

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**CYTOKINE DETECTION IN HUMAN TEARS IN
VARIOUS ANTERIOR SEGMENT EYE CONDITIONS
(WITH SPECIAL REGARD TO PENETRATING
KERATOPLASTY)**

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1. Introduction and literature review

Changes in the quality and quantity of the tears are important parameters in the various anterior segment eye diseases. This information can furnish characteristic data in the course of different eye conditions. Human tears contain many specific and nonspecific immune components.

The continuous interactions between the cells of the immune system take place via direct cell-cell contacts and via cytokines. In these cell communications, polypeptide regulators (glucoproteins/proteins/peptides) play very important roles. In the course of inflammatory reactions, cytokines are crucial mediators of intercellular communication. Certain cytokines, alone or in combination with others, are known to act as activators of inflammatory cells and to provide early signals for the initiation and amplification of mucosal inflammatory responses.

Cytokines play a role in maintaining the integrity of the normal cornea. They take part in the regulation of the inflammatory and immunological reactions of the ocular surface. Their precise ophthalmological roles in inflammatory and postoperative processes still await clarification.

Interleukin-6 (IL-6) is a pluripotent proinflammatory cytokine: besides its inflammatory role, it can reduce inflammation, restore the ocular immune privilege and prevent ocular surface inflammation. IL-6 has been detected in the aqueous humor, in tears and in the cornea, and has been implicated in the pathogenesis of ocular surface inflammatory and other pathologic processes, such as bullous keratopathy, keratitis, keratoconus, corneal ulceration, contact lens wear and uveitis. IL-6 may also play a crucial role in the development of postoperative inflammation after cataract surgery, since it has been shown to stimulate collagen synthesis and may therefore be involved in corneal wound healing.

Interleukin-8 (IL-8) is one of the most specific cytokines, a chemokine. High concentrations of IL-8 have been detected in closed-eye tears and in the tears of patients during contact lens wear, in allergic conjunctivitis and in Sjögren's syndrome keratoconjunctivitis.

Interleukin-1 (IL-1) consists of the products of two genes, IL-1-alpha and IL-1-beta-polypeptide. IL-1 β has been regarded as a proinflammatory cytokine capable of initiating the inflammatory cascade. IL-1 induces the centripetal movement of the Langerhans cells and mediates keratocyte apoptosis. This multifunctional cytokine is an active player in inducing corneal tissue damage and contributing to tissue repair. High tear concentrations of IL-1 have been detected in rosacea and conjunctivochalasis.

Tumor necrosis factor (TNF) is a proinflammatory cytokine. It has multiple biological functions, among the most important which are the induction of fibroblast proliferation and its role in angiogenesis, both important features in corneal wound healing. TNF- α can further enhance the expression of IL-8, thereby prolonging inflammatory processes. TNF- α induces the synthesis of IL-10, one of the major anti-inflammatory cytokines.

Interleukin-10 (IL-10) is essential for maintenance of the anterior chamber-associated immune deviation (ACAID). IL-10 has an angiogenic potential and decreases the expression of MHC class II in monocytes/macrophages, thus interfering with their antigen-presenting function. IL-10 also suppresses the production of other proinflammatory cytokines, such as TNF- α , IL-1 β and IL-8.

Interleukin-12 (IL-12) is an important regulator of the inflammatory response. Its biologically active form is the 70 kDa heterodimer (IL-12p70), which consists of 2 subunits (40 kDa -p40; and 35 kDa -p35). The role of IL-12 in the transplant rejection of various tissues and organs has already been examined.

Corneal transplantation is the most frequently performed, but, in terms of its immunobiology still the least understood form of allogenic transplantation. Keratoplasty is one of the most successful forms of tissue transplantation. The high success rate is a consequence of the special corneal microenvironment. This includes the fact that some recipient corneas are avascular, the absence of antigen-presenting Langerhans cells in the central part of the cornea, the low expression of the major MHC antigens, the local production of the immunosuppressive cytokines, and the expression of the Fas ligand. Despite the

immunologically privileged nature of the cornea, immune-mediated graft rejection remains the major cause of unsuccessful human corneal allograft transplantation

User-friendly and informative investigations are necessary in the post-keratoplasty period in order to detect the rejection process early, as far as possible before the onset of clinical symptoms. With an appropriate method, the immunosuppressive therapy could be individual, and started at the appropriate time and the number of transplant rejections could be decreased. A better understanding of cytokine secretion and functions upon graft rejection may allow improved therapeutic and preventive treatment modalities.

At the Department of Ophthalmology in Debrecen we have performed examinations on tears after keratoplasty since 1979, in order to study the transplant rejection process.

The importance, the variations in quantity and the pathologic changes in the various cytokines in the postoperative periods following penetrating keratoplasty (PKP) are unknown. It is assumed that the levels of the different cytokines are not only changed because of transplant rejection. Noncomplicated (nonrejected) PKP can with be modeled three basic types of pathologic tears: (1) the surgical trauma can be characterized by different postoperative situations; (2) reflex tearing caused by irritation of the sutures after PKP can be described by inflammation-free corneal foreign bodies (resulting in hypersecretion); and (3) the postoperative inflammation after PKP can modify the cytokine levels in the tears, and thus acute bacterial conjunctivitis can be used to model hypersecretion.

Cytokine and chemokine expressions in the course of corneal transplant rejection have been studied at both mRNA and protein levels in animal models, and at the protein level in the aqueous humor in humans. The activity of immune cells causing graft rejection after PKP has been indirectly characterized by the determination of cytokine levels in the aqueous humor. In cases of immune rejection, it could also be worthwhile to measure the cytokine levels in the noninvasively collected tears and to determine the cytokines which participate in the destruction of the different cornea layers.

2. Aims

- I. To determine the levels of IL-6 and IL-8 in tears collected from the eyes of patients with different irritative eye diseases and postoperative situations, in order to acquire information on the immunological changes occurring during the early postoperative period following various forms of eye surgery, including PKP.
- II. To determine the changes in the concentrations of inflammatory cytokines (IL-1 β , IL-6, TNF- α , IL-8, IL-10 and IL-12p70) in postoperative tear samples from PKP patients without corneal rejection, during 1 year postoperatively.
- III. To identify the cytokines that play roles in the corneal rejection process; to determine the difference between the multiple cytokine patterns in tear samples collected from patients with or without corneal rejection following PKP. To compare the changes in the concentrations of inflammatory cytokines (IL-1 β , IL-6, TNF- α , IL-8, IL-10 and IL-12p70) in postoperative tear samples from PKP patients with and without corneal rejection, during 1 year postoperatively.

3. Patients and methods

Prospective studies were performed at the Department of Ophthalmology, Medical and Health Science Center, University of Debrecen. None of the subjects were taking any medication that could interfere with tear production, and none suffered from any disease of known immunological origin. Following the tenets of the Helsinki Declaration, informed written consent was signed by all participants.

3.1. Patients groups

3.1.1. The subjects were divided into four groups.

Group I contained 11 patients with acute bacterial conjunctivitis. The mean age of the patients was 46.9 years (range 25-76 years, SD 18.3). The diagnosis was based on the clinical symptoms. Conjunctival culturing was not performed. Tear samples were taken at the first visit before any eye drops were instilled.

Group II contained 12 patients 1 day postoperatively after uncomplicated cataract extraction. The mean age of the patients was 67.3 years (range 44-83 years, SD 11.4). Seven patients underwent standard phacoemulsification with foldable lens implantation. Five patients were operated on with extracapsular cataract extraction and implantation of a PCL.

Group III comprised 7 patients with irritation due to the presence of a corneal foreign body. The mean age was 44.3 years (range 38-61 years, SD 7.8). Tear samples were taken at the beginning of their first examination before any eye drops were instilled. Excessive tearing due to irritation was noted, but the eyes were not hyperemic, inflamed or infected.

Group IV involved 30 patients 1 week after PKP. The mean age was 54.7 years (range 17-82 years, SD 19.4). The indications for PKP included various corneal diseases: keratoconus (9 eyes), pseudophakic bullous keratopathy (10 cases), failed graft (rekeratoplasty, 4 cases), keratouveitis (1 eye) and corneal vascularization (6 cases). All donor materials were preserved in Optisol-GS (Bausch&Lomb, USA) for a maximum of 7 days. Postoperative treatment included local corticosteroid eyedrops (prednisolone acetate) and local antibiotic eyedrops (neomycin) 5 times a day. Three eyes needed an additional subconjunctival steroid

injection, and 5 patients received systemic anti-inflammatory therapy (iv. or oral corticosteroid). Tear samples were collected early in the morning, before the first eye drops were instilled.

We also recruited 52 normal controls. The mean age of the controls was 54.8 years (range 21-83 years, SD 21.9).

3.1.2./3.1.3. In a prospective design, nonstimulated tears were collected from the affected eye of each of 9 patients at regular intervals for 1 year following PKP. In 3 cases with transplant rejection, tear collection was performed until the rejection events occurred (83, 216 and 422 days postoperatively). The mean age of the patients was 45.0 years (range 18-70 years, SD 14.2). The indications for PKP were: keratoconus, transplant rejection/2nd PKP, bullous keratopathy, Salzmann's nodular degeneration, congenital hereditary endothelial dystrophy and pannus corneae. All donor material was preserved in Optisol-GS (Bausch&Lomb, USA) for at most 7 days. Routine medication (local corticosteroids and antibiotics) was applied after transplantation. Five high-risk patients received systemic anti-inflammatory therapy (i.v. (initial dose: 1mg/bw/day) or oral (initial dose: 0.5 mg/bw/day) corticosteroid) to prepare them for rekeratoplasty or due to recipient vascularization. As long as the patients remained at the Department of Ophthalmology (1 week postoperatively), the tear samples were collected early in the morning, before the first eye drops were instilled. At the ophthalmological controls, the samples were collected in the morning, more than 1 h after the administration of the local treatment.

3.2. *Tear collection*

3.2.1./3.2.2./3.2.3. Before tear collection, the anterior ocular status of each subject was carefully assessed; a slit-lamp under low illumination was used to avoid reflex tearing. Tear samples were collected without stimulation, under nontraumatic conditions, using sterilized glass capillary tubes, from the lower tear

meniscus, without touching the lid margin or the conjunctiva. The duration of collection was in all cases exactly 2 min, and the total volume of the collected tears was registered. The collected tear samples were frozen within 15 min, without centrifugation, and stored at –80 °C until further analysis.

3.2.2./3.2.3. To avoid pipetting and dilution errors, collected tear samples of < 4 µl were excluded. In some cases, dry eye did not allow tear collection. Tear samples were collected in the morning before, and 1, 3 and 7 days after the operation, between 7.30 and 8.00 a.m., just before the first eye drops were instilled, and then at every ophthalmological control. At the beginning of the rejection episode, sampling was performed before any additional medication.

3.2.3. Corneal endothelial rejection was diagnosed by the onset of an acute inflammatory episode combined with endothelial precipitates and/or stromal edema with increased central corneal thickness.

3.3. *Cytokine measurements*

3.3.1. The total protein (TP) concentration in the tear samples was determined by using the bicinchoninic acid protein-assay kit from Pierce (Rockford, IL, USA). Next, the concentrations of IL-6 and IL-8 were determined with commercially available human ultrasensitive, solid-phase enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Inc. Nivells, Belgium). The sensitivity of both the IL-6 and the IL-8 ELISA was <0.1 pg/ml, and the detection covered the range 0.16-10 and 0.39-25 pg/ml, respectively.

3.3.2./3.3.3. The concentrations of 6 inflammatory cytokines (IL-8, IL-1 β , IL-6, TNF- α , IL-10 and IL-12p70) were measured via the cytometric bead array (BD Biosciences Pharmigen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, 15 µl of tear sample (in some cases a diluted sample) or standard reagent was added to 15 µl of capture Ab-bead reagent. This mixture was incubated for 30 min and 15 µl of detector Ab-phycoerythrin conjugate was then added, followed by incubation for 2.5 h at room temperature and washing to remove any unbound reagent before data acquisition. Two-color flow cytometric analysis was performed with a FACSArray™ cytometer (BD Biosciences

Immunocytometry Systems, San Jose, CA, USA). Data were acquired and analyzed with the BD cytometric bead array software (FCAP Array 1.0.1 program, SoftFlow Kft., Pécs). Standard curves were generated by using the reference cytokine concentrations supplied by the manufacturer. During the preparation of the human cytokine standards, additional dilutions were prepared to achieve higher sensitivity. The assay sensitivities were 0.04 pg for TNF- α , IL-8, IL-1 β , IL-12p70 and IL-6, and 0.02 pg for IL-10.

3.4. *Special calculation method*

3.4.1. The rates of release of the cytokines (IL-6 and IL-8) into the tears were calculated from the concentration (pg/ μ l) and the volume of tears (μ l) collected in 2 min. The TP release was calculated from the TP concentration (μ g/ μ l) and the volume of tears (μ l) collected in 2 min.

3.5. *Statistical analysis*

3.5.1. The results for the different groups were compared with each other, as well as with the normal controls. Linear regression was used for the subsequent analysis of the data. The statistical package applied was STATA Version 8.2. The significance criterion was set at $\alpha=0.05$. The variables were transformed to improve normality by using the method with the best effect. Age, TP, IL-6 and IL-8 release and these rates were log-transformed. The tear volume and the IL concentrations were square root transformed. All measured concentrations (TP, IL-6 and IL-8) were adjusted for age and tear volume and the calculated releases were adjusted for age.

3.5.2. The overall concentrations of cytokines determined throughout 1 year after 9 cases of noncomplicated PKP were calculated by using locally weighted regression analysis of outcomes against the day of follow-up in patients.

3.5.3. Tear volumes, cytokine concentrations and their ratios to that of IL-10 were compared by Wilcoxon's rank-sum test in tear samples from patients with rejection vs. those with an uncomplicated engraftment. The group-specific overall

concentrations of cytokines determined throughout the overall time course were calculated by using locally weighted regression analysis of outcomes against the day of follow-up in patients with and without rejection. Statistical significance was set at $p<0.05$.

4. Results

4.1. The volume of tear samples collected within 2 min ranged from 2.2 μl (1 normal control) to 121.7 μl (1 patient in the group with bacterial conjunctivitis). The maximum collected tear volume was therefore 55 times more than the minimum. Since the 2-min collected tear volumes were significantly higher ($p<0.001$) in the patient groups (due to reflex tearing and increased transudation) compared to the controls (basic tear production), calculation of the release of TP and cytokine took place to compare the release data to each other.

In the normal controls, the mean level of IL-6 was 110 pg/ml (SD 142). The IL-6 concentrations were significantly higher than this in the tears of the patients with bacterial conjunctivitis (366 pg/ml (SD 296), $p<0.001$) and after PKP (170 pg/ml (SD 235), $p=0.040$). The IL-6 concentrations were 189 pg/ml (SD 184) after cataract operation and 109 pg/ml (SD 72) in the tears of the patients with a corneal foreign body, with no statistical differences as compared with the controls.

The IL-6 release was 20 pg (SD 21) in the tears of patients with bacterial conjunctivitis; 5 pg (SD 5.2) after cataract operation; 6.3 pg (SD 4.6) with corneal foreign body; and 6.6 pg (SD 6.7) after PKP. A significantly elevated IL-6 release was observed in all the patient groups as compared with the normal controls (0.6 pg (SD 0.7)) (bacterial conjunctivitis, corneal foreign body and PKP groups: $p<0.001$; cataract operation group: $p=0.003$).

In the normal controls, the mean level of IL-8 was 572 pg/ml (SD 637). The IL-8 concentration in the bacterial conjunctivitis group was significantly higher than this (661 pg/ml (SD 550); $p=0.04$). The IL-8 concentration was 171 pg/ml (SD 144) in the tears of the patients after cataract operation; 203 pg/ml (SD 170)

in those with a corneal foreign body; and 220 pg/ml (SD 277) after PKP, which were not significantly different from the controls. In the normal controls, the IL-8 release was 2.8 pg (SD 2.9). The IL-8 release into the tears was significantly higher in three of the patient groups than in the normal control subjects (bacterial conjunctivitis: 31 pg (SD 35), p=0.001, corneal foreign body: 13 pg (SD 13), p=0.03, PKP: 8.6 pg (SD 5.4) p<0.001). In the cataract operation group, however, the IL-8 release (4.4 pg (SD 4.2) was not significantly higher than in the controls (p=0.053). No significant differences were observed between the patient groups when the concentrations and the releases of IL-6 and IL-8 were compared.

The extents of the release of IL-6 and IL-8 within 2 min were related to the TP measured in the various patient groups and compared with those for the normal controls. The ratio of IL-6/TP release was significantly higher in all the patient groups (bacterial conjunctivitis: p=0.01, cataract operation: p=0.048, corneal foreign body and PKP: p<0.01) than in the control samples. The IL-6/TP concentration ratio was significantly higher in the bacterial conjunctivitis (p=0.04) and corneal foreign body (p=0.03) groups than in the controls. Neither the IL-8/TP release ratio, nor the IL-8/TP concentration ratio was significantly higher in the patient groups than in the controls. Neither the IL-6/IL-8 release ratio, nor the IL-6/IL-8 concentration ratio was statistically higher in the patient groups than in the controls.

4.2. A total of 76 tear samples were collected from 9 patients during 1 year following noncomplicated PKP. Two were predicted to be high-risk, and 7 low-risk PKPs. The alterations in the concentrations of the cytokines in each eye during the postoperative period exhibited the same pattern. The operation itself caused a pronounced release of all cytokines. The most pronounced increases were observed on day 1 for IL-6 (25-fold) and IL-8 (5-fold). Similar biphasic IL-10 and TNF- α responses were seen during the 1 year observation period. By 1 year, the IL-1 β , IL-6 and IL-8 concentrations had declined to the pretransplantation levels.

4.3. Altogether 105 tear samples were collected from the patients. At 14 months after the operation, 9 patients presented clear grafts, whereas in 3 cases there was endothelial rejection of the corneal graft. All 3 rejected grafts had been predicted to be high-risk PKPs. The onset of immune rejection after transplantation was at 216, 422 or 83 days.

Among the 9 unrejected grafts, 2 had been expected to involve high-risk, and 7 low-risk PKP. The tear sample volume collected from the patients with corneal rejection did not differ significantly from that from those with uncomplicated corneal grafts ($p=0.01$).

The cytokine concentrations varied widely, but exhibited the same alteration pattern in each eye during the postoperative period. Early cytokine responses induced by the stress caused by the operation were evident in all grafts, regardless of the occurrence of corneal rejection. During the early postoperative phase (days 1 to 3), the levels of all tested cytokines (IL-1 β , IL-6, TNF- α , IL-8, IL-10 and IL-12p70) rose, probably as a result of tissue injury rather than an allogeneic response.

The most pronounced increases were observed on day 1 for IL-6 (~ 25-fold) and IL-8 (nearly 5-fold), regardless of the occurrence of corneal rejection.

The proinflammatory cytokine IL-1 β , 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-12p70 response was followed by a decline in both complicated and uncomplicated grafts.

In uncomplicated corneal grafts, the similar biphasic IL-10 and TNF- α responses observed were presumably associated with the postoperative healing process. The slow IL-10 and TNF- α level decreases gave way to a second cytokine release peak at about 1 year after PKP, although TNF- α was always detected at low levels, even upon rejection.

In the tears from the corneal rejection patients, the IL-6 and IL-8 concentrations increased ($p=0.009$ and $p=0.01$, respectively), whereas those of

IL-10, TNF- α and IL-12p70 decreased significantly ($p=0.008$, $p=0.006$ and $p=0.0009$, respectively) relative to the uncomplicated corneal grafts, while the IL-1 β concentration did not change significantly ($p=0.383$).

As the balance of the pro- and anti-inflammatory cytokines determines the inflammatory status of the eye, we calculated the ratios of the IL-6, TNF- α and IL-8 concentrations to that of IL-10. In the patients with endothelial rejection, IL-6/IL-10 and IL-8/IL-10 were significantly higher ($p=0.023$ and $p=0.015$, respectively), while TNF- α /IL-10 was significantly lower ($p=0.045$) throughout the examination period than in those with uncomplicated grafts.

5. Discussion

As a result of our examinations, we obtained data on the complex immunological events in cases of different inflammatory anterior segment eye diseases and postoperative situations. It has been shown that these reactions can be evaluated reliably from human tear fluid. During the first part of our study, we confirmed that cytokines are crucial mediators of the intercellular communication in the normal wound healing in the different postoperative situations and also in cases of acute bacterial conjunctivitis and irritant corneal foreign body. In the second part of our study, the special immunobiological aspects were shown after uncomplicated PKP, and we then determined the concentrations and the changes in the concentrations of several cytokines involved in corneal transplant rejection.

In the first step, we compared the levels of total protein and two cytokines in human tears collected from the eyes of patients with different anterior segment eye conditions and of normal individuals. The results of the present study confirm that IL-6 and IL-8 are normal constituents of the human tear fluid. It has been suggested that both cytokines could also play a role in the maintenance of the ocular surface homeostasis.

In the case of ocular surface epithelium-derived proteins, the lacrimal gland fluid would act as a diluting and flushing agent, reducing the protein levels with increasing tear secretion volume. It has been suggested that the concentrations of tear fluid components should be evaluated as a function of the actual secretion rate of the lacrimal gland (tear flow rate). The volumes of tear samples collected within 2 min were significantly higher ($p<0.001$) in the patient groups than in the controls.

In our study, the 2-min collected tear volumes within the patient groups differed appreciably. This depends on the large variations in individual sensitivity and the variability of the disease stages, resulting in various diluting effects. The different volumes of the tear samples collected from normal and from diseased eyes gave comparable cytokine release values, whereas the simple concentration values were not usable. The rate of release of the cytokine into the tears was calculated from the concentration and the volume of tears collected in 2 min. Moreover, our data indicated that the TP, IL-6 and IL-8 releases were influenced by age, both in the controls and in the patient groups.

We studied three basic types of pathologic tears to model the noncomplicated, nonvascularized PKP:

- (1) The postoperative situation after PKP is always characterized by an increased permeability of the conjunctival vessel walls, resulting in the transudation of plasma proteins in the tears. Patients 1 day after cataract extraction were enrolled for this purpose.
- (2) Reflex tearing caused by irritation of the sutures after uncomplicated PKP could be described by inflammation-free corneal foreign bodies.
- (3) The rare postoperative inflammation after PKP could obscure the immunological signs of transplant rejection. The presence of acute bacterial inflammation provokes cytokine production, and this type of conjunctivitis can therefore be used as a "positive control" in IL determinations. In our study, besides PKP, these three basic types of anterior segment eye conditions were examined and the changes in the concentration and the release of IL-6 and IL-8 were demonstrated.

A considerable increase in IL-6 release was observed in all the patient groups as compared with the normal controls, although the concentration of IL-6 was significantly higher only in the tears of eyes with acute bacterial conjunctivitis or after PKP. We did not observe significant differences in IL-6 release between the patient groups. This could be a consequence of the low statistical power due to the limited numbers of subjects involved in the patient groups.

IL-6 is an important mediator in the pathogenesis of intraocular and ocular surface inflammatory processes. IL-6 stimulates the collagen synthesis of the keratocytes, and promotes the healing process of the corneal epithelium. The significantly increased IL-6 release after PKP could therefore be a consequence of the normal corneal wound healing.

The results of the current investigation suggest that IL-6 has an important role not only in the ocular surface inflammatory processes, but also in postoperative situations, such as postoperative inflammation after cataract surgery and PKP. IL-6 may be a highly sensitive indicator of various types of irritative eye diseases and inflammatory and infectious conditions. The IL-6 release into the tears was highest of all in the patients with conjunctivitis, but the IL-6 release was significantly higher in all the patient groups than in the control samples.

IL-8 release was significantly higher in all the patient groups as compared with the control samples, except in the cataract operation group. Only the patients with acute bacterial conjunctivitis were characterized by an IL-8 concentration significantly higher than in the controls. In the other patient groups, the IL-8 concentrations were even lower, presumably because of the dilution effect of reflex tearing.

The increased release of IL-6 and IL-8 into the tear fluid of the various patient groups seemed to be associated in part with a higher tear-fluid secretion rate. Our results may support the assumption that the protein release is a more reliable indicator of changes in the production of locally produced proteins than simple concentration values, especially when the tear flow rates differ considerably.

According to our philosophy, we examined the immunological aspects of noncomplicated, nonrejected PKP, and studied its demonstrability in postoperative tear samples. Little is known concerning the normal and pathological levels and the importance of the different cytokines in the post-PKP period. To identify the mediators playing roles in the rejection process, it was necessary to review which cytokines participate in the normal postoperative events, and in the complex course of the corneal wound healing.

The constitutive release of 6 cytokines (IL-1 β , IL-6, TNF- α , IL-8, IL-10 and IL-12p70) into the tears throughout 1 year after noncomplicated PKP was established. The early cytokine responses could be attributed to the physical damage to the cornea, a result of tissue injury and the presence of suture material, rather than an allogeneic response. The levels of IL-1 β , TNF- α , IL-10 and IL-12p70 remained constantly high after transplantation. The biphasic IL-10 and TNF- α responses were associated with the postoperative healing process, like the elevated IL-6 concentration. The present study indicated out the complex immunological events after PKP.

Previous studies did not demonstrate an increase in IL-6 concentration in the aqueous humor of patients without corneal rejection. In contrast, we confirmed low IL-6 concentrations even in the tears of patients with uncomplicated clear grafts. IL-6 had reached the background level by 1 year postoperatively. For this, we suggest several possible explanations. IL-6 has the crucial functions of keeping the corneal button clear, stimulating collagen synthesis and supporting corneal epithelial healing. The significant increase in IL-6 release after PKP could indicate the normal corneal wound healing process. IL-6 is part of the endogenous anti-inflammatory system. The increased IL-10 levels of the tears could be an indicator of graft tolerance. The graft tolerance effect is one reflection of the anti-inflammatory modification. In our study, IL-1 β had reached the background level by 6 months postoperatively.

In summary, our results demonstrated the complex immunological events following PKP, and the postoperative physiological role and importance of the different inflammatory cytokines.

The immunological events and the rejection process after PKP are intensively researched fields, but a number of questions are still unanswered. Our next project was to determine the roles of the different cytokines in the corneal rejection process following PKP.

During the early postoperative phase, the levels of all the tested cytokines rose and then rapidly declined, regardless of the occurrence of corneal rejection. Our results showed that the tears of patients who undergo corneal transplant rejection present significantly higher levels of IL-6 and IL-8 and lower levels of IL-10, TNF- α and IL-12p70 than the tears of transplanted patients without rejection during 1 year postoperatively.

Our study confirmed the increase in IL-6 in the aqueous humor of patients with corneal rejection by demonstrating a significantly increased IL-6 concentration in the tears from patients with graft rejection relative to those without. IL-6 has the crucial functions of keeping the corneal button clear, stimulating collagen synthesis and supporting corneal wound healing, as well as being part of the endogenous anti-inflammatory system.

Our study proved, that maintenance of the corneal graft tolerance by IL-10 is essential for the prevention of transplant rejection. It is well known that IL-10 is essential for the maintenance of the ACAID. This anti-inflammatory cytokine has a role in angiogenesis. In our study, the IL-10 concentration was significantly lower in the tears of patients with rejection relative to those with nonrejection. The reduced level of IL-10 in the tears of patients with endothelial rejection could be an important attribute of the pathophysiology of transplant rejection. Accordingly, we hypothesized that the increased IL-10 levels of the tears in the eyes with clear corneal grafts could be an indicator of graft tolerance, whereas during rejection IL-10 can act as an inhibitory factor for T-helper type 1 responses. IL-10 decreases the expression of MHC class II in monocytes/macrophages, thereby interfering with their antigen-presenting function. IL-10 also modulates monocytes by suppressing the production of other proinflammatory cytokines (TNF- α , IL-1 β and IL-8).

In our human study, second IL-1 β and IL-8 concentration peaks coincided with the onset of graft rejection. IL-1 β has been considered a multifunctional,

proinflammatory cytokine in the cornea, capable of initiating the inflammatory cascade and inducing corneal tissue damage, while also contributing to tissue repair. This could explain why we found no significant difference in IL-1 β level in tears from complicated and uncomplicated grafts. Although the IL-1 β levels were not related to the transplantation outcome, it seems that this cytokine is an active player in corneal rejection, which inspires us to perform further examinations. Furthermore, IL-1 β , IL-8 and TNF- α are also involved in neovascularization, and both TNF- α and IL-1 β act as autocrine factors that can further enhance the expression of IL-6 and IL-8. The literature draws attention to the enhanced expression of the proinflammatory cytokines (IL-1 β and TNF- α) in high-risk cornea transplantation. In other studies, the corneal TNF- α expression was found to be higher at both the mRNA and the protein level in the aqueous humor and serum from hosts with rejected corneal allografts. In contrast, we observed significantly decreased TNF- α levels in the tears from patients with corneal rejection relative to those with uncomplicated corneal grafts. It has been suggested that TNF- α can induce apoptosis via the susceptibility of the corneal endothelial and epithelial cells.

The induction of immunity to graft antigens in the draining lymph nodes after cornea transplantation occurs via an IL-12 and INF- γ -dependent mechanism. A significant upregulation of IL-12 mRNA was observed in rejected corneal allografts in rats. The local delivery of IL-12p40 results in the partial inhibition of activated T-cell infiltration and the release of Th1 cytokines, both playing critical roles in corneal allograft rejection, but not sufficient to prevent rejection. Our results reveal a significant decrease in IL-12p70 in the tears of patients with corneal rejection as compared with uncomplicated grafts.

The different tear samples could be comprehensively characterized with the balance of pro- and anti-inflammatory cytokines. Disruption of the balance of pro- and anti-inflammatory cytokines may lead to transplant rejection and decreased corneal graft tolerance. The reduced levels of IL-10 and TNF- α and the increased levels of IL-6 and IL-8 in the tears of patients with rejection results in a trend toward increased IL-6/IL-10 and IL-8/IL-10 ratios, and decreased TNF- α /IL-10.

Despite our results, the present study involves certain limitations: 1) the comparatively low number of patients involved in the tear sampling; 2) some tear samples had cytokine concentrations (IL-10, IL-12 and TNF- α) near the detection limit; and 3) the treatments in the complicated and noncomplicated cases were not completely similar. Our stratified statistical data analysis revealed that the systemic administration of steroids did not exert a confounding effect on our results. None of the examined cytokines could be used as a marker of transplant rejection in human tears. Further investigations are needed to identify the cytokine combination that could really be used as a marker of transplant rejection. Despite the difficulties inherent in the examination and the discussion, this study is the first step toward the establishment of the immunological analysis of tears from patients undergoing PKP that could be used as a predictor of the clinical status and the need for preventive therapy.

6. Summary of new results

- I. We have confirmed the enhanced release of IL-6 and IL-8 into the tears in various anterior segment eye diseases and in postoperative conditions. These two cytokines may be used as an indicator of various irritative anterior segment eye diseases, including PKP.
- II. The constitutive release of 6 cytokines throughout 1 year after uncomplicated PKP was established. The early cytokine responses could be attributed to the physical damage to the cornea, a result of tissue injury and the presence of suture material. The present study highlighted the complex immunological events after PKP.
- III. The enhanced release of IL-6 and IL-8 into the tears of patients with corneal graft rejection concomitant with decreased concentrations of IL-10, TNF- α and IL-12p70 may possibly serve as an indicator of the rejection process.

7. Usefulness of the scientific results

Corneal transplantation is one of the most commonly performed tissue transplantations. Corneal graft rejection is a significant complication of corneal transplantation and in recent decades its frequency has not changed substantially in either the high-risk or the low-risk cases.

Determination of the levels of the different cytokines in the tears of transplanted patients and examination of the changes in the cytokine concentrations furnishes an opportunity to obtain data from the pathologic intercellular communications before the rejection process. On the basis of our present human studies relating to tear examinations, we have outlined for the first time the complex immunebiological events characteristic of graft rejection after PKP. This study is a first step toward establishment of the typical changes in cytokine secretion before the rejection process. This may allow improved therapeutic and preventive individual treatment modalities, serving for the good of the patients.

8. Publications

This thesis is built upon the following publications:

1. **Fodor M.**, Facskó A., Rajnavölgyi É., Hársfalvi J., Bessenyei E., Kardos L., Berta A.: Enhanced release of IL-6 and IL-8 into tears in various anterior segment eye diseases. *Ophthalmic Res* 2006; 38: 182-188. **(IF:1.01)**
2. **Fodor M.**, Facskó A., Rajnavölgyi É., Hársfalvi J., Berta A.: Interleukin-6 meghatározása humán könnyből a szem elülső szegmentumát érintő állapotokban. *Szemészet* 2007; 144: 61-64.
3. **Fodor M.**, Gogolák P., Rajnavölgyi É., Berta A., Kardos L., Módis L., Facskó A.: Long-term kinetics of cytokine responses in human tears after penetrating keratoplasty. *J Interferon Cytokine Res* 2009; 29: 375-380. **(IF:2.667)**
4. **Fodor M.**, Berta A., Rajnavölgyi É., Gogolák P., Facskó A.: Pro- és anti-inflammatorikus citokinek meghatározása perforáló keratoplasticán átesett betegek könnyéből. *Szemészet* 2009; 146: 49-52.

Citable abstracts in relation to the theses:

1. **Fodor M.**, Facskó A., Rajnavölgyi É., Berta A.: Interleukin-6 meghatározása humán könnymintákból különböző elülső szegmentum betegségekben. *Szemészet* 2003; 140(S):84.
2. **Fodor M.**, Facskó A., Rajnavölgyi É., Berta A.: Interleukin-8 meghatározása humán könnyből a szem elülső szegmentumát érintő állapotokban. *Szemészet* 2007; 144(S): 41-42.
3. **Fodor M.**, Berta A., Rajnavölgyi É., Gogolák P., Facskó A.: Citokinek vizsgálata könnyből perforáló keratoplastica után. *Szemészet* 2009; 146(S): 29-30.

Posters

1. **Fodor M.**, Rajnavölgyi É., Hársfalvi J., Facskó A., Berta A.: Detection of interleukin-6 in human tears in various anterior segment eye conditions.

- 2007.06.9-12. Joint Congress of European Society of Ophthalmology and American Academy of Ophthalmology (SOE/AAO); Bécs.
2. **Fodor M.**, Nagy V., Pflieger Gy., Tornai I., Berta A.: Hepatitis C vírus okozta kétoldali NAION esete. 2008.05.29-31. Magyar Szemorvos Társaság Kongresszusa; Pécs.
 3. **Fodor M.**, Nagy V., Pflieger Gy., Tornai I., Berta A.: Hepatitis C vírus okozta kétoldali NAION esete. 2009.04.20. DAB Szemklinikai előadások; Debrecen.

Presentations in relation to the thesis:

1. **Fodor M.**, Facskó A., Rajnavölgyi É., Berta A.: Interleukin-6 meghatározása humán könnymintákból különböző elülső szegmentum betegségekben, 2003.08.29. Magyar Szemorvostársaság Kongresszusa, Cornea szekció; Budapest.
2. **Fodor M.**, Facskó A., Rajnavölgyi É., Hárnfalvi J., Berta A.: Humán könnyvizsgálatok protein assay segítségével, 2003.11.28. Továbbképző előadás, SOTE I; Budapest.
3. **Fodor M.**: Interleukinok (IL-6, IL-8) meghatározása humán könnymintákból különböző elülső szegmentum betegségekben, 2004.04.29. DAB Szemklinikai előadások; Debrecen.
4. **Fodor M.**, Berta A.: IL-6 and IL-8 in various anterior segment eye diseases, 2004.10.15. Congress of the European Contact Lens Society of Ophthalmologists (ECLSO); Budapest.
5. **Fodor M.**, Facskó A., Rajnavölgyi É., Berta A.: Interleukin-8 meghatározása humán könnyből a szem elülső szegmentumát érintő állapotokban. 2007.06.23. Magyar Szemorvostársaság Kongresszusa; Debrecen.
6. **Fodor M.**: Könny-citokinmeghatározások a szem elülső szegmentumának betegségeiben. 2009.04.20. DAB Szemklinikai előadások; Debrecen
7. **Fodor M.**, Berta A., Rajnavölgyi É., Gogolák P., Facskó A.: Citokinek vizsgálata könnyből perforáló keratoplastica után. 2009.06.26. Magyar Szemorvostársaság Kongresszusa; Budapest.

8. **Fodor M.**: Citokinek vizsgálata könnyből perforáló keratoplastica után. 2009.06.29. DEOEC A klinikai orvostudományok doktori iskola 2009. évi Ph.D. szimpóziuma. Debrecen.

Other publications:

1. Tsorbatzoglou A., **Fodor M.**, Vámosi P., Németh G., Berta A.: Tapasztalataink glaucomás szemeken végzett phacoemulsificatioval. Szemészet 2003; 140:136-138.
2. **Fodor M.**, Tsorbatzoglou A., Vámosi P., Berta A.: Phacoemulsificatio hatására bekövetkezett szemnyomásváltozás a korai postoperativ szakban nem glaucomás betegeken. Szemészet 2003; 140: 33-35.
3. **Fodor M.**, Berta A., Módis L.: A könnyfilm-beszáradi teszt szerepe a száraz szem diagnosztikájában. Szemészet 2007; 144: 191-195.
4. Módis L., **Fodor M.**, Berta A.: A conjunctivális impressziós citológia szerepe a száraz szem diagnózisában. Szemészet 2007; 144: 171-175.
5. **Fodor M.**, Nagy V., Berta A., Tornai I., Pflieger G.: Hepatitis C virus presumably associated consecutive anterior ischemic optic neuropathy. Eur J Ophthalmol 2008; 18: 313-315. (**IF: 1.018**)
6. Módis L., Kettesy B., Szalai E., **Fodor M.**, Berta A.: Endothelialis keratoplasztikával szerzett tapasztalataink. Szemészet 2009; 146: 35-41.

Other abstracts:

1. Tsorbatzoglou A., **Fodor M.**, Vámosi P., Berta A.: Glaucomás betegeken végzett phacoemulsificatio során szerzett tapasztalataink. Szemészet 2002; 139(S):51.
2. **Fodor M.**, Tsorbatzoglou A., Vámosi P., Berta A.: Phacoemulsificatio hatására bekövetkezett szemnyomásváltozás a korai postoperativ szakban nem glaucomás betegeken. Szemészet 2002; 139(S):53.

Other presentations:

1. **Fodor M.**, Berta A., Módis L.: Morfológiai vizsgálómódszerek a szárazszeműség korszerű diagnosztikájában. 2002.01.25. Továbbképző előadás, SOTE I.; Budapest. (Pályamunka: A száraz szem magyarországi, 2004 évi kutatási pályázata: I. díj.)
2. **Fodor M.**, Tsorbatzoglou A., Vámosi P., Berta A.: Phacoemulsificatio hatására bekövetkezett szemnyomásváltozás a korai postoperativ szakban nem glaucomás betegeken. 2002.04.13. Magyar Műlencse Implantációs és Refraktív Sebészeti Társaság Kongresszusa (SHIOL); Keszthely.
3. Tsorbatzoglou A., **Fodor M.**, Vámosi P., Berta A.: Phacoemulsificatio hatására bekövetkezett szemnyomásváltozás a korai postoperativ szakban glaucomás betegeken. 2002.04.13. Magyar Műlencse Implantációs és Refraktív Sebészeti Társaság Kongresszusa (SHIOL); Keszthely.
4. **Fodor M.**: A száraz szem konzervatív terápiája III. A-vitamin. 2004.11.11. Továbbképző előadás, DEOEC Szemklinika; Debrecen.
5. **Fodor M.**, Németh G., Tornai I., Szalczer L., Dinya Z., Kenyeres A., Felszeghy Sz., Újvári T., Módis L., Berta A., Facskó A.: PCL csere transzparencia csökkenés miatt. 2008.03.28. Magyar Műlencse Implantációs és Refraktív Sebészeti Társaság Kongresszusa (SHIOL); Keszthely.
6. **Fodor M.**: A ROP etiopathogenese és terápiás lehetőségei. 2008.10.18. Továbbképző előadás, DEOEC Szemklinika; Debrecen.

Scientometric parameters

Number of in extenso publications:	3
as author:	3
Cumulative impact factor:	4,695
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