Soluble Fas Ligand and Transforming Growth Factor β₂ in the Aqueous Humor of Patients With Endothelial Immune Reactions After Penetrating Keratoplasty

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Background: Excellent long-term prognosis of penetrating corneal grafts has been explained by the immunological privilege of the cornea and the anterior chamber. In animal models the secretion of transforming growth factor β₂ (TGF-β₂) into the anterior chamber and the expression of the Fas ligand on corneal endothelial cells were identified as important for the integrity of the immunological privilege.

Objective: To determine the TGF-β₂ and soluble Fas ligand (sFasL) levels in the aqueous humor of patients after penetrating keratoplasty (PK) who have and who do not have immune reactions.

Methods: Anterior chamber puncture was performed in 13 patients who had a cataract without PK (group 1), in 31 patients after PK who did not have immune reactions (group 2), and in 12 patients after PK newly diagnosed as having endothelial immune reactions (group 3). Total TGF-β₂ and sFasL were determined via enzyme-linked immunosorbent assay.

Results: Transforming growth factor β₂ was detected in all patients, irrespective of the underlying condition; there was no difference in TGF-β₂ levels between the different groups (P= .89, analysis of variance). None of the patients in group 1, 11 of 31 patients in group 2, and 8 of 12 patients in group 3 had detectable sFasL concentrations (P=.002, χ² test). Soluble Fas ligand averaged (mean [SD]) 20.8 (31.1) pg/mL in group 2, and 38.1 (33.2) pg/mL (P<.01, analysis of variance) in group 3.

Conclusions: It appears that total TGF-β₂ is maintained at high steady-state levels, while the level of sFasL is upregulated in patients who underwent PK, particularly in the advent of graft rejection.

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SINCE THE first description by Paufique et al¹ in 1949 of an immune reaction in a patient after penetrating keratoplasty (PK), a variety of animal keratoplasty models have contributed to the understanding of the immunological cascade and the immunological privilege of the cornea and the anterior chamber.²-⁶ Excellent long-term prognosis of penetrating corneal grafts has been explained by the immune privilege of the cornea and the anterior chamber.²-⁶ In animal models, the secretion of transforming growth factor β (TGF-β) by corneal endothelial cells, cells in the trabecular meshwork, or cells of the ciliary body was identified to be important for the integrity of the immunological privilege.⁷-¹² More recently, expression of Fas ligand (FasL) on corneal endothelial cells was described, and a role for this molecule in the immune privilege was deemed possible.¹³-¹⁶ Transforming growth factor β is a polypeptide cytokine with inhibitory activity on T lymphocytes.¹⁷ About 80% to 90% of the biological TGF-β activity in the aqueous humor of rabbits and humans is due to TGF-β₂.¹⁸ Antiserum samples to TGF-β₂ were demonstrated to reverse the inhibitory activity of the aqueous humor.¹⁸ These findings led to the paradigm of TGF-β₂ as the major molecule guarding the immune privilege.

Fas and its ligand (FasL) are transmembrane proteins that are responsible for peripheral lymphocyte homeostasis during immune responses.¹⁹ Interaction between Fas+ cells and FasL+ cells leads to apoptosis of Fas+ cells.¹³,¹⁴ Abundant amounts of FasL are found in the retina, the uvea, and the cornea of mice and humans.¹³ In the cornea, FasL, expressed on human endothelial and epithelial cells, was demonstrated to be capable of killing Fas+ lymphoid cells.¹⁵ In the mouse keratoplasty model, FasL+ orthografts were accepted at a rate of 45%, whereas FasL- grafts were all rejected.¹⁵ A soluble form of Fas ligand (sFasL) is released by the shed-
ming of membrane-bound FasL. Soluble FasL has been demonstrated to be released by activated lymphocytes.13,19 Likewise, the corneal endothelium has been suggested to be capable of releasing sFasL.20 Soluble FasL is most probably incapable of inducing apoptosis.21 On the contrary, it may even interfere with induction of apoptosis by membrane-bound FasL.21

Disturbance of the immunological privilege may be followed by immune reactions leading to irreversible graft failure. Immune reactions after PK are observed in up to 18% of normal-risk patients and in up to 75% of high-risk patients.22-25 In the clinical setting it has not yet been investigated if TGF-β and/or sFASL levels in the aqueous humor correlate with immune reactions and graft survival. If this was the case, the determination of TGF-β, and sFasL levels in the aqueous humor might contribute to establish a risk profile for every patient who undergoes PK. Hence, preventive measures such as systemic immunosuppression might be administered individually. In this study, therefore, TGF-β, and sFasL concentrations in the aqueous humor of patients with PK who had and who did not have immune reactions were determined.

**METHOD**

Anterior chamber puncture was performed in 56 patients. In group 1, 13 patients who had a cataract but who were not receiving anti-inflammatory medication and who had no history of eye disease except cataract were examined. Group 2 consisted of 31 patients after PK without any sign of an immune reaction. All patients of groups 1 and 2 were referred to the clinic for cataract surgery. Group 3 comprised 12 patients after PK with a newly diagnosed endothelial immune reaction. All invasive procedures were performed after 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. Detailed information on the patients is given in Table 1.

**PK, POSTOPERATIVE THERAPY, AND FOLLOW-UP**

All keratoplasties were performed with modified Franschetti trephines. For graft fixation a double-running cross-stitch suture with nylon 10.0 was used.26 After surgery topical gentamicin sulfate, 5 mg 4 times daily, was administered at least until the graft was covered by a complete epithelial layer. Then 1% prednisolone acetate eye drops were given 5 times daily and tapered during the first 5 postoperative months. Systemic corticosteroids were administered for only 3 weeks postoperatively. Oral azetabolamide was administered at a daily dose of 250 mg twice daily for 5 days postoperatively. In high-risk keratoplasties systemic mycophenolate mofetil (1 g, twice daily) or systemic cyclosporine (aiming at trough levels between 120 and 150 ng/mL) was administered for 6 months postoperatively. Postoperative slitlamp examinations of the control grafts were scheduled at 6 weeks and at 4, 12, and 18 months postoperatively, and thereafter once a year.

**GRAFTS**

All grafts were preserved in organ culture according to the guidelines of the European Eye Bank Association.27 Preoperative evaluation of the graft endothelium was performed in hypotonic solution under the phase-contrast microscope the day before PK. Detailed graft data are given in Table 1.

**IMMUNE REACTIONS**

In all patients of group 3 endothelial immune reactions were diagnosed at the slitlamp via endothelial precipi-
tates and stromal edema. All patients received 1% prednisolone acetate eye drops every hour until elimination of all precipitates. Furthermore, a subconjunctival injection with + mg of betamethasone acetate was administered. Additionally, all patients received systemic corticosteroids at a daily oral dose of 1 mg of flucortolone per kilogram of body weight, which was tapered over 3 weeks.

**IMMUNOSUPPRESSIVE THERAPY AT THE TIME OF ANTERIOR CHAMBER PUNCTURE**

None of the patients in group 1 received immunosuppressive therapy at the time of anterior chamber puncture. In contrast, 8 of the 31 patients in group 2 received 1 to 2 drops of 1% prednisolone acetate per day as the only immunosuppression at the time of puncture (Table 1 and Table 2). Furthermore, all patients were treated with 1 g of mycophenolate mofetil twice daily or systemic cyclosporine aiming at trough levels between 120 and 150 ng/mL (Table 1 and Table 2). In 5 patients in group 3, 1 to 2 drops of 1% prednisolone acetate per day were administered as the only immunosuppression and in 3 patients systemic immunosuppression was performed in the same manner as in group 2 (Table 1).

**ANTERIOR CHAMBER PUNCTURE**

Anterior chamber puncture was performed under the operation microscope using topical (group 3) or retrobulbar (groups 1 and 2) anesthesia within 24 hours after referral of the patients to the clinic. All eyes were rinsed with sterile solution (BSS; Alcon Pharma, Freiburg, Germany) prior to anterior chamber puncture. A paracentesis lancet was used to penetrate the cornea in an avascular peripheral area over a length of 1 mm. Contact to limbal or peripheral corneal vessels was completely avoided. Aqueous humor (0.05-0.1 mL) was drawn into conventional tuberculine syringes without contact to intraocular structures.

**DETERMINATION OF TOTAL TGF-β2 AND sFasl CONCENTRATIONS**

All samples were immediately frozen at –70°C until cytokine determination. Prior to analysis, TGF-β2 was activated to the immunoreactive form by incubation in 1N hydrochloric for 10 minutes at room temperature, then neutralized in 1.2N sodium hydroxide/0.5M HEPES buffer as previously described. Levels of TGF-β2 were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Minn) as previously described. The ELISA for the level of TGF-β2 was performed in all the samples from study groups 1 and 2, but in only 11 of 12 samples from group 3.

Likewise, the concentration of sFasl was detected by ELISA. Therefore, the Tanaka et al assay,28 conceived to detect more than 50 pg/mL of sFasl in plasma, was optimized to detect 16 to 500 pg/mL of sFasl in aqueous humor. This was possible owing to the low protein concentrations in the aqueous humor that resulted in very low background. To ensure the reproducibility of the standard curve, 5 independent standard curves were pipetted. The ELISA for sFasl was performed in all samples from study groups 2 and 3, but in only 12 of 13 samples from group 1.

**CALCULATION OF ENDOTHELIAL CELL LOSS AND STATISTICAL EVALUATION**

At every clinical control time, central graft endothelium was evaluated using a noncontact specular microscope (Robo Noncon, Konan, Japan). This specular microscope calculates endothelial cell density automatically in the variable frame mode after the examiner marks the centers of adjacent cells. In the past, it was proven to deliver reliable and reproducible results.29 For calculation of endothelial cell loss only patients with at least 3 postoperative endothelial cell density values at different control times were included in the study. Reduction of endothelial cell density was assumed to follow the dynamics of first-order decay over follow-up. A scatterplot of the logarithmically transformed cell density values against...
Figure 1. Levels of transforming growth factor β2 (TGF-β2) in the study groups (P=.89, analysis of variance). Group 1 indicates 13 patients with a cataract without penetrating keratoplasty; group 2, 31 patients after penetrating keratoplasty without immune reactions; and group 3, 11 patients after penetrating keratoplasty with newly diagnosed endothelial immune reactions. The bar represents SD.

RESULTS

Transforming growth factor β2 was detected in all patients, irrespective of the underlying condition. The total TGF-β2 level (mean [SD]) averaged 1294 (624) pg/mL in group 1, 1197 (513) pg/mL in group 2, and 1221 (587) pg/mL in group 3, respectively (P=.89, ANOVA) (Figure 1). Thus, TGF-β2 was detected in the aqueous humor of all patients, and levels were independent from PK and the presence of immune reactions.

Soluble FasL was detected in the aqueous humor of none of the patients in group 1 (0 of 12 patients), but in 11 of 31 patients in group 2, and in 8 of 12 patients in group 2 (P=.002, χ² test). Thus, the incidence of detectable sFasL was higher in patients with PK who had immune reactions than in those without immune reactions, and for both groups was higher than in the controls (patients with cataracts). Soluble FasL concentrations in group 2 averaged 20.8 (31.1) pg/mL and 38.1 (33.2) pg/mL in group 3 (P<.01, ANOVA) (Figure 2). In group 2 a range of 0 to 95 pg/mL was noted that was similar to that in group 3 with 0 to 92 pg/mL. This explains the overlap of the bars in Figure 2.

A statistically significant correlation between donor and patient parameters of group 2 such as donor age, interval from death to graft excision, amount of time the graft was in organ culture, preoperative cell density of the graft, patient age, indication for surgery, graft diameter, amount of time since PK, immune reactions prior to anterior chamber puncture, endothelial cell loss prior to anterior chamber puncture or further immune reactions, and TGF-β2 or sFasL concentrations could not be shown (Table 2 and Table 3). Only patients receiving systemic immunosuppressive therapy at the time of anterior chamber puncture had statistically significantly higher sFasL concentrations than patients without systemic immunosuppression (P<.05, t test for independent variables).

In the control group (group 1) levels of TGF-β2 averaged 1294 pg/mL. Tripathi et al have previously reported a mean TGF-β2 level of 1480 pg/mL in a comparable control group. In patients without immune reactions after PK (group 2), higher levels of TGF-β2 might have been expected so as to exert an augmented “immunosuppression” on T lymphocytes. In patients with newly diagnosed endothelial immune reactions after PK (group 3), lower TGF-β2 levels might have been expected if graft rejection was the result of a failure of the immunological privilege. In case of a longstanding immune reaction, on the other hand, an up-regulation of TGF-β2 would have been conceivable, to restrain immunological graft destruction. The results of the study, however, could not detect any differences between the 3 study groups.
The high steady-state levels of TGF-β2 in the aqueous humor are compatible with its proposed role in maintenance of the immunological privilege. However, the anterior chamber appears to be unable to regulate the total TGF-β2 level to react to immunological challenges. The results may seem to contrast with the findings of King et al, who, in a rat model of keratoplasty, found the level of TGF-β2 to be increased in eyes that had immune reactions. In their study, however, only TGF-β2 levels of the graft, but not of the aqueous humor, were determined.

The question if levels of active TGF-β2 in the aqueous humor are regulated cannot be answered by our experiments. Animal experiments in endotoxin-induced uveitis revealed a regulation of the active but not of the total TGF-β2 level in the aqueous humor. Whether the same is true for PK has not yet been investigated, neither in the rat nor mouse model nor clinically. Since the concentration of active TGF-β2 is influenced by freezing, the determination has to be performed immediately after anterior chamber puncture. Under experimental conditions such an approach is possible since many samples can be obtained almost simultaneously from different animals. In a large keratoplasty center, approximately 1 or 2 patients per week fit the study criteria. Therefore, samples have to be collected over a long period of months to years. From a practical viewpoint, the availability of a laboratory for single determinations of active TGF-β2 is influenced by freezing, the determination has to be performed immediately after anterior chamber puncture. Under experimental conditions such an approach is possible since many samples can be obtained almost simultaneously from different animals. In a large keratoplasty center, approximately 1 or 2 patients per week fit the study criteria. Therefore, samples have to be collected over a long period of months to years. From a practical viewpoint, the availability of a laboratory for single determinations of active TGF-β2 is influenced by freezing, the determination has to be performed immediately after anterior chamber puncture. Under experimental conditions such an approach is possible since many samples can be obtained almost simultaneously from different animals.

For the determination of sFasL the Tanaka et al assay, conceived to detect more than 50 pg/mL sFasL in plasma, was proven to detect 16 to 500 pg/mL of sFasL in aqueous humor. The concentration of sFasL could not be detected in the control group (group 1). This is in contrast to Sugita et al, who report detectable concentrations of sFasL in 11 of 20 healthy controls. In their study, concentrations of sFasL averaged 273 pg/mL. In contrast, the concentrations of sFasL were found in only 6 of 17 patients with uveitis, with a mean concentration of 132 pg/mL. We found much lower sFasL values. Particularly, none of the controls had detectable concentrations of sFasL in the anterior chamber. The discrepancy between the study by Sugita et al and ours may have been caused by the circumstances of anterior chamber puncture and by the assay. The amount of sFasL to be detected in the aqueous humor can be influenced by contact of the syringe to intraocular structures, by contamination with blood from limbal or peripheral corneal vessels, by interference of the tear film with sFasL concentrations, or by the assay. In our study, only 0.05 to 0.1 mL was drawn into tuberculin syringes. This leaves sufficient aqueous humor in the anterior chamber to avoid contact of the syringe to sFasL containing intraocular tissues. Second, in the present study all eyes were rinsed with a sterile solution (BSS) prior to anterior chamber puncture to exclude disturbances by the tear film. Third, blood-tinged samples were excluded from the study. To what extent these contaminants can be excluded in the Sugita et al study is impossible to say. Critical amounts of up to 0.2 mL aqueous humor were drawn by that group. The antibodies used by the Sugita group, NOK-1 and NOK-2, do not differentiate between membrane-bound FasL and sFasL; thus, that minor admixture of the much heavier membrane-bound FasL might significantly falsify the results.

In the present study, we were able to show that the concentration of sFasL was undetectable in the aqueous humor of the controls but was detectable in recipients with PK (groups 2 and 3). Maximum concentrations were 95 pg/mL. In patients with endothelial immune reactions (group 3) sFasL concentrations were higher than in those without immune reactions (group 2). It thus seems that, unlike total TGF-β2 level, the concentration of sFasL is up-regulated in some patients after PK, particularly in the scenario of corneal graft rejection. While it is tempting to believe that sFasL may play a role in immunological privilege and its failure, just what this role might be remains a matter of speculation. Since in group 3 the ratio of high-risk patients was higher than in group 2, we cannot answer the question whether elevated sFasL concentrations are a reflection of the degree of preoperative inflammation. On the other hand, overexpression of sFasL in the patients with immune reactions after PK might be compensatory to suppress inflammation. These aspects should be investigated in further studies in which preoperative and postoperative sFasL concentrations of patients with PK are available.

Various graft and patient parameters might influence TGF-β2 and sFasL concentrations in the aqueous humor after PK. However, a correlation between donor age, interval from death to graft excision, amount of time the graft was in organ culture, preoperative cell den-

Table 3. Correlation Between Selected Variables and Transforming Growth Factor β2 (TGF-β2) and Soluble Fas Ligand (sFasL) Concentrations for Study Group 2 Using the Pearson Product-Moment Correlation Coefficient for Parametric Bivariate Correlation

<table>
<thead>
<tr>
<th>Variable†</th>
<th>r for TGF-β2 Level</th>
<th>P Value for TGF-β2 Level</th>
<th>r for sFasL Level</th>
<th>P Value for sFasL Level</th>
<th>r for sFasL Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age, y</td>
<td>−0.24</td>
<td>.21</td>
<td>0.04</td>
<td>.85</td>
<td></td>
</tr>
<tr>
<td>Interval from death to graft excision, h</td>
<td>0.26</td>
<td>.19</td>
<td>−0.02</td>
<td>.92</td>
<td></td>
</tr>
<tr>
<td>Amount of time the graft was in organ culture, d</td>
<td>−0.27</td>
<td>.16</td>
<td>−0.30</td>
<td>.13</td>
<td></td>
</tr>
<tr>
<td>Preoperative cell density of the graft, cells/mm²</td>
<td>−0.17</td>
<td>.39</td>
<td>−0.10</td>
<td>.63</td>
<td></td>
</tr>
<tr>
<td>Patient age, y</td>
<td>0.15</td>
<td>.42</td>
<td>0.15</td>
<td>.44</td>
<td></td>
</tr>
<tr>
<td>Graft diameter, mm</td>
<td>0.17</td>
<td>.37</td>
<td>0.05</td>
<td>.81</td>
<td></td>
</tr>
<tr>
<td>Amount of time from PK to AC puncture, d</td>
<td>−0.40</td>
<td>.83</td>
<td>−0.08</td>
<td>.67</td>
<td></td>
</tr>
<tr>
<td>Endothelial cell loss prior to puncture, 10⁻⁶ d⁻¹</td>
<td>0.01</td>
<td>.99</td>
<td>−0.48</td>
<td>.20</td>
<td></td>
</tr>
</tbody>
</table>

*PK indicates penetrating keratoplasty; AC, anterior chamber.

†Donor age, interval from death to graft excision, amount of time the graft was in organ culture, preoperative cell density, patient age, graft diameter, amount of time from PK to AC puncture, and endothelial cell loss prior to puncture.
sity of the graft, patient age, indication for surgery, graft diameter, amount of time since PK, immune reactions prior to anterior chamber puncture, or endothelial cell loss prior to anterior chamber puncture on TGF-β2, or sFasL concentrations in the aqueous humor after PK was excluded with appropriate statistical methods. For this analysis, only patients of group 2 were included to exclude the presumed influence of the immunological destructive process. It has to be borne in mind, however, that the number of patients subjected to this analysis is small (31 of 31 patients with detectable levels of TGF-β2 and 110 of 31 patients with detectable concentrations of sFasL). Recipients of systemic immunosuppressive therapy at the time of anterior chamber puncture had statistically significantly increased concentrations of sFasL, while this did not affect TGF-β2 levels. Without preoperative data on sFasL concentrations in these patients, the importance of this observation cannot be explained at the moment.

Another intention of the present study had been to evaluate if concentrations of TGF-β2 or sFasL in the aqueous humor might predict immune reactions in the further course after PK. To this end, we unfortunately cannot supply any data, since after an observation period after anterior chamber puncture of almost half a year none of the group 2 patients has gone on to develop irreversible graft failure.

In summary, TGF-β2 was very abundant in the aqueous humor, while sFasL concentrations were low. Soluble FasL but not TGF-β2 concentrations were increased in the aqueous humor of patients after PK. The elevation of the concentration of sFasL was even more pronounced in patients with PK who had immunological reactions. The high levels of TGF-β2 in the anterior chamber support the notion of TGF-β2 as the champion of the immunological privilege. Further studies must explore the significance of sFasL in protecting the integrity of the immunological privilege of the eye, and the mechanisms of its regulation. Furthermore, the role of further cytokines (e.g., interleukins 1, 2, 4, 6, 8, 10, 12; interferon gamma; tumor necrosis factor α; α-melanocyte-stimulating hormone; vasoactive intestinal peptide; calcitonin gene-related peptide; or macrophage inhibition factor) involved in the immunological cascade should be analyzed. The number of patients appropriate for such determinations and willing to participate in such studies, however, is rather small. Furthermore, the amount of aqueous humor taken from a single patient is so small that on average only 2 cytokines can be determined. Therefore, further studies in this field will take many years.

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