Active transforming growth factor-β₂ is increased in the aqueous humor of keratoconus patients

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Purpose: The secretion of transforming growth factor-β₂ (TGF-β₂) into the aqueous humor was identified to be important for the maintenance of the immunological privilege in the anterior chamber of the eye. Therefore, prognosis of penetrating corneal grafts might depend on intraocular levels of TGF-β₂. In this study, we determined active TGF-β₂ levels in eyes with pathological corneas prior to penetrating keratoplasty (PK).

Methods: Anterior chamber puncture was performed in 38 cataract patients without PK (group I), in 15 keratoconus eyes undergoing PK (group II), and in 66 eyes with various indications for PK (group III). About 0.05-0.1 ml of aqueous humor were harvested from each patient. Analysis of active TGF-β₂ was started via ELISA within three h following puncture.

Results: Average active TGF-β₂ concentration was 39.9±24.3 pg/ml (median 41.1 pg/ml) in group I, 78.9±53.8 pg/ml (median 85.8 pg/ml) in group II, and 31.5±37.7 pg/ml (median 23.6 pg/ml) in group III. The differences between group II and groups I and III were statistically significant (p<0.001).

Conclusions: The highest levels of active TGF-β₂ were found in keratoconus eyes. This finding might partly explain the excellent prognosis of keratoconus eyes regarding PK. Further (prospective) studies are being carried out to discover whether active TGF-β₂ might serve as predictive parameter for immune reactions following PK.

METHODOLOGY

Patients: In group I, we examined 38 patients without a history of eye disease except cataract. Group II consisted of 15 keratoconus eyes. Group III comprised 66 eyes with various indications for PK. For detailed information on the study groups see Table 1. Glaucoma and diabetes mellitus as accompanying diseases were excluded from the study.

All invasive procedures were performed with properly obtained written informed consent in adherence to the Declaration of Helsinki for research involving human subjects as well as to the principles outlined by the Committee on Publication Ethics. Research was approved by the local ethics committee.

Anterior chamber puncture: Prior to anterior chamber puncture all eyes were rinsed with sterile solution (BSS®; Alcon Laboratories Inc., Houston, TX). A paracentesis lancet was used to penetrate the cornea in an avascular peripheral 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 humor (0.05-0.1 ml) was drawn with conventional tuberculin syringes without contact to intraocular structures.

Determination of active transforming growth factor-β₂: The concentration of TGF-β₂ in the aqueous humor was determined using a Sandwich-ELISA (DuoSet Elisa Development Kit, human TGF-β₂, R&D Systems Europe, Wiesbaden, Germany) within three h following puncture. The samples were not frozen but stored at 4 °C within this time period.
The 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 sample volumes were small, 50 µl of each sample were diluted 1:2 with the reagent diluent and were determined afterwards. The concentration of the capture antibody was four µg/ml and of the detection antibody 200 ng/ml. The sensitivity of that ELISA was 14 pg/ml of TGF-β2, so concentrations below 14 pg/ml were regarded as 0 pg/ml. The optical density was read using an automated platereader (GENios, Tecan, Crailsheim, Germany) with a 450 nm filter. Concentrations were calculated with the Magellan software (Tecan, Crailsheim, Germany).

Statistical analysis: Statistical evaluation was performed using SPSS 11.0 for Windows. Chi² test, Fisher exact test, and multifactorial ANOVA were applied. Correlations were evaluated for statistical significance with Pearson’s test. A p-value of <0.05 was considered statistically significant.

RESULTS

The average active TGF-β2 concentration was 39.9±24.3 pg/ml (median 41.1 pg/ml) in group I, 78.9±53.8 pg/ml (median 85.8 pg/ml) in group II, and 31.5±37.7 pg/ml (median 23.6 pg/ml) in group III (Figure 1, for further details see Table 2). The differences between group II and groups I and III were statistically significant (p<0.001, ANOVA). If the sensitivity value (14 pg/ml) of the ELISA used for these analyses was set as 14 pg/ml but not as 0 pg/ml, the results did not reveal any statistically significant difference compared to the results presented here.

A statistically significant association between TGF-β2 and age was observed (p=0.03, r=-0.27, Pearson correlation) with older patients showing lower levels of active TGF-β2. Furthermore, we found statistically significant differences between groups I, II, and III regarding age (p<0.001, ANOVA) where the youngest patients belonged to the keratoconus group. Thus, we performed a two-factorial ANOVA to confirm the differences of active TGF-β2 levels between the three groups by monitoring age as a potential confounding factor and still found a statistically significant difference (p=0.03, ANOVA).

A statistically significant correlation between sex and the level of active TGF-β2 was not observed (p=0.77).

Statistically, patients in group I received significantly more topical anesthesia than patients in groups II and III. However, independently from the underlying disease, we could not find a statistically significant correlation between TGF-β2 levels and sex. Patients in group I received significantly more topical anesthesia than patients in groups II and III. However, independently from the underlying disease, we could not find a statistically significant correlation between TGF-β2 levels and sex.

Comparison of clinical data on study groups showing that patients with keratoconus (group 2) were younger than patients in study group 1 and 3 whereas patients in group 1 received more topical anesthesia than patients in group 2 and 3. The asterisk indicates a p<0.05 (t-test or χ² test).
and the kind of anesthesia (r=-0.03, p=0.72, Pearson correlation).

**DISCUSSION**

*Transforming growth factor-β2 levels in keratoconus eyes:* Levels of active TGF-β2 in aqueous humor were statistically significantly higher in keratoconus eyes than in controls or in patients with various other indications for keratoplasty. Differences between keratoconus eyes and eyes with herpes simplex virus keratitis or corneal scars did not reach statistical significance, but sample sizes in those groups were low. Therefore, the analysis need to be repeated with larger sample sizes to allow for a correct statistical interpretation.

Like Yamamoto et al. [9], we observed a negative correlation between TGF-β2 levels and age with older patients having lower concentrations of TGF-β2 in the aqueous humor. Nevertheless, keratoconus patients revealed statistically significantly higher levels of TGF-β2 than the other groups even when correction for age as a confounding factor was done. However, the p-value changed from p<0.001 to p=0.03. We also found statistically significant differences for the kind of anesthesia between the study groups and the different diseases leading to PK (see Table 1). However, we could not find a statistically significant correlation between active TGF-β2 levels and the kind of anesthesia (r=-0.03, p=0.72, Pearson correlation). Furthermore, there are no data in the literature about the influence of different kinds of anesthesia on TGF-β2 levels: hence, the kind of anesthesia might not be responsible for the differences in TGF-β2 levels observed in this study.

High levels of active TGF-β2 might be a consequence of high total protein levels in the aqueous humor. However, in a previous study, we were able to show that total protein levels are elevated to an identical extent in eyes with and without immune reactions following PK (analysis according to the method of Bradford, unpublished data). Therefore, total protein levels cannot explain differences of TGF-β2 levels in aqueous humor. Due to small sample volumes total protein analysis was not possible in this study.

Analysis of keratoconus corneas by Saghizadeh et al. [10] regarding TGF-β2 mRNA yielded the same values as in healthy subjects. An earlier study of Zhou et al. [11] found enhanced expression of TGF-β in keratoconus corneas. In both studies the evaluation was carried out immunohistologically and only Saghizadeh et al. [10] performed TGF-β subtyping. It remains unknown whether there is a correlation between intracorneal levels of TGF-β2 mRNA and intracameral levels of active TGF-β2.

*Influence of steroids on transforming growth factor-β2 levels:* Although only a few patients in group III received either topical or systemic steroids at the time of sampling, there might be an influence of steroids on active TGF-β2 levels in the aqueous humor. On the one hand, topical or systemic steroids might reduce levels of active TGF-β2 in the aqueous humor as it could be shown that locally produced hydrocortisone and corticosterone inhibited the proliferation of a TGF-β sensitive cell line in vitro [12]. On the other hand, Wallentin and Lundberg [13] found that topical steroid treatment did not reduce levels of bFGF and active TGF-β in rats following cataract extraction. Furthermore, Wakefield et al. [14] reported that members of the steroid hormone family are able to increase TGF-β levels probably by regulating the translatability of TGF-β1 mRNA or by post-translational processing events. A significant fraction (25-90%) of the induced TGF-β has been detected in its biologically active rather than latent form.

### Table 2. Concentrations of active transforming growth factor-β2 in the aqueous humor

<table>
<thead>
<tr>
<th>Indication</th>
<th>n</th>
<th>Active TGF-β2</th>
<th>Comparison to keratoconus</th>
<th>Comparison to cataract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cataract</td>
<td>38</td>
<td>39.9±24.3 (41.1)</td>
<td>p&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td>Keratoconus</td>
<td>15</td>
<td>78.9±53.8 (85.8)</td>
<td>-</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Fuchs dystrophy</td>
<td>16</td>
<td>40.2±31.8 (37.8)</td>
<td>p=0.007</td>
<td>p=1.0</td>
</tr>
<tr>
<td>Bullous keratopathy</td>
<td>11</td>
<td>11.9±14.3 (0)</td>
<td>p&lt;0.001</td>
<td>p=0.033</td>
</tr>
<tr>
<td>Repeat keratoplasty</td>
<td>16</td>
<td>40.3±37.2 (31.8)</td>
<td>p=0.011</td>
<td>p=1.0</td>
</tr>
<tr>
<td>Herpes simplex virus keratitis</td>
<td>6</td>
<td>60.5±72.1 (46.7)</td>
<td>p=0.695</td>
<td>p=0.489</td>
</tr>
<tr>
<td>Corneal scars</td>
<td>3</td>
<td>39.4±34.1 (57.9)</td>
<td>p=0.170</td>
<td>p=1.0</td>
</tr>
<tr>
<td>Various</td>
<td>14</td>
<td>12.9±26.6 (0)</td>
<td>p&lt;0.001</td>
<td>p=0.03</td>
</tr>
</tbody>
</table>

The statistical analysis was ANOVA. The sensitivity of the ELISA was 14 pg/ml of TGF-β2 therefore, concentrations below 14 pg/ml were regarded as 0 pg/ml. If more than 50% of the samples had TGF-β2 levels below the detection limit, the resulting median was presented as 0 pg/ml.
form. They concluded that the use of the members of the steroid hormone superfamily for inducing TGF-β as an immunosuppressive growth inhibitor might even have therapeutic potential [14]. Whether topical or systemic steroid treatment has a direct influence on active TGF-β levels in the aqueous humor has to be investigated in further studies.

**Transforming growth factor-β, levels in glaucomatous eyes:** Various recent studies found increased levels of TGF-β2 in the aqueous humor of glaucoma patients suggesting an effect of TGF-β in the pathogenesis of glaucoma [15-17]. However, keratoconus patients do not have an increased risk for glaucoma. One explanation for this could be that the results of these studies might have been influenced by cryopreservation of the analyzed samples as we could recently show that freezing and thawing leads to partial activation of TGF-β2 [8]. Another explanation could be the distribution of TGF-β2 in the anterior chamber. In glaucoma, for example, cells of the trabecular meshwork are observed to be the main producers of TGF-β2 and are, at the same time, the most affected cell type [17]. In corneal disorders, endothelial cells might be responsible for increased or decreased levels of TGF-β2 and might be affected regarding their proliferative potential [18]. Therefore, the effects of TGF-β2 in the anterior chamber probably depend on the production site and on the cells being mainly affected by locally produced TGF-β2.

**Influence of transforming growth factor-β2 on the pathogenesis of keratoconus:** Elevated levels of TGF-β2 in aqueous humor and corneal tissue [11] of keratoconus eyes might also be associated with its pathogenesis. On the one hand, TGF-β2 diminishes various chemokines. On the other hand, it elevates metalloproteinases such as TIMP-1 [19], which are suspected to be involved in the pathogenesis of keratoconus. Girard et al. reported that TGF-β2 modulates metalloproteinase expression of corneal stromal cells in vitro [20]. Parkin and coworkers, however, found no TGF-β2 effect on the in vitro activity of the keratocytic metalloproteinases derived from keratoconus corneas [21].

**Influence of transforming growth factor-β2 on graft prognosis following penetrating keratoplasty:** As TGF-β2 maintains parts of the anterior chamber-associated immune deviation, which promotes corneal allograft survival [1], increased TGF-β2 levels might partly explain the good prognosis of PK in patients with keratoconus. In the long term, we observed that more than 90% of penetrating grafts in keratoconus eyes remained clear. This is in contrast to all other indications for PK such as bullous keratopathy or even Fuchs’ endothelial dystrophy [22,23]. However, keratoconus eyes do not have significantly less endothelial immune reactions than eyes with Fuchs’ endothelial dystrophy [22]. This could be explained by a reduced slow chronic endothelial cell loss due to clinically invisible immune reactions in keratoconus eyes [23]. As a result, high TGF-β2 levels might establish an immunosuppressive climate in the anterior chamber, reducing that chronic endothelial cell loss in the absence of clinically significant immune reactions. Another explanation is that the improved prognosis of keratoconus eyes following PK could result from better endothelial reserves of keratoconus eyes compared to Fuchs’ endothelial dystrophy or bullous keratopathy. However, besides its immunosuppressive effects, TGF-β2 keeps the corneal endothelium in a non-proliferative state if cell contact is lost [18]. So, following PK keratoconus eyes with elevated levels of active TGF-β2 might have a worse proliferative potential of endothelial cells compared to corneal disorders with lower TGF-β2 levels in the aqueous humor. This favors an immunological effect of high TGF-β2 levels leading to improved clear graft survival of keratoconus eyes following PK.

**Perspectives:** As TGF-β2 is not the only cytokine maintaining the immunological privilege of the anterior ocular segment, future analysis will have to comprise further cytokines and growth factors in the aqueous humor like somatostatin, thrombospondin, or alpha-melanocyte stimulating hormone prior to and following PK. Future studies with long term follow-up will probably show what happens to TGF-β2 levels following PK and whether high preoperative levels of TGF-β2 are associated with less immune reactions and good long term clear graft survival.

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**REFERENCES**


