

# Total protein concentration and T-cell suppression activity of aqueous humour before and after penetrating keratoplasty

J-S Mo<sup>1</sup>, P Maier<sup>2</sup>, D Böhringer<sup>2</sup>, H Reinshagen<sup>3</sup>, R Sundmacher<sup>2</sup> and T Reinhard<sup>2</sup>

## Abstract

**Purpose** We wished to determine whether immune privilege parameters assayed in aqueous humour (AqH) are relevant to the fate of penetrating keratoplasty (PK) in humans.

**Methods** AqH was collected in 28 patients before PK (prospective cohort), in 6 patients with no history of graft rejection undergoing cataract surgery after PK (acceptors), in another 6 patients undergoing treatment of an acute endothelial immune reaction (rejectors), and in 65 controls undergoing uncomplicated cataract extraction. AqH was tested for total protein concentration and the ability to suppress T-cell activation.

**Results** AqH protein concentrations of acceptors and rejectors post-PK were elevated ( $2.7 \pm 0.8$  and  $2.7 \pm 0.7$  mg/ml, respectively) compared with pre-PK AqH level and cataract controls ( $1.0 \pm 0.1$  mg/ml,  $P = 0.01$ ). All AqH samples suppressed T-cell activation, irrespective of source and timing of AqH removal.

**Conclusion** Assays of immune privilege markers in AqH suggest that PK surgery may result in a sustained loss of integrity of the blood–aqueous barrier. Although trends were evident, values of immune privilege markers determined pre- and post-PK were not statistically significantly different between the study groups. However, further prospective studies determining additional immune privilege markers have to be conducted in order to find out whether these markers might serve as predictive parameters for immune reactions following PK.

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**Keywords:** keratoplasty; aqueous humour; immune privilege; blood–aqueous barrier

## Introduction

At 5 years after penetrating normal-risk keratoplasty (PK), up to 95% of the grafts are clear.<sup>1</sup> Nevertheless, most individuals are likely to be forced to undergo at least a second keratoplasty in their lifetime, as clear graft survival might ultimately be limited by late endothelial failure due to chronic endothelial cell loss: although inter-individual variability is high, ~17% of central endothelial cell density is lost annually, even in the absence of any clinically evident endothelial immune reaction or any other known damaging factor to the corneal endothelium.<sup>2,3</sup> Individual extrapolation<sup>4</sup> implies a life span that might be potentially limited to only around 20 years in most cases, as central endothelial cell density values  $< \sim 400$  cells per  $\text{mm}^2$  are incompatible with clear graft survival.<sup>5</sup> Although some influencing factors on the individual severity of chronic endothelial cell loss have been established,<sup>2</sup> the main causative mechanisms remain elusive. A clinically invisible immune reaction directed against endothelial cells is a promising candidate, but immunological involvement has not yet been proven.<sup>6</sup>

Additionally, 23% of penetrating normal-risk keratoplasties suffer from at least one clinically evident endothelial immune reaction within the first 5 years.<sup>1</sup> These acute graft reactions further reduce central endothelial density.<sup>7</sup> Even if endothelial immune reactions are reversible because of intense and early treatment,<sup>8</sup> the life

<sup>1</sup>Department of Ophthalmology, University of Louisville School of Medicine, Louisville, KY, USA

<sup>2</sup>University Eye Hospital, Freiburg, Germany

<sup>3</sup>Eye Hospital Pallas, Olten, Switzerland

Correspondence: P Maier, University Eye Hospital, Killianstr. 5, 79117 Freiburg, Germany  
Tel: +49 761 270 40010;  
Fax: +49 761 270 40630.  
E-mail: philip.maier@uniklinik-freiburg.de

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spans of affected grafts are reduced dramatically.<sup>7</sup> Therefore, *a priori* identification of patients at risk for aggressive loss of endothelial cells is desirable and would pave the way for secondary and tertiary prevention.

Studies carried out in experimental animals have shown that deterioration of ocular immune privilege may contribute to corneal allograft rejection and/or severe chronic endothelial cell loss. As there are markers of immune privilege integrity that can be assessed in aqueous humour (AqH),<sup>9,10</sup> a study of these immune privilege integrity markers before and after PK seems warranted.

Transforming growth factor (TGF)- $\beta$ 2, for example, is one of the most important factors in AqH to maintain the immunosuppressive climate in the anterior chamber that has a role in immune privilege of the eye.<sup>11</sup> We found that active<sup>12</sup> but not total<sup>13</sup> TGF- $\beta$ 2 is decreased in the AqH of patients actively suffering from an endothelial immune reaction. Furthermore, TGF- $\beta$ 2 is statistically significantly increased in the AqH of patients with keratokonus compared with patients with various other corneal diseases.<sup>14</sup> This may partially explain the excellent prognosis of patients with keratokonus following PK.

In the present study, two putative immune privilege integrity markers are evaluated:

- (1) *Total AqH protein concentration*: The amount of protein in AqH is an index marker for the integrity of the blood–aqueous barrier, as this barrier normally prevents random diffusion of high-molecular-weight blood components out of the blood vessels.<sup>15,16</sup> A defective blood–aqueous barrier, in turn, would facilitate enrichment of AqH for complement factors, circulating antibodies,<sup>17,18</sup> or metabolites exerting toxicity towards the corneal endothelium.<sup>19</sup>
- (2) *Capacity of AqH to suppress in vitro the activation of T cells*: The immunosuppressive activity of AqH on T-cells stimulated *in vitro* by anti-CD3 screens for

the overall functional integrity of the immunosuppressive microenvironment that helps to confer immune privilege on the anterior chamber and indirectly on the corneal allograft.

By examining the AqH obtained from patients before PK, at the time of graft rejection (rejectors), and at the time of cataract extraction post-PK (acceptors), we determined whether the abnormality of one or more markers of immune privilege integrity in AqH correlated with the occurrence of graft rejection, and whether the PK itself alters ocular immune privilege.

## Materials and methods

### Study design

Anterior chamber puncture was performed in 28 patients before PK, who were prospectively followed regarding the occurrence of immune reaction; in 6 patients post-PK, with no history of graft rejection and undergoing cataract surgery (acceptors); and in another 6 patients post-PK, who were undergoing treatment of an acute phase of an endothelial immune reaction (rejectors). Data on patients are given in Table 1. As normal controls, 65 patients undergoing uncomplicated cataract surgery provided normal AqH. AqH samples were assayed for total protein content and capacity to suppress T-cell activation *in vitro*. Data were collected after obtaining written informed consent in adherence to the Declaration of Helsinki for research involving human subjects. The local Ethics Committees approved the research.

### Anterior chamber paracentesis

Anterior chamber puncture was performed under the operation microscope after administering topical or retrobulbar anaesthesia within 24 h after referral of the

**Table 1** Patients, organ culture and donor data

	Group 1: <i>prospective cohort</i>	Group 2: <i>acceptors</i>	Group 3: <i>rejectors</i>	P-value
<i>n</i>	28	6	6	—
Time interval from keratoplasty to puncture (days)	—	311.5 ± 71.6	222.8 ± 29.0	0.28 <sup>a</sup>
Percentage with up to two HLA mismatches (A/B/DR)	72.2	16.7	50.0	0.27 <sup>b</sup>
Percentage normal risk keratoplasties	64.7	66.7	33.3	0.28 <sup>b</sup>
Age of patients (years)	63.8 ± 4.1	61.7 ± 3.6	57.0 ± 8.6	0.63 <sup>a</sup>
Age of donors (years)	67.0 ± 0.4	69.7 ± 5.0	58. ± 7.8	0.24 <sup>a</sup>
Post-mortem time (h)	19.2 ± 4.6	14.8 ± 5.7	21.6 ± 8.9	0.54 <sup>a</sup>
Storage time in organ culture (days)	17.2 ± 0.6	18.5 ± 1.4	16.2 ± 0.9	0.20 <sup>a</sup>
Endothelial cell density after organ culture (cells/mm <sup>2</sup> )	2457.7 ± 61.0	2231.0 ± 45.5	2381.3 ± 80.3	0.14 <sup>a</sup>
Follow-up (days)	551.3 ± 40.7	697.3 ± 70.5	518.7 ± 120.6	0.23 <sup>a</sup>

Mean ± SEM statistical tests for comparison of group 2 and group 3.

<sup>a</sup>ANOVA.

<sup>b</sup> $\chi^2$ -test.

patients to the clinic. All eyes were rinsed with a sterile solution (BSS) before anterior chamber puncture. A paracentesis lancet was used to penetrate the cornea in an avascular peripheral area over a length of 1 mm. Contamination from limbal or peripheral corneal vessels was avoided carefully. AqH (0.05–0.1 ml) was collected in conventional tuberculin syringes, transferred to plastic vials, and immediately frozen (–50 °C).

### *Penetrating keratoplasty*

Only donor corneas that met the EEBA criteria<sup>20</sup> were used for surgery. All corneas had been held in organ culture for at least 10 days. The data of donors are given in Table 1.

### *Assessment of rejection-free clear graft survival*

Visits were scheduled 6 weeks, 4 and 12 months postoperatively. Long-term follow-up was scheduled yearly.

Immune reactions were diagnosed when either endothelial precipitates or stromal oedema was present in the graft. In case of graft rejection the anterior chamber was infused with corticosteroids<sup>8</sup> for therapeutic reasons immediately in those patients. Afterwards all patients suffering from the immune reactions were treated with corticosteroid eye drops (prednisolone-21-acetate 1%) every hour, and the dosage was tapered individually until all precipitates were eliminated. Furthermore, a subconjunctival injection with betamethasone-21-acetate was performed. In severe cases, where extensive corneal oedema was present, systemic corticosteroids at a daily oral dose of 1 mg flucortolone per kg body mass were administered additionally and tapered within 3 weeks.

### *Analysis of AqH samples*

#### *Measurement of total protein concentration*

Protein levels in AqH were analysed using a protein assay kit (BCA; Pierce, Rockford, IL, USA). Bovine serum albumin was used as a standard.

#### *Effect of AqH on T-cell proliferation stimulated in vitro by anti-CD3mAb*

T-cell proliferation was assayed in a miniculture system as described previously.<sup>21</sup> Spleens were removed from naive BALB/c mice and pressed through nylon mesh to produce single-cell suspensions. Red blood cells were lysed with Tri-NH<sub>4</sub>Cl. T cells were subsequently purified by passing through a T-cell enrichment column (R&D Systems, Minneapolis, MN, USA). The enriched T cells were suspended in serum-free medium composed of RPMI 1640 medium, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml

penicillin, 100 µg/ml streptomycin (all from BioWhittaker, Lancaster, MA, USA), and 100 mM 2-ME (Sigma Chemicals, St Louis, MO, USA), and supplemented with 0.1% bovine serum albumin (Sigma, Chemicals), insulin, transferrin, and selenium (ITS +) culture supplement (1 µg/ml iron-free transferrin, 10 ng/ml linoleic acid, 0.3 ng/ml Na<sub>2</sub>Se, and 0.2 µg/ml Fe[NO<sub>3</sub>]<sub>3</sub> (Collaborative Biomedical Products, Bedford, MA, USA). A 96-well V-shaped bottom plate (Corning, NY, USA) was used in the assay. Twenty-five thousand (2.5 × 10<sup>4</sup>) enriched T cells in 10 µl serum-free medium and 5 µl of AqH or PBS were added to each well and cultured for 1 h. Then 10 µl hamster anti-mouse CD3e IgG (2C11, 2.5 µg/ml; PharMingen, San Diego, CA, USA) in 10 µl serum-free medium (or 10 µl serum-free medium alone, as negative control) was added to the well. The cells were pulsed with 2.5 µl 20 Ci/ml [3H]thymidine for the final 8 h of the 48-h incubation (37 °C, 5% CO<sub>2</sub>–95% humidified air mixture), and recovered using a cell harvester (model 96, Tomtec, Orange, CT, USA). Thymidine [3H] incorporation was measured in counts per minute, using a liquid scintillation counter (Betaplate 1205; Wallac, Gaithersburg, MD, USA). Each AqH sample was distributed to three wells of the culture and average of the counting from the three wells was regarded as the final reading of the sample.

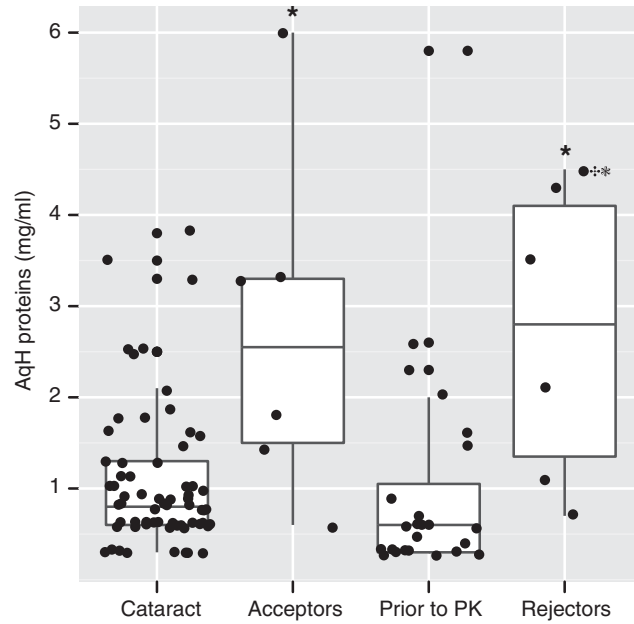
### *Statistical analysis*

Statistical analysis was performed in order to detect differences between groups in the two individual integrity markers defined above. Total protein concentration and ability of AqH samples to suppress *in vitro* stimulation of T cells were compared between groups by means of Student's *t*-test.

## **Results**

### *Assay of protein content of AqH samples*

AqH collected from control patients undergoing cataract extraction showed a mean total protein concentration of 1.0 ± 0.1 SEM mg/ml (*n* = 65, Figure 1). Similarly, the mean protein concentration in AqH from patients before PK was also 1.0 ± 0.3 SEM mg/ml (*n* = 24, Figure 1). By contrast, AqH samples obtained from eyes with rejected cornea grafts (*n* = 6) and from eyes with accepted cornea grafts (*n* = 6) showed elevated levels of total protein, each with a mean value of ~2.7 mg/ml (Figure 1). Although there is no statistically significant difference between the protein levels of AqH from eyes with rejected grafts and those from eyes with accepted grafts, the protein levels in both groups were significantly higher than the protein levels in cataract control AqH and pre-PK AqH (Figure 1).



**Figure 1** Box plot of total protein concentrations in AqH. AqH samples were obtained from cataract controls ( $n = 65$ ), from patients at the time of subsequent cataract surgery (acceptors,  $n = 6$ ), from patients before PK ( $n = 28$ ), and during rejection (rejectors,  $n = 6$ ). Values in the box-plot diagram are given as median, 25/75% percentiles (boxes), 10/90% percentiles (bars) and individual values (dots) of the total protein concentration. \* indicates mean values statistically significantly greater than cataract controls,  $P < 0.05$ .

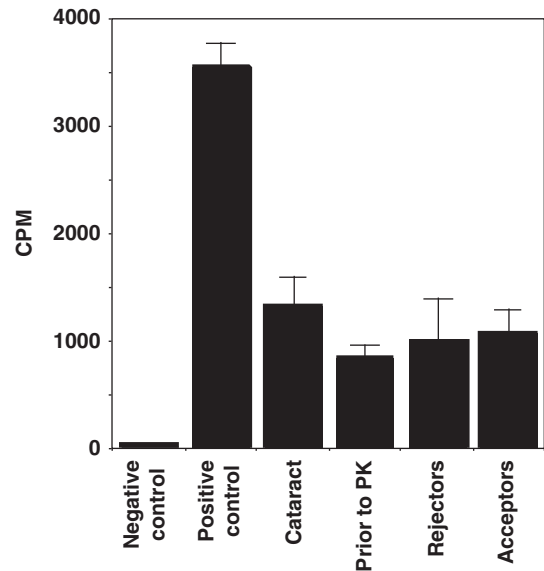
These results indicate that breakdown of the blood–ocular barrier (as revealed by elevated protein levels in AqH) is a typical sequel of PK, and that this condition persists for an extended period of time post surgery, irrespective of whether the graft is rejected or accepted.

**Assay of effects of AqH on T-cell proliferation**

AqH samples were also tested for their capacity to suppress proliferation of murine T-cells stimulated *in vitro* with the mitogenic antibody anti-CD3. As the results displayed in Figure 2 reveal, AqH from cataract control eyes suppressed T-cell proliferation profoundly (37% of positive control). AqH from pre-PK donor eyes, as well as from rejector and acceptor eyes similarly suppressed T-cell activation (all  $< 37\%$  of positive control). There is no statistically significant difference among these four groups. These findings indicate that the capacity of AqH to suppress T-cell activation *in vitro* is not altered by the disease processes creating the need for PK or by the PK surgical procedure itself, nor by the ultimate fate of the graft.

**Discussion**

It is anticipated that ocular surgeries such as PK cause a breakdown in the blood–ocular barrier. In fact, short-term disintegration of the blood–aqueous barrier



**Figure 2** Effects of AqH on T-cell proliferation. Murine T cells (BALB/c,  $2.5 \times 10^4$ ) were cultured with anti-CD3 antibody (2C11, positive control) or without 2C11 (negative control), or with anti-CD3 antibody plus AqH ( $5 \mu\text{l/well}$ ) from the different patient groups described in the legend to Figure 1. After 48 h of incubation and 8 h of pulse with [ $^3\text{H}$ ]thymidine, radioisotope incorporation was measured in counts per minute. Values represent mean  $\pm$  SEM. No statistically significant differences between the study groups were observed.

after PK has been observed after PK<sup>22–24</sup> by means of laser flare tyndallometry, which provides a non-invasive estimation of the total protein concentration in AqH.<sup>24</sup> Massive disruption of the barrier, as diagnosed from increased anterior chamber flare-tyndallometry values, has been associated with an increased risk of developing rejection episodes.<sup>24</sup> However, our results on total protein content during graft rejection could not confirm this association. Our results rather indicate that a compromised blood–aqueous barrier, based on significantly elevated protein concentrations in AqH, is a persistent feature of eyes following PK, irrespective of whether the grafts are accepted or rejected. Therefore, the finding of increased AqH protein concentration in only the post-PK specimens indicates that this is a postsurgical and not allospecific effect on account of the more traumatic surgery compared with cataract extraction. Moreover, our findings imply that reestablishment of a blood–aqueous barrier is not readily achieved after PK.

Our finding that AqH protein concentrations remain abnormally high even after 1 year post-PK is striking because previous studies have reported a return within less than a year to a normal blood–aqueous barrier based on AqH laser flare values.<sup>22–24</sup> It is possible that direct measurement of the AqH concentration of protein may prove to be a more sensitive indicator of the integrity of the blood–aqueous barrier than AqH laser flare. In contrast to our results, Van Gelderen *et al.*<sup>25</sup> found that 5 months following PK, the total protein content in the AqH of patients with accepted grafts was lower compared with patients with graft rejection or herpetic stromal keratitis. Furthermore, we cannot conclude that the observed breakdown of the blood–aqueous barrier is specifically found following PK as we did not include a second control group (eg, patients following cataract).

It is of further interest that the capacity of AqH to suppress T-cell activation *in vitro* is still a characteristic of AqH from patients who had undergone PK before, as this kind of AqH contains high levels of proteins that have leaked through a compromised blood–aqueous barrier. Recent studies of AqH removed from the eyes of mice with experimentally induced inflammation in the anterior chamber have yielded a similar result. AqH from the eyes of mice with experimental autoimmune uveitis recovers its capacity to suppress T-cell activation *in vitro* even while the intraocular inflammation accelerates.<sup>26</sup> Similarly, AqH from the eyes of mice in which anterior uveitis has been induced with an intravitreal injection of bacterial lipopolysaccharide is profoundly inhibitory of T-cell activation *in vitro*.<sup>21</sup> The immunosuppressive factor closely linked with suppression in these uveitic eyes is active TGF- $\beta$ . This is in line with previous reports that

suggest that high levels of TGF- $\beta$  may be protective for immune reactions after PK.<sup>10,12</sup> The pan-T-cell suppressor activity measure in this experimental setting does not seem to be an adequate parameter to characterize the status of the immune privilege following PK. However, there are many more factors that influence the immune privilege of the anterior chamber that may be helpful in explaining immune reactions following PK.<sup>27</sup> Analysing the levels of individual cytokines or cytokine patterns in the AqH for example might give more specific information on the disturbances of the immune privilege in the anterior chamber.<sup>12,13,28–31</sup>

It was disappointing to learn that total protein content and capacity to suppress T-cell proliferation by AqH failed to correlate with graft rejection. As studies conducted in mice have already demonstrated, the eye subjected to trauma or an inflammatory insult can call upon multiple, nested strategies to maintain local immunosuppression (suppression of T-cell proliferation). Therefore, any assay of AqH that simply assesses global immunosuppression will fail to distinguish strategies that promote graft acceptance from strategies to avoid graft rejection. Furthermore, the low number of samples in this investigation regarding patients following PK, with only six patients in each group, is another limitation regarding the interpretation of the presented results. We hope that future studies targeting other immunomodulatory factors in AqH (such as various interleukins, thrombospondin, and alpha-melanocyte-stimulating hormone) may prove to be helpful in predicting the risk of immune reactions following PK.

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## Summary

### What was known before

- Despite the immunological privilege of the anterior ocular segment immune reactions are still the major reason for graft failure following penetrating keratoplasty. However, the mechanisms leading to graft rejection are not fully understood. Until now there are only clinically defined risk factors helping to decide who is at high risk for graft rejection and who is not.

### What this study adds

- The analysis of aqueous humour from patients with and without immune reactions following keratoplasty revealed that the blood–aqueous barrier is broken down for more than 1 year after surgery. Furthermore, the capability of aqueous humour to suppress T-cell activation did not change after keratoplasty compared with controls independently of the occurrence of immune reactions. Finally, determination of total protein content and the capability to suppress T-cell activation in aqueous humour could not help to differentiate between patients with and without immune reactions, and so more specific immunological parameters in aqueous humour need to be analysed in the future.
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## Conflict of interest

The authors declare no conflict of interest.

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