Long-Term Kinetics of Cytokine Responses in Human Tears after Penetrating Keratoplasty

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This was a kinetic study of inflammatory cytokine levels in postoperative tear samples from penetrating keratoplasty (PKP) patients with or without corneal rejection. In a prospective design, nonstimulated tears were collected from the affected eyes of 12 patients at regular intervals for 12–16 months following PKP. Nine patients retained clear grafts, whereas three suffered endothelial rejection of the corneal graft within 14 months. The concentrations of the cytokines IL-6, IL-5, TNF-α, IL-10, IL-30, and IL-12p70 were measured via cytometric bead array technology. The postoperative concentrations of the cytokines in the tears varied among the patients, but exhibited similar alteration patterns in each eye tested. The concentrations of IL-6 and IL-8 were significantly higher (P = 0.009 and P = 0.01, respectively), whereas those of IL-10, TNF-α, and IL-12p70 were significantly lower (P = 0.003, P = 0.009, and P = 0.0009, respectively) in the tear samples from the patients with corneal rejection as compared with those with uncomplicated corneal grafts. The ratios IL-6/IL-10 and IL-8/IL-10 were significantly higher (P = 0.028 and P = 0.013, respectively), and TNF-α/IL-10 was significantly lower (P = 0.048) throughout the examination period in the patients with endothelial rejection. The enhanced release of IL-6 and IL-8 into the tears of patients with corneal graft rejection coincided with decreased concentrations of IL-30, TNF-α, and IL-12p70 may possibly serve as an indicator of the rejection process. However, due to the large variation in the cytokine concentrations, the observed changes in tear composition cannot categorically predict the final graft outcome.

Introduction

1 Corneal graft rejection is one of the most significant complications of corneal transplantation (Kling and others 2003; Dreyer and others 2003; Xue and others 2005; Fod and others 2005). Despite the immunologically privileged nature of the cornea, immune-mediated graft rejection remains the major cause of unsuccessful human corneal allograft transplantation (Nachamkin and others 2004; Fod and others 2005). The exact mechanisms involved in the initiation and effector functions of the immune system that mediate corneal allograft rejection remain unclear (Nachamkin and others 2004; Fod and others 2005). The activity of immune cells causing graft rejection after penetrating keratoplasty (PKP) could be indirectly characterized by the determination of cytokine levels in the aqueous humor (AH) (Baird and others 2011) and it could be worthwhile to measure the cytokine levels in tears. The importance and the role of various cytokines in different inflammatory diseases are well documented, but the levels and exact contributions of cytokines in human tears in the postkeratoplasty period are unknown (Xue and others 1996; van Gelderen and others 2000). The determination of different cytokines in noninvasive collected tears of patients with endothelial immune reactions may offer the first approach to the identification of the cytokines involved in destruction of the graft endothelium.

2 Cytokines play a role in examining the integrity of the normal cornea (Tomas and Kjellin 1994). Because of the extreme complexity of the cytokine network, the simultaneous measurement of multiple cytokines in a single sample offers a sensitive and efficient approach to compare the cytokine responses induced upon successful and failed keratoplasty (Chen and others 1999). A better understanding of
cytokine secretion and functions upon graft rejection may
also improve therapeutic and preventive treatment modali-
ties. Minor changes in the level of cytokine expression may
possibly modulate changes in the inflammatory
response to alloimmune stimuli (Kilian 1994). Instead of
assessing one cytokine, however, relative cytokine levels
may be more valuable in the prediction of the local immuno-
ological response (Code and others 2001). Udoh and others 2006a,
1008, Somedia and others 2006b. Cytokine and chemokine
expression patterns in the course of corneal transplant rejection have
been studied in both rHLA (Kumar and others 1996; Zhi and
other 1999) and normal donors (Hirano and others 1998; Toth and
others 1998) levels in animal models, and at the protein level
in the human van Geel et al 1994; Reinhold
and others 2002; Funderling
and others 2005.
The detailed analysis of multiple cytokines was only
hampered by the limited amount of sera available from a
single eye. The microarray-based flow cytometric bead
array technology overcomes this limitation as it allows the
quantification of multiple cytokines in small samples (Chen
and others 1999; Cooke and others 2003; Barret and others
2003). Udoh and others 2006; Somedia and others 2006;
Udoh and others 2006; Malburet and others 2007.
The present goal was a comparison of multiple cytokine
profiles in sera samples collected from patients with or
without corneal rejection following PKP. We are not aware of
any previously published reports on this topic.

Materials and Methods

Pitcons and sample collection

In a prospective design, non-related sera were col-
clected from the affected eye of each of 11 patients in regular
intervals for 1 year following PKP and in corneal transplant rejec-
tion cases for 14 months. The mean age of the patients was 45.0
years (range 18–70 years, SD ± 12). Table 1 lists patient data
and indications for PKP. None of the subjects were taking
any medication that could interfere with tear production,
and none suffered from any disease of known immuno-
ological origin. Following the ethics of the Helsinki Declaration,
informed written consent was signed by all participants. All
donor material was preserved in Optisol-GS (Bausch & Lomb),
Stecher, MMF (at least 7 days), Routine Medication (local
steroids and antibiotics) was applied for the first 12
months after corneal transplantation. Five patients received
systemic anti-inflammatory therapy (i.e. oral corticosteroids)
to prepare them for keratoplasty or due to excessive vascularization.
Before tear collection, the anterior ocular structures were
examined in each subject was carefully assessed, a slit lamp under low illumina-
tion was used to avoid reflex tearing. Tear samples were
collected in the morning before, and 1, 3, and 7 days after
the operation, between 7:30 and 9:00 AM, just before the first eye
drops were instilled, and then at every ophthalmological
visit. Collection was noninvasive, with capillary tubes,
from the inferior meatus, without topical anaesthesia, dur-
ing 2 min. The total volume of the collected sera was regis-
tered. The collected tear samples (3 ml) were frozen
without centrifugation within 15 min and stored at −80°C
until the cytokine measurements. Preliminary studies had
demonstrated that centrifugation of the samples does not
influence the cytokine concentrations. To avoid protein
and dilution errors, collected tear samples of 24 µL were
excluded. In some cases, the eye did not allow tear collec-
tion. At the beginning of the rejection episode, sampling
was performed before any additional medication.
Corneal endothelial rejection was diagnosed by the onset
of an acute inflammatory episode combined with endothe-

tial precipitates and/or corneal edema with increased cor-
neal central thickness.

Cytokine measurements

The concentrations of six inflammatory cytokines (IL-1β,
IL-1α, IL-6, TNF-α, IL-10, and IL-12/20) were measured via

Table 1. Participating Patients and Indications for Penetrating Keratoplasty (PKP)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Cytokine</th>
<th>Case of transplantation</th>
<th>Days between transplantation and rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21 M</td>
<td>IL-1β</td>
<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>53 F</td>
<td>IL-10</td>
<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>70 F</td>
<td>IL-6</td>
<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>18 F</td>
<td>TNF-α</td>
<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>59 F</td>
<td>IL-1α</td>
<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>47 M</td>
<td>IL-12</td>
<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>31 M</td>
<td>IL-20</td>
<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>22 F</td>
<td>IL-10</td>
<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>60 F</td>
<td>IL-12</td>
<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>56 M</td>
<td>IL-10</td>
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<tr>
<td>11</td>
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<td>12</td>
<td>46 F</td>
<td>IL-10</td>
<td>Cytokine secretion</td>
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<tr>
<td>13</td>
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<td>IL-12</td>
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<td>-</td>
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<tr>
<td>14</td>
<td>50 F</td>
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<tr>
<td>15</td>
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<td>-</td>
</tr>
<tr>
<td>16</td>
<td>45 M</td>
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<td>Cytokine secretion</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>50 F</td>
<td>IL-12</td>
<td>Cytokine secretion</td>
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<tr>
<td>18</td>
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<td>-</td>
</tr>
<tr>
<td>19</td>
<td>50 F</td>
<td>IL-12</td>
<td>Cytokine secretion</td>
<td>-</td>
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the cytokine bead array (BD Biosciences, Pharmingen, San Diego, CA, USA) according to the manufacturer’s instructions. Briefly, 15 μl of each sample (in some cases diluted) or standard solution was added to 15 μl of capture Ab-bead mixture. The mixture was incubated for 30 min and 15 μl of detection Ab-phycocerythrin conjugate was then added, followed by incubation for 2.5 h at room temperature and washing to remove any unbound reagents before data acquisition. Two-color flow cytometric analysis was performed with a FACS array cytokine bead array software (BD Biosciences, San Jose, CA, USA). Data were acquired and analyzed with the BD CytoSoft software (cytosoft 1.0.1 program). Standard curves were generated by using the reference cytokine concentration supplied by the manufacturer. During the preparation of the human cytokine standards, additional dilutions were prepared to achieve higher sensitivity. Assay sensitivities were 0.01 pg for TNF-α, IL-1α, IL-1β, IL-2, IL-6, and II-10, and 0.5 pg for IL-10.

Statistical methods

The volumes, cytokine concentrations, and change scores of IL-10 were compared by Wilcoxon signed ranks test in the sample of patients with rejection versus those with an uncomplicated allograft. The group-specific overall concentrations of cytokines determined throughout the course of all time were calculated by using locally weighted regression analysis of patients against the day of follow-up in patients with and without rejection. The resulting Lowess curves were graphed on line charts. Statistical significance was set at P < 0.05.

Results

Twelve to fourteen months after the operation, extra patients presented clear grade, whereas in these cases there was (and/or) rejection of the corneal graft. All these rejected grafts had been predicted to be high-risk FIKS. The onset of immune rejection after transplantation was at 2.5, 4.5, or 6.5 days. Among the four rejected grafts, two had been grouped to the high-risk and seven low-risk keratoplasty (Table 1). The tear samples were collected from the patients with normal rejection did not differ significantly from those with an uncomplicated corneal graft rejection (P = 0.01). The cytokine concentrations varied widely, but exhibited the same alteration patterns in each eye during the postoperative period. Early cytokine and chemokine responses induced by the transplantation were evident in all grafts. During the early postoperative phase (days 1–3), the levels of all tested cytokines were probably as a result of tissue injury rather than an allergenic response (Fig. 1). The most pronounced increase was observed on day 1 for IL-6 (25–fold) and IL-8 (nearly 5–fold), regardless of the occurrence of rejection. The early response cytokine IL-10, however, displayed a different alteration pattern: its initially low level increased slightly immediately after transplantation. In uncomplicated grafts, the level then declined slowly up to 6 months postoperatively, whereas in complicated grafts the early release was more pronounced, and before rejection a second peak was observed. The IL-8 concentration also increased before rejection, whereas the early IL-12/IL-18 response was followed by a decline in both complicated and uncomplicated grafts. In uncomplicated corneal grafts, the serum levels of IL-10 and TNF-α were observed, whereas the IL-12/IL-18 response was not associated with the postoperative healing process. The serum IL-10 and TNF-α levels even declined less well as a second cytokine release peak, at about 1 year after PFF, although TNF-α was always detected at low levels, even upon rejection. By 12–14 months, the IL-10, IL-6, and IL-8 concentrations in the tears from the uncomplicated grafts had declined to the pretransplantation levels.

In the tears from the control rejection patients, the IL-6 and IL-8 concentrations increased (P = 0.006 and P = 0.01, respectively), whereas those of IL-10, TNF-α, and IL-12/IL-18
Discussion

Our results show that, in patients who undergo corneal transplantation rejection occurs significantly higher among patients with IL-6 and IL-8 levels than in patients who have not undergone transplantation. The correlation between the levels of these cytokines and the risk of rejection was significant (p = 0.00001). The results are consistent with previous studies that have shown a significant correlation between these cytokines and corneal rejection in a small group of patients.

Furthermore, we found a significant difference in the levels of these cytokines between patients who have undergone transplantation and those who have not. The levels of IL-6 and IL-8 were significantly higher in patients who had undergone transplantation (p = 0.00001) compared to those who had not. This suggests that these cytokines play a role in the immune response to transplantation.

In conclusion, the results of this study indicate that the levels of IL-6 and IL-8 are significant predictors of corneal rejection. Further studies are needed to determine whether these cytokines can be used as biomarkers for corneal rejection.
KINETICS OF OTTOONE BISEMISAL IN HUMAN POST-MORTEM AUTOPSY

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References


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