

Decreased active TGF- β ₂ levels in the aqueous humour during immune reactions following penetrating keratoplasty

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LABORATORY STUDY

Abstract

Purpose The secretion of transforming growth factor (TGF)- β ₂ into the aqueous humour is important for maintaining the immunological privilege of the anterior ocular segment that promotes corneal allograft survival. Levels of total TGF- β ₂, however, were similar in eyes with and without immune reactions following penetrating keratoplasty (PK). In this study, we tested the hypothesis whether the activated form of TGF- β ₂ is decreased in eyes with immune reactions following PK.

Methods Anterior chamber puncture was performed in 38 cataract patients without PK (group I), in 10 patients without immune reaction following PK (group II), and in 10 patients following PK with newly diagnosed endothelial immune reaction (group III). About 0.05–0.1 ml of aqueous humour were harvested from each patient. Analysis of active TGF- β ₂ was started via ELISA within 3 h following puncture.

Results Average active TGF- β ₂ concentration was 38.9 pg/ml \pm 24.3 in group I, 47.4 pg/ml \pm 23.1 in group II, and 12.7 \pm 26.0 pg/ml in group III. The differences between groups I and III ($P=0.005$) and groups II and III ($P=0.008$) were statistically significant.

Conclusion The lowest levels of active TGF- β ₂ were measured in eyes with newly diagnosed endothelial immune reactions. This suggests that TGF- β ₂ has a protective effect on corneal grafts following PK. Further (prospective) studies are being carried out to discover whether active TGF- β ₂—determined prior to PK—might serve as a predictive

parameter for the occurrence of immune reactions.

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Introduction

Good long-term prognosis of penetrating corneal grafts has been explained by the immunological privilege of the cornea and the anterior chamber.^{1–4} However, corneal graft rejection is a significant complication in corneal transplantation and one of the most important reasons for graft failure.^{5,6}

The secretion of transforming growth factor- β (TGF- β) by corneal endothelial cells, cells of the trabecular meshwork, or the ciliary body has been identified to be an important factor for the maintenance of that immunological privilege.^{7–12}

TGF- β is a polypeptide cytokine with inhibitory activity on T-lymphocytes, and about 80–90% of this activity in the aqueous humour is due to the isoform TGF- β ₂.^{13,14} Antisera to TGF- β ₂ have been demonstrated to reverse the inhibitory activity of aqueous humour.¹⁴

Low levels of TGF- β ₂, therefore, might be associated with an elevated risk for immune reactions following penetrating keratoplasty (PK). In a previous study, we found that total TGF- β ₂ levels were similar in eyes with and without immune reactions following PK.¹⁵ However, more than 90% of total TGF- β ₂ cannot exert its biological functions via binding to its

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receptor as it is secreted as an inactive precursor (latent TGF- β complex). This precursor (200 kDa) is complexed with latency-associated peptide and bound to latent TGF- β -binding protein. To activate the latent form of TGF- β , the complexed peptides need to be removed extracellularly via proteolytic cleavage.¹⁶ The exact mechanisms by which latent TGF- β 2 is activated physiologically are not completely understood. One model of activation has been proposed in which latent TGF- β is released from the extracellular matrix by proteases, localised to cell surfaces, and activated for example by thrombospondin-1¹⁷ or specific integrins.¹⁸ In previous studies, we found that total TGF- β 2 levels in the aqueous humour of patients with and without immune reaction are comparable¹⁵ and that in the aqueous humour, only about 2–5% of total TGF- β 2 are available in its activated form.¹⁹ Furthermore, we found highest levels of active TGF- β 2 in keratoconus patients, who have the best prognosis following PK.²⁰ We therefore tested the hypothesis whether active TGF- β 2 is decreased in eyes with immune reaction compared to eyes without immune reaction following PK.

Patients and methods

Patients

In group I, we examined 38 cataract patients without anti-inflammatory medication and without a history of eye disease except cataract. None of the included patients suffered from glaucoma or diabetes. In this group, patients received either topical (92.1%), retrobulbar, (5.3%) or general anaesthesia (2.6%).

Group II consisted of 10 patients undergoing cataract extraction following PK with no signs of an immune reaction. In this group, one patient developed secondary glaucoma and one patient developed diabetes mellitus type 2 during follow-up.

Group III comprised 10 patients following PK with a newly diagnosed endothelial immune reaction. For therapeutic reasons, irrigation of the anterior chamber with corticosteroids was done immediately in those patients.²¹ None of the included patients suffered from glaucoma or diabetes. For the treatment of endothelial immune reactions in 30% of cases systemic and in 100% of cases topical steroids were started before puncture of the anterior chamber.

In cases of a high-risk keratoplasty, patients received long-lasting systemic immunosuppression either with cyclosporin A (CSA) or mycophenolate mofetil (MMF), which was started immediately after surgery. This was the case in five patients of group II (3 \times repeat keratoplasty, 1 \times herpetic keratitis, 1 \times acanthamoeba keratitis) and in two of group III (1 \times repeat keratoplasty,

1 \times herpetic keratitis). At the time of puncture of the anterior chamber, four patients (2 \times CSA, 2 \times MMF) in group II and one patient (1 \times MMF) in group III were still on systemic immunosuppression.

Detailed information on patients in groups II and III is given in Table 1.

All invasive procedures were performed with properly obtained written informed consent in adherence to the Declaration of Helsinki for research involving human subjects. Research was approved by the local ethics committee.

Penetrating keratoplasty, post-operative therapy, follow-up

All keratoplasties were performed with mechanical trephines. To fix the grafts, we used a double-running cross-stitch suture with Nylon 10.0.²² Gentamycin ointment was administered following surgery at least until the graft was covered by a complete epithelial layer. Then, prednisolone-21-acetate 1% eye drops were given five times daily and tapered during the first five post-operative months. Systemic corticosteroids were administered for only 3 weeks post-operatively. Oral acetazolamide was administered at a daily dose of 2 \times 250 mg for five days post-operatively. Follow-up examinations of the graft were scheduled 6 weeks, 4, 12, and 18 months post-operatively, and thereafter once a year.

High-risk cases were defined by an increased risk for immune reactions (eg having three or four quadrants with deep vascularisation, limbal-stem-cell insufficiency, severe atopic dermatitis,²³ herpes-keratitis^{24,25} history of previous keratoplasty, or positioning of the graft close to the limbus).²⁶

Grafts

Grafts were either kept in hypothermic storage or preserved in organ culture according to the guidelines of the European Eye Bank Association.²⁷ Detailed graft data are provided in Table 1.

Immune reactions

In all patients of group III, endothelial immune reactions were diagnosed clinically via endothelial precipitates and stromal oedema at the slit lamp.

Anterior chamber puncture

Prior to anterior chamber puncture all eyes were rinsed with sterile solution (BSS). A paracentesis lancet was used to penetrate the cornea in an avascular peripheral

Table 1 Grafts' and patients' data on study groups II and III

	Group II acceptors (n = 10)	Group III rejectors (n = 10)	P-value	Statistical test
Donor age (years)	57.6 ± 11.5	53.0 ± 22.0	0.57	t-test
Interval from death to graft excision (h) ^a	32.4 ± 14.6	18.2 ± 11.7	0.03	t-test
<i>Kind of graft storage</i>			0.14	χ^2 test
Organ culture (%)	90	50		
Hypothermic storage (%)	10	50		
Duration of organ culture (days)	20.4 ± 7.5	21.4 ± 5.4	0.81	t-test
Graft diameter (mm)	7.99 ± 0.22	8.05 ± 0.47	0.72	t-test
Preoperative cell density of the graft (cells/mm ²)	2485 ± 303	2380 ± 142	0.49	t-test
Recipient age (years)	55 ± 7.8	57.2 ± 18.4	0.73	t-test
Patient sex (female)	60	60	1	χ^2 test
<i>Diagnosis leading to PK</i>				
Fuchs' dystrophy (%)	30	50		
Keratoconus (%)	10	10		
Herpetic keratitis (%)	10	10	0.60	χ^2 test
Bulluos keratopathy (%)	0	10		
Corneal dystrophy	0	10		
Acanthameba keratitis (%)	10	0		
Corneal scar (%)	10	0		
Repeat keratoplasty (%)	30	10		
<i>Anesthesia</i>				
General (%)	20	0	0.31	χ^2 test
Retrolbulbar (%)	30	30		
Topical (%)	50	70		
Percentage of high-risk PK	50	20	0.35	χ^2 test
Whole follow-up period after PK (days)	941.9 ± 469.1	1063.3 ± 396.4	0.54	t-test
Follow-up after anterior-chamber puncture (days)	515.9 ± 162.5	534.1 ± 80.5	0.76	t-test
Time period between PK and anterior- chamber puncture (days)	426.0 ± 446.0	529.2 ± 400.7	0.59	t-test
Ratio with systemic steroids at puncture (%)	0	30	0.21	χ^2 test
Ratio with only topical steroids at puncture (%) ^a	50	100	0.04	χ^2 test
Ratio with antibiotics at puncture (%)	20	20	1	χ^2 test
<i>Ratio with systemic immunosuppression (CSA or MMF) at puncture (%)</i>	40	10	0.23	χ^2 test
CSA	20	0		
MMF	20	10		
Incidence of immune reactions following puncture (%)	0	20	0.45	χ^2 test
Ratio of clear grafts in whole follow-up (%)	100	70	0.21	χ^2 test

PK = keratoplasty.
^aP < 0.05.

area over a length of 1 mm. Contact with limbal or peripheral corneal vessels was completely avoided. If there was any bleeding observed, the sample was not used for further analysis. Aqueous humour (0.05–0.1 ml) was drawn into conventional tuberculine syringes without coming into contact with intraocular structures. Following this puncture, surgery was continued as usual.

In cases of IR irrigation of the anterior chamber with steroids followed.

Determination of active TGF- β_2

The concentration of active TGF- β_2 in the aqueous humour was determined by using a Sandwich-ELISA

(DuoSet Elisa Development Kit, human TGF- β_2 , R&D Systems Europe, Wiesbaden, Germany) within 3 h following puncture. Until determination by ELISA, all samples were kept at 4°C. ELISA was handled according to the manufacturer's manual except that the sample volume and the concentrations of the capture and the detection antibodies were modified. Because each sample volume was barely above 100 μ l, 50 μ l of each sample were diluted 1:2 with the reagent diluent and determined afterwards. The concentration of the capture antibody used was 4 μ g/ml, and of the detection antibody was 200 ng/ml. The sensitivity of that ELISA was 14 pg/ml of active TGF- β_2 . Therefore, concentrations below 14 pg/ml were regarded as 0 pg/ml. Optical density was read using an automated platereader (GENios, Tecan, Crailsheim, Germany) with a 450 nm filter. Concentrations were calculated using Magellan software (Tecan). Due to small sample volumes, an additional determination of total TGF- β_2 following acidic activation was not possible.

Statistical analysis

Statistical evaluation was performed using SPSS 11.0 for Windows. The paired *t*-test, χ^2 and ANOVA were used. Correlations were determined with the Pearson's correlation coefficient. *P*-values <0.05 were regarded as statistically significant.

Results

Average active TGF- β_2 concentration was 39.9 pg/ml \pm 24.3 (median 41.1 pg/ml) in group I, 46.7 pg/ml \pm 21.9 (median 45.2 pg/ml) in group II, and 12.7 pg/ml \pm 26.0 (median 0 pg/ml) in group III. The differences between groups I and III (*P* = 0.005, ANOVA), and groups II and III (*P* = 0.008, ANOVA) were statistically significant (Figure 1).

The group of acceptors (II) consisted of 10 patients with the average concentration of active TGF- β_2 being above the detection level in 9 of 10 measurements, whereas active TGF- β_2 levels in the aqueous humour of the 10 patients in group III with newly diagnosed immune reaction was below the sensitivity value of the ELISA in 70% of cases.

As active TGF- β_2 levels in the aqueous humour decrease with age (26), we performed a two-factorial ANOVA to confirm the difference between group III and groups I and II by monitoring age as a potential confounding factor, and still found a statistically significant difference (*P* < 0.01, ANOVA).

There were no significant correlations between gender and levels of active TGF- β_2 .

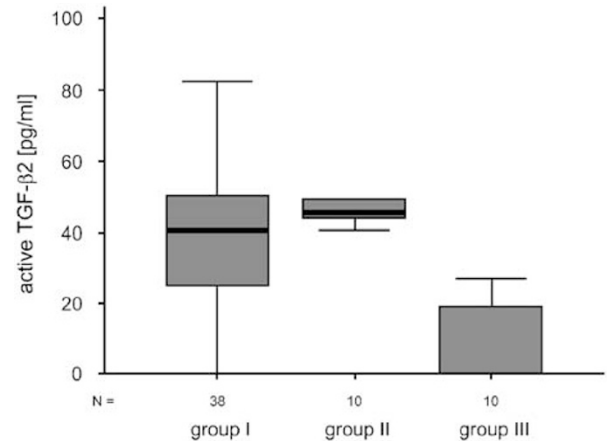


Figure 1 Active TGF- β_2 prior to cataract surgery (group I, *n* = 38, average active TGF- β_2 concentration 39.9 pg/ml \pm 24.3) and following penetrating keratoplasty without endothelial immune reaction (group II, *n* = 10, average active TGF- β_2 concentration 46.7 pg/ml \pm 21.9) as well as with endothelial immune reaction (group III, *n* = 10, average active TGF- β_2 concentration 12.7 pg/ml \pm 26.0). The differences between group III and groups I (*P* = 0.005, ANOVA) and II (*P* = 0.008, ANOVA) were statistically significant. Box plot diagrams given as median values, 25/75% percentiles (boxes), 10/90% percentiles (bars), and outliers (circles) of TGF- β_2 levels.

We could not find statistically significant differences for various grafts' properties with potential influence on post-operative endothelial cell density between groups II and III. However, in group III, the time from death to corneoscleral excision was significantly lower than in group II (*P* = 0.03, *t*-test).

Regarding clinical attributes, we found a statistically significant difference for the ratio of patients with topical steroids at the time of puncture with significantly more patients in group III being treated with topical steroids (*P* = 0.04, χ^2 test).

We did not find a statistically significant correlation for the time period between PK and puncture of the anterior chamber and the levels of active TGF- β_2 in groups II and III (*r* = -0.05, *P* = 0.89 for group II, *r* = -0.06, *P* = 0.86 for group III, Pearson's correlation).

All patients' and grafts' data are summarised in Table 1.

Had the sensitivity value (14 pg/ml) of the ELISA used for these analyses been set at 14 pg/ml instead of 0 pg/ml, it would have made no statistical differences to the results presented here.

Discussion

TGF- β_2 has been identified as a major factor maintaining the immunological privilege of the cornea and anterior chamber.¹⁻⁴ In a previous study, however, similar levels

of total TGF- β ₂ were observed in eyes with and without endothelial immune reactions following PK.¹⁵ Therefore, in this study, we determined levels of active TGF- β ₂ in the aqueous humour of patients with and without immune reaction following PK. As we could show that cryopreservation of aqueous humour increases active TGF- β ₂ levels probably due to partial activation of TGF- β ₂ by freezing and thawing,¹⁹ we analysed active TGF- β ₂ levels in all samples within 3 h following puncture.

Levels of active TGF- β ₂ were statistically significantly decreased in the aqueous humour of patients suffering from an endothelial immune reaction (group III) compared to patients without immune reactions following PK (group II), whose active TGF- β ₂ levels were similar to control patients without PK (group I).

As an endothelial immune reaction is usually accompanied by mild anterior uveitis, reduced levels of active TGF- β ₂ might be explained by dilution due to influx into the anterior chamber as part of the breakdown of the blood–aqueous barrier. If this was the case, total protein levels and total TGF- β ₂ levels were supposed to be decreased as well. However, we could show that total TGF- β ₂ levels¹⁵ as well as total protein levels (unpublished data) are comparable in the aqueous humour of patients with and without immune reaction. Furthermore, Min *et al*²⁸ found that active TGF- β ₂ levels were normal in patients with uveitic secondary glaucoma. Therefore, decreased active TGF- β ₂ levels during an endothelial immune reaction do not seem to be a simple result of an intraocular inflammation but a result of an immunological process in the anterior ocular segment.

Yamamoto *et al*²⁹ reported that there is a negative association between TGF- β ₂ levels and age with older patients having lower concentrations of TGF- β ₂ in the aqueous humour. Nevertheless, statistical analysis revealed significantly lower active TGF- β ₂ levels in patients suffering from an endothelial immune reaction compared to those without immune reaction when correcting for age as a confounding factor is done.

In this study, patients with a newly diagnosed endothelial immune reaction received statistically significant more topical steroids at the time of puncture than patients without endothelial immune reaction. On the one hand, topical or systemic steroids might reduce levels of active TGF- β ₂ in the aqueous humour as it could be shown that locally produced hydrocortisone and corticosterone inhibited the proliferation of a TGF- β ₂-sensitive cell line *in vitro*.³⁰ On the other hand, Wallentin and Lundberg³¹ found that topical steroid treatment did not reduce levels of bFGF and active TGF- β in rats following cataract extraction. Furthermore, Wakefield *et al*³² reported that members of the steroid hormone family are able to increase TGF- β ₁ levels probably by

regulating the translatability of TGF- β ₁ mRNA or by post-translational processing events. A significant fraction (25–90%) of the induced TGF- β was in its biologically active rather than in its latent form. They concluded that the use of members of the steroid hormone superfamily might even have therapeutic potential by inducing TGF- β as an immunosuppressive growth inhibitor. Whether topical or systemic steroid treatment has a direct influence on active TGF- β ₂ levels in the aqueous humour has to be investigated in further studies.

Patients in group II received more systemic immunosuppression (20% CSA, 20% MMF) compared to patients in group III (10% MMF). However, the difference was not statistically significant. The reason why patients in group II received more systemic immunosuppression than in group III, was that in this group, more high-risk keratoplasties were performed where systemic immunosuppression with CSA or MMF was started in the first place (see Table 1). Within group II, we could not find a statistically significant difference for TGF- β ₂ levels between patients with or without systemic immunosuppression (median 48.3 pg/ml *vs* 44.4 pg/ml, respectively). However, sample size is small and an effect of systemic immunosuppression with CSA or MMF cannot be excluded by this analysis. Therefore, further research is needed to find out whether systemic CSA or MMF might influence TGF- β ₂ levels in the aqueous humour as there are no data in the literature covering this question until now.

Primary open-angle glaucoma has been associated with increased levels of active TGF- β ₂ in the anterior chamber.^{33–36} Increased levels of active TGF- β ₂ have been reported as well in diabetes^{28,37} especially following photocoagulation.²⁹ To avoid influences on active TGF- β ₂ levels in the aqueous humour by these frequently accompanying diseases, we decided at the beginning of this study that glaucoma and diabetes were exclusion criteria. However, during follow-up in group II, one patient developed secondary glaucoma and one patient developed diabetes mellitus type II. As we found comparable TGF- β ₂ levels in group II and in the control group (group I), a significant effect of these two cases with diseases potentially increasing TGF- β ₂ levels seems to be unlikely.

The time from death to corneoscleral excision was statistically significantly lower for grafts transplanted in group III compared to those in group II. This might have a positive influence on the chronic endothelial cell loss following PK in group III.³⁸ Furthermore, there was a difference for the kind of graft storage. In group II, more grafts were kept in hypothermic storage compared to group II (90 *vs* 50%, respectively). However, death to corneoscleral excision time and the storage method have

never been reported to be correlated to an increased risk for the occurrence of endothelial immune reactions. On contrary, a loss of Langerhans cells has been observed during storage of corneal transplants, which was independent from preservation medium (organ culture or hypothermic storage) and preservation time.³⁹ Therefore, these grafts' attributes should neither influence active TGF- β 2 levels in the aqueous humour nor increase the frequency of immune reactions following PK.

In corneal transplantation, immune reactions mostly occur between 12 and 24 months following PK^{40,41} and afterwards endothelial immune reactions are rarely observed. Therefore, during this time, an immunosuppressive environment in the anterior chamber is exceptionally important, which could be maintained by high levels of active TGF- β 2. One could speculate that levels of active TGF- β 2 decrease with time following PK as the host might develop some kind of graft-induced tolerance to the donor tissue. If this assumption was correct, higher levels of active TGF- β 2 prior to PK would lead to better clear graft survival. This is furthermore supported by the fact that we found highest levels of active TGF- β 2 in keratoconus patients, who have the best prognosis following PK.²⁰ We received samples of patients from group II (426.0 ± 446.0 days) about 100 days earlier than those from group III (529.2 ± 400.7 days). This might have an influence on active TGF- β 2 levels in the aqueous humour. However, the difference in follow-up time was not statistically significant ($P = 0.59$, t -test). Furthermore, we could not find a statistically significant correlation for the time period between PK and puncture of the anterior chamber and active TGF- β 2 levels, but small sample size of only 10 patients in each group has to be considered. So, further studies are necessary, to find out if there is a decrease of active TGF- β 2 over time following PK.

Besides total or active TGF- β 2, various factors are involved in the maintenance and regulation of the immunological privilege of the anterior chamber, which have to be investigated in future studies. Thrombospondin, for example, is a potent physiologic regulator of TGF- β activation⁴² and somatostatin⁴³ as well as α -melanocyte stimulating hormone⁴⁴ have been identified as important regulators of the anterior chamber associated immune deviation.

The findings of this study might be interpreted as hinting at a primarily altered immunological privilege in eyes that develop immune reactions following PK. The question thus arises whether only eyes with decreased TGF- β 2 levels develop endothelial immune reactions following PK. If that is the case, active TGF- β 2 might serve as a predictive parameter if determined prior to PK. Then, decreased TGF- β 2 levels should force the

surgeon to administer effective (topical and/or systemic) immunomodulative measures in the long run. This has to be found out in further prospective studies with long-lasting clinical follow-up.

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