TGF-β signaling to prevent scar formation after filtering glaucoma surgery.

Scar formation is the most frequent cause of failure following glaucoma filtering surgery, but the pathophysiological mechanisms of filtering bleb scarring are not fully elucidated. The cytokine transforming growth factor-β (TGF-β), is pivotal in wound healing and scar formation in general [1,2]. All three TGF-β isoforms have been identified in the eye [3,4] with TGF-β-2 being the predominant isoform associated with ocular scarring diseases such as proliferative vitreoretinopathy and cataract formation [5,6]. In a mouse model of conjunctival scarring, TGF-β-2 was strongly expressed in the stroma of the wounded area [7] and a TGF-β-2-specific neutralizing antibody prevented conjunctival scarring after glaucoma surgery in a rabbit model [8]. However, a recent phase III clinical trial failed to demonstrate a significant effect of TGF-β-2-specific antibodies in the prevention of filtering bleb scarring [9] and raised some reservations concerning the targeting of TGF-β.

In the early phases of wound healing, TGF-β is secreted by inflammatory cells [10] and acts as a chemoattractant. In the long-term, TGF-β promotes the transdifferentiation of fibroblasts to highly contractile myofibroblasts, which deposit extracellular matrix proteins and serve as the main agents of scarring if present persistently. TGF-β binds to a heterodimeric receptor complex, consisting of two serine-threonine kinase receptors designated TGF-β type I and II receptor, and leads to the activation of several intracellular signaling pathways. The tissue distribution of TGF-β receptor-bearing cells in normal conjunctiva and scarred filtering blebs is currently unclear.

Furthermore, scarring is associated with alterations in the extracellular matrix composition. TGF-β induces the expression of the ED-A fibronectin splice variant [11] as a preconditon for TGF-β-mediated myofibroblast transdifferentiation. Studies in vitro revealed that TGF-β-induced myofibroblast transdifferentiation was averted by the blocking of ED-A fibronectin with specific antibodies [11]. The role of ED-A fibronectin in filtering bleb scarring has not been addressed.

To gain further insight into the possible role of TGF-β in filtering bleb scarring in human eyes in vivo, we studied the cell type-specific distribution of TGF-β-RII and the presence of ED-A fibronectin in normal conjunctiva and scarred filtering blebs.

METHODS

Tissue samples: Conjunctival specimens were obtained during standard intraocular surgery after comprehensive information and written consent of the selected patients. The tenets of the Declaration of Helsinki were followed, and an institutional ethics committee approval had been granted. Native conjunctival tissue was gained from four patients.
undergoing strabismus surgery while hypertrophic scar tissue was obtained from seven patients undergoing filtering bleb revision surgery after multiple preceding operations (Table 1). All specimens were embedded in Tissue Tek cryopreservant and snap frozen in liquid nitrogen.

**Tissue processing and immunofluorescent staining:** The following antibodies were used: rabbit anti-TGF-β-RII (L-21, Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal mouse anti-PECAM-1 (DAKO, Glostrup, Denmark) to detect vascular endothelial cells; anti-α-SMA, anti-FN, anti-ED-A-FN (Sigma, St. Louis, MO), and anti-O-linked sialoglycoprotein (D2–40, Signet Laboratories, Dedham) to detect lymphatic endothelium; and mouse anti-prolyl-4-hydroxylase (DAKO) directed against an enzyme involved in posttranslational collagen modification to detect activated fibroblasts. Secondary antibodies were labeled with Alexa-488 or Alexa-568 (Molecular Probes, Leiden, The Netherlands). Cryostat sections of 6 µm thickness were prepared, air dried, fixed in pure acetone for 20 min at −20 °C, and blocked for 1 h in TRIS buffered saline (TBS, pH 7.6), containing 4% normal goat serum (NGS, Dianova, Hamburg, Germany). Antibodies were diluted in TBS, containing 1% NGS plus 0.1% Triton X-100. The primary antibodies were incubated overnight at 4 °C in a humidified chamber. Secondary antibodies were applied sequentially for 1 h at room temperature. Following each incubation, the specimens were washed three times in TBS and finally mounted in Vectashield (Vector, Burlingame, CA). The stained slides were viewed with a laser scanning confocal microscope (TCS SP-2, Leica, Bensheim, Germany).

### Table 1. Patient Characteristics

| Age [yrs] | Sex | Diagnosis                  | Previous Surgery [#of proc.] | Time after previous surgery [mo] | History of topical medication |
|-----------|-----|----------------------------|-------------------------------|---------------------------------|------------------------------|
| 14        | f   | Strabismus                 | 0                            | -                              | -                            |
| 29        | f   | Strabismus                 | 0                            | -                              | -                            |
| 6         | m   | Strabismus                 | 0                            | -                              | -                            |
| 28        | m   | Strabismus                 | 0                            | -                              | -                            |
| 53        | m   | Glaucoma / POAG            | 4                            | 2                              | + / multiple Allergies       |
| 8         | m   | 2º Glaucoma after injury   | 5                            | 1                              | + Hx of 6 years              |
| 62        | m   | 2º Glaucoma after injury   | 5                            | 1                              | + / Hx of 16yrs              |
| 79        | f   | Glaucoma / PEX             | 1                            | 2                              | + / Hx of 3yrs               |
| 37        | f   | Glaucoma / juvenile        | 4                            | 8                              | + / Hx of 14yrs              |
| 53        | m   | Glaucoma / uveitic         | 2                            | 2                              | + / Hx of 5yrs               |

Survey of patient characteristics including age, sex, reason for surgery (strabismus surgery or glaucoma surgery), type of glaucoma, number and time after previous surgery, history of topical medication. All patients undergoing revision glaucoma surgery received topical mitomycin C treatment. Abbreviations: POAG: primary open angle glaucoma; PEX: pseudoexfoliation glaucoma.

**RESULTS**

*Increased cell density in scarred filtering blebs:* In normal conjunctiva, the subepithelial stroma contained sparse fibroblasts and occasional small vascular structures (Figure 1A, arrows). Specimens of scarred filtering blebs showed a high density of spindle-shaped cells, presumably fibroblasts, several vascular structures, and areas of inflammatory cell infiltrates (Figure 1B). Only one scar specimen and all normal specimens contained an epithelial layer. To avoid leakage and allow for proper wound closure after revision surgery, care was taken to spare the conjunctival epithelial layer, which explains the absence of epithelium in most scar specimens.

*TGF-β receptor II expression in native conjunctiva and scarred filtering blebs:* To assess TGF-β receptor expression and identify TGF-β-responsive cell types, immunofluorescent double stains were performed. Vascular endothelial cells were stained with Alexa-488, while fibroblasts, identified by their spindle shape, were labeled with Alexa-568. The resulting images were analyzed using a laser scanning confocal microscope to determine the spatial distribution of TGF-β receptor II expression in relation to the various cell types within the filtering bleb tissue.
stained with an antibody recognizing PECAM-1, and activated fibroblasts were detected using an antibody against the enzyme prolly-4-hydroxylase. TGF-β-RII expression was strong in epithelial cells (Figure 2A) and in vascular endothelial cells (Figure 2A,B,C). Activated, prolly-4-hydroxylase-positive fibroblasts were absent in normal conjunctiva, and a faint staining for TGF-β-RII was rarely seen in non-vascular areas of the normal conjunctival stroma (Figure 2A and Figure 3A).

Specimens of scarred filtering blebs were rich in vascular structures as detected by PECAM-1 staining (Figure 2B,C). PECAM-1 positive cells stained for TGF-β-RII (Figure 2B,C), but non-vascular spindle-shaped cells were also expressing this receptor (Figure 2C, arrows). The latter appear to be activated fibroblasts as detected by an anti-prolyl-4-hydroxylase antibody (Figure 3B,C). These cells were abundant in scarred filtering blebs, and most of them expressed TGF-β-RII (Figure 3B,C, arrows). In scarred filtering blebs, fibroblasts also expressed α-SMA (Figure 4B,C), which was detected in perivascular cells as well. Lymphatic endothelium was not detected (Figure 4E,F).

No staining was observed when the anti-TGF-β-RII antibody was preadsorbed to a specific control peptide, or when non-immune rabbit IgG was used as a primary antibody as well as when primary antibodies were omitted (data not shown).

**Fibronectin and ED-A fibronectin in native conjunctiva and scarred filtering blebs:** To study fibronectin extracellular matrix composition in conjunctival tissue, we performed double stains using antibodies against total fibronectin and the ED-A fibronectin splice variant. Fibronectin was found throughout the subepithelial stroma in both the normal conjunctiva and scarred filtering blebs (Figure 5A,B). In normal conjunctiva, expression of the ED-A fibronectin splice variant was restricted to perivascular areas and basal epithelial cells (Figure 5A). In contrast, ED-A fibronectin was present throughout the stroma of scarred filtering blebs (Figure 5B).

**DISCUSSION**

Scar formation remains a serious problem following filtering glaucoma surgery. The cytokine TGF-β, promotes the transdifferentiation of human tenon fibroblasts to
myofibroblasts in vitro and has been detected in conjunctival epithelium and subepithelial extracellular matrix after glaucoma surgery [12,13]. However, data on the localization of the respective TGF-β receptors in conjunctival tissue have been lacking. A recent phase III clinical trial, which failed to show an advantage of a topical administered antibody against TGF-β-2 over placebo has raised some reservations regarding the effectiveness of TGF-β-antagonizing strategies to prevent filtering bleb scarring. To further address the relevance of TGF-β in human filtering bleb scarring in vivo, we assessed the cell type-specific localization of TGF-β receptors in normal conjunctiva and scarred filtering blebs. Furthermore, we examined the distribution of ED-A fibronectin as an indicator for TGF-β-induced extracellular matrix alterations.

TGF-β receptors were detected in conjunctival epithelial cells and in vascular endothelial cells of both the normal conjunctiva and scarred filtering blebs. This is consistent with studies of cutaneous squamous epithelium [14] where TGF-β exerts homeostatic and antiproliferative effects [15]. The significance of TGF-β in angiogenesis is also well established although the exact effects of TGF-β on vascular endothelial cells have not fully unraveled. Transgenic mice deficient in TGF-β-1, TGF-β receptor 1 (ALK1), TGF-β receptor 2 (ALK4), or the accessory TGF-β-binding protein endoglin, suffer from defects in angiogenesis and vascular malformations (reviewed in [16,17]). An increase in vascularity is an important clinical sign of impending filtering bleb failure [18].

Most importantly, we provide evidence for a substantially different distribution of TGF-β receptors and ED-A fibronectin in the connective tissue of the normal conjunctiva and scarred filtering blebs. As detected by immunofluorescence, TGF-β receptors were virtually absent in fibroblasts of normal conjunctiva while they were abundant and strongly expressed in activated fibroblasts of scarred filtering blebs. Similarly, ED-A fibronectin was confined to perivascular areas in normal conjunctiva but abundantly present in all the scarred filtering blebs. In light of previous studies by other investigators, these data are consistent with TGF-β-induced alterations and indicate a state of enhanced TGF-β responsiveness in scarred filtering blebs. A strong TGF-β receptor expression can result from stimulation by TGF-β [19,20] and has been demonstrated in subcutaneous fibroblasts of granulation tissue and hypertrophic cutaneous scars [14] as well as in an animal model of excisional wound repair [21]. Furthermore, pronounced TGF-β receptor expression results in enhanced TGF-β responses [22]. The sparse staining for TGF-β-RII in fibroblasts of normal conjunctiva does not exclude TGF-β receptor expression but rather indicates a low expression level close to the limit of immunofluorescence detection. Human tenon fibroblasts, derived from normal conjunctiva, respond to TGF-β in vitro

![Figure 4](http://www.molvis.org/molvis/v14/a17)

Figure 4. Localization of α-smooth muscle actin (SMA) and the absence of lymphatic endothelium in scarred filtering bleb tissue. Serial sections were double labeled for TGF-βRII (A, D, and G; green) and α-SMA (B, C), O-linked sialoglycoprotein (E, F), and PECAM-1 (H, I, red). α-SMA is colocalized to TGF-βRII in stromal fibroblasts and perivascular cells (A, B, C). Vascular structures expressing TGF-βRII (H) were negative for markers of lymphatic endothelium (E). Some collagen fiber autofluorescence is present in the red channel (B, E, H). Scale bar represents 50 µm.

![Figure 5](http://www.molvis.org/molvis/v14/a17)

Figure 5. Expression of fibronectin and the ED-A fibronectin splice variant. Fibronectin is abundant in normal conjunctiva (A) and in scarred filtering blebs (B). In contrast, expression of the fibrogenic ED-A fibronectin splice variant is restricted to perivascular areas in normal conjunctiva (A), but expands throughout the tissue in scarred filtering blebs (B). Specimens were double-stained for total fibronectin (green) and the fibronectin isoform, ED-A (red). Scale bar represents 50 µm.
Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002; 3:349-63. [PMID: 11988769]

Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. N Engl J Med 1994; 331:1286-92. [PMID: 7935686]

Lutty GA, Merges C, Threlkeld AB, Crone S, McLeod DS. Heterogeneity in localization of isoforms of TGF-beta in human retina, vitreous, and choroid. Invest Ophthalmol Vis Sci 1993; 34:477-87. [PMID: 7680639]

Pasquale LR, Dorman-Pease ME, Lutty GA, Quigley HA, Jampel HD. Immunolocalization of TGF-beta 1, TGF-beta 2, and TGF-beta 3 in the anterior segment of the human eye. Invest Ophthalmol Vis Sci 1993; 34:23-30. [PMID: 8425829]

Connor TB Jr, Roberts AB, Sporn MB, Danielpourg D, Durt LL, Michels RG, de Busstos S, Enger C, Kato H, Lansing M, Hayashi H, Glaser BM. Correlation of fibrosis and transforming growth factor-beta type 2 levels in the eye. J Clin Invest 1989; 83:1661-6. [PMID: 2708527]

Hales AM, Chamberlain CG, McAvoy JW. Cataract induction in lenses cultured with transforming growth factor-beta. Invest Ophthalmol Vis Sci 1995; 36:1709-13. [PMID: 7601651]

Cordeiro MF. Role of transforming growth factor beta in conjunctival scarring. Clin Sci (Lond) 2003; 104:181-7. [PMID: 12546640]

Mead AL, Wong TT, Cordeiro MF, Anderson IK, Khaw PT. Evaluation of anti-TGF-beta2 antibody as a new postoperative anti-scarring agent in glaucoma surgery. Invest Ophthalmol Vis Sci 2003; 44:3394-401. [PMID: 12882787]

CAT-152 0102 Trabeculectomy Study Group. Khaw P, Grehn F, Hollo G, Overton B, Wilson R, Vogel R, Smith Z. A phase III study of subconjunctival human anti-transforming growth factor beta(2) monoclonal antibody (CAT-152) to prevent scarring after first-time trabeculectomy. Ophthalmology 2007; 114:1822-30. [PMID: 17908591]

Wahl SM, Hunt DA, Wakefield LM, McCartney-Francis N, Wahl LM, Roberts AB, Sporn MB. Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci USA 1987; 84:5788-92. [PMID: 2886992]

Serini G, Bochaton-Piallat ML, Ropraz P, Geinoz A, Borsi L, Zardi L, Gabbiani G. The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. J Cell Biol 1998; 142:873-81. [PMID: 9700173]

Saika S, Yamanaka O, Baba Y, Kawashima Y, Shirai K, Miyamoto T, Okada Y, Ohnishi Y, Ooshima A. Accumulation of transforming growth factor-beta1. J Cell Biol 1998; 142:873-81. [PMID: 7601651]

Hayashi H, Glaser BM. Correlation of fibrosis and transforming growth factor-beta3 in the anterior segment of the human eye. Invest Ophthalmol Vis Sci 1993; 34:23-30. [PMID: 8425829]

Schmid P, Itin P, Cherry G, Bi C, Cox DA. Enhanced expression of transforming growth factor-beta type I and type II receptors in wound granulation tissue and hypertrophic scar. Am J Pathol 1998; 152:485-93. [PMID: 9466575]

Ten Dijke P, Goumans MJ, Lebrin F, Valdimarsdottir G. Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways. Trends Cardiovasc Med 2003; 13:301-7. [PMID: 14522471]
17. Lebrin F, Deckers M, Bertolino P, Ten Dijke P. TGF-beta receptor function in the endothelium. Cardiovasc Res 2005; 65:599-608. [PMID: 15664386]
18. Picht G, Grehn F. Classification of filtering blebs in trabeculectomy: biomicroscopy and functionality. Curr Opin Ophthalmol 1998; 9:2-8. [PMID: 10180508]
19. Bloom BB, Humphries DE, Kuang PP, Fine A, Goldstein RH. Structure and expression of the promoter for the R4/ALK5 human type I transforming growth factor-beta receptor: regulation by TGF-beta. Biochim Biophys Acta 1996; 1312:243-8. [PMID: 8703994]
20. McWhirter A, Colosetti P, Rubin K, Miyazono K, Black C. Collagen type I is not under autocrine control by transforming growth factor-beta 1 in normal and scleroderma fibroblasts. Lab Invest 1994; 71:885-94. [PMID: 7807970]
21. Gold LI, Sung JJ, Siebert JW, Longaker MT, Type I. RI) and type II (RII) receptors for transforming growth factor-beta isoforms are expressed subsequent to transforming growth factor-beta ligands during excisional wound repair. Am J Pathol 1997; 150:209-22. [PMID: 9006337]
22. Garner WL, Karmiol S, Rodriguez JL, Smith DJ Jr, Phan SH. Phenotypic differences in cytokine responsiveness of hypertrophic scar versus normal dermal fibroblasts. J Invest Dermatol 1993; 101:875-9. [PMID: 8245516]
23. Meyer-Ter-Vehn T, Gebhardt S, Sebald W, Buttmann M, Grehn F, Schlunck G, Knaus P. p38 inhibitors prevent TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts. Invest Ophthalmol Vis Sci 2006; 47:1500-9. [PMID: 16565385]
24. Van Obberghen-Schilling E, Roche NS, Flanders KC, Sporn MB, Roberts AB. Transforming growth factor beta 1 positively regulates its own expression in normal and transformed cells. J Biol Chem 1988; 263:7741-6. [PMID: 3259578]
25. Discher DE, Janney P, Wang YL. Tissue cells feel and respond to the stiffness of their substrate. Science 2005; 310:1139-43. [PMID: 16293750]
26. Muro AF, Chauhan AK, Gajovic S, Iaconcig A, Porro F, Stanta G, Baralle FE. Regulated splicing of the fibronectin EDA exon is essential for proper skin wound healing and normal lifespan. J Cell Biol 2003; 162:149-60. [PMID: 12847088]