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Cytokine Profile in Tears of Patients following Autokeratoplasty-Immunological Implications

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Abstract

Aim: To examine the immunological processes following penetrating keratoplasty and compare the kinetics of the changes in cytokine concentrations in tears of patients with allo- and autokeratoplasty.

Methods: "Patient A" had choroidal melanoma and corneal button of this enucleated eye was used for autokeratoplasty. "Patient B" had previous operation causing reduced endothelial cell count in the blind donor eye, used to replace the opaque cornea of the fellow eye having corneal transplant rejection. The control group consisted of 10 eyes after penetrating keratoplasty with clear corneal grafts. The concentration of tumor-necrosis-factor α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, IL-13, IL-17, IL-18, interferon (IFN)-γ, chemokine (C-X-C motif) ligand (CXCL)8/IL-8, CXCL10/IP-10 and chemokine (C-C motif) ligand 5 (CCL5)/RANTES in tear samples were measured by cytometric bead array technology at regular time intervals.

Results: The concentration of IL-1β, IL-10, IL-13, IL-17 and IFNγ in tear samples of Patient A (uncomplicated autokeratoplasty) was below, while in Patient B (complicated autokeratoplasty) was above the control-based empirical trend line. IL-6, CXCL8 and CCL5 concentrations were mainly below trend line in Patient A and above in Patient B. CXCL10 concentration was continuously above empirical trend in patient A, and always below in patient B. IL-18 and TNF-α concentrations did not differ from empirical trend in autokeratoplasty.

Conclusion: Cytokine concentrations in tears from uncomplicated penetrating contralateral autokeratoplasty differed markedly from penetrating keratoplasty, whereas those from complicated autokeratoplasty were similar to those observed in allografts. Reasons other than allogeneic response, such as primary graft failure due to insufficient donor endothelial cell count, tissue injury or healing process itself are probably involved in long-term immunological response.

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Introduction

Corneal transplantation is the most commonly performed solid organ transplantation in the World [1-2]. Cornea graft failure is common and allograft rejection remains the predominant cause of poor long-term survival (60% at 5 years compared to 80% for renal transplantation) [2-4].

In the unique event of having the patient's own fellow eye as a donor for corneal tissue, the risk of immunologic rejection is practically avoided [5]. Autokeratoplasty has been performed using either the ipsilateral or contralateral cornea as donor tissue [5-8]. Contralateral autokeratoplasty (AKP) is a very rare surgical procedure, indicated in patients requiring corneal transplantation in one eye and having no vision in the contralateral eye with normal cornea. Previous studies on AKP are limited published as small case series of one to four patients [1, 9-12]. One of the first reports on autokeratoplasty comes from our department half a century ago [13]. Autokeratoplasty, when indicated, gives unique insight into the mechanisms beyond rejection following penetrating keratoplasty (PKP).

Cytokines, as important mediators of the immune response, play an essential role in corneal alloimmunity. Little information is available on the molecular mediators of corneal graft rejection in humans [14-16]. Recent studies have implicated a pathophysiological role for cytokines and chemokines in graft dysfunction after lung and kidney transplantation, gaining popularity in discovering anti-cytokine and chemokine therapy for primary graft dysfunction [17-18]. Studying the role of different cytokines in the postoperative period of keratoplasty, syngeneic animal corneal transplantation models have been rarely used [19-21] and human autografted cases are actually absent. Analysis of human tears on the ocular surface may provide important information about the mechanisms of graft rejection without the risk of anterior chamber sample collection. Because of the rarity of human AKP, no studies have been published to date on the cytokine response after autografting tested in tears.

Our goal was to determine whether the concentrations of a carefully selected panel of cytokines and chemokines studied in tears from human corneal autokeratoplasty differ from those obtained after allokeratoplasty. Furthermore, we wanted to determine the kinetics of the cytokine release in tears of patients following AKP and to test whether partial autograft dysfunction unrelated to any immune reaction can induce altered cytokine release.

Patients and Methods

Patients

In a prospective design, nonstimulated tear samples were collected from two patients at regular time intervals before and after AKP. Both surgical procedures were performed by one specialist (A.B.) under local, retrobulbar anaesthesia. There were no surgical complications in either of the eyes. "Patient A" had unilateral large choroidal melanoma

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and the corneal button of this enucleated eye was used for the AKP to treat central macular corneal opacities obscuring the visual axis without any vascularization or inflammation. The mean endothelial cell count was 2650 cells/mm^2. The visual outcome was limited by amblyopia and age-related macular degeneration (corrected distance visual acuity: 0.3 and 0.06, one and four years after autokeratoplasty, respectively). "Patient B" had a previous operation causing reduced endothelial cell count in the donor eye. The mean endothelial cell count was 1800 cells/mm2. The abnormal but clear cornea from this blind eye was used to replace the opaque cornea of the fellow eye because of corneal transplant rejection. The decision to do an autologous corneal transplantation and to use a donor graft from the previously operated fellow eye was made clinically and by a well documented request by the patient. Patient B had a non-immune graft failure after surgery. One year postoperatively therapeutic contact lens had to be used to offset a partial decompensation of the autograft but no secondary glaucoma or other complications have occured. The follow-up was 1 year and the corrected distance visual acuity was 0.1. Table 1 summarizes the data and indications for AKP of the two patients. After AKP, the patients were initially treated with topical corticosteroid/antibiotic combination for 3 weeks. Later, the eye-drops were discontinued to minimize the development of corticosteroid responsive pressure changes and wound healing inhibition.

	"Patient A"	"Patient B"
Age (years) / sex	66 / male	57 / male
Preoperative best-corrected visual acuity in the recipient eye	Count fingers	0.02
Preoperative best-corrected visual acuity in the donor eye	Excentric 0.06	No light perception
Preoperative diagnosis in recipient eye	Corneal leucoma, amblyopia	Transplant rejection; corneal perforation and subsequent penetrating keratoplasty was performed
Cause of vision loss in donor eye	Intraocular melanoma	Retinal detachment
Preoperative endothelial cell count in the donor eye (cells/mm2)	2650	1800
Postoperative (1 years) corrected distance visual acuity in the recipient eye	0.3	0.1
Reason for decreased visual acuity in recipient eye postoperatively	Amblyopia, age-related macular degeneration	Corneal edema
Status of the graft at the end of the follow-up period in the recipient eye	Clear	Partially decompensated with therapeutic contact lens

Table 1: Characteristics of the patients undergoing penetrating contralateral autokeratoplasty.

The control group consisted of 10 eyes of 10 patients after penetrating allokeratoplasty with clear corneal grafts. Characteristics of the controls, including primary indications for corneal allotransplantation are shown in Table 2. None of the subjects were taking any medication that could interfere with the tear production or suffered from any disease of known immunological origin. All control donor material was preserved in Optisol-GS (Bausch & Lomb, USA) for at most 7 days. There were no surgical or postoperative complications in either of the eyes. Routine medication (local corticosteroids and antibiotics) was applied for the first 12 months after corneal transplantation. Two patients received systemic anti-inflammatory therapy (i.v. or oral corticosteroid) to prepare them for rekeratoplasty or due to recipient vascularization.

Following the tenets of the Declaration of Helsinki, informed written consent was signed by all participants prior to corneal transplantation. Research was approved by our local ethics committee.

	Sex	Age	Preoperative diagnosis
1	Female	34	Macular corneal dystrophy
2	Female	32	Keratoconus
3	Female	46	Re-keratoplasty due to large astigmatism
4	Male	50	Vascularized corneal leucoma
5	Male	21	Keratoconus
6	Female	79	Fuchs' endothelial dystrophy (FED)
7	Male	64	Macular corneal dystrophy
8	Female	57	Pseudophakic bullous keratopathy (PBK)
9	Male	34	Keratoconus
10	Male	57	Autograft donor

Table 2: Characteristics of the controls undergoing penetrating allokeratoplasty.

Sample collection

Before every tear collection, the anterior ocular status of each subject was carefully assessed; a slit-lamp examination under low illumination was performed to avoid reflex tearing. Tear samples were collected in the morning before and after the operation, between 7.30 and 8.00 a.m., just before the first eye drops were instilled, and then at each ophthalmological visit during the one-year follow-up. Non traumatic tear collection was carried out with capillary tubes from the inferior meniscus, without topical anesthesia for 2 min; the total volume of the collected tears was registered. The samples were immediately transferred to Eppendorf tubes and frozen at –80 °C without centrifugation within 15 min from collection. Preliminary studies had demonstrated that centrifugation of the samples does not influence the cytokine concentrations. To avoid pipetting and dilution errors, collected tear samples of < 4 µl were excluded. In some points of time, dry eye did not allow tear collection.

Cytokine measurements

The concentration of cytokines (TNF-α, IL-1β, IL-6, IL-10, IL-13, IL-17, IL-18, IFNγ, IL-8/CXCL8, IP-10/CXCL10 and RANTES/CCL5) was measured by the Cytometric Bead Array method. Combined FlowCytomix™ Simplex Kits were used with the appropriate FlowCytomix Basic Kit with minor modifications of the manufacturer's instructions (eBioscience, Bender MedSystems GmbH, Vienna, Austria). Briefly, 15 µl of tear samples (in some cases diluted samples) or serial dilutions of mixed standard cytokines were added to 15 µl suspension of fluorescent cytokine capture beads on multiwell filter microplates. 15 µl of biotin conjugated anti-cytokine antibody was added to the wells, than the plates were incubated for 2 hours on a microplate shaker. Plate wells were emptied and washed with a vacuum filtration manifold. Phycoerythrin conjugated streptavidin was added to the wells followed by additional incubation for 1 hour and washing

as described above. 150 µl assay buffer was applied to the wells, then multiparametric data acquisition was performed on a FACS Array cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA). Data were analyzed with the FlowCytomix Pro 2.3 software. Additional serial dilutions of the standard were applied to obtain better sensitivity and therefore, modified standard curves were generated in the analysis. The detection limits were: TNF-α: 3.2 pg/ml; IL-1β: 4.2 pg/ml; IL-6: 1.2 pg/ml; IL-10: 1.9 pg/ml; IL-13: 4.5 pg/ml; IL-17A: 2.5 pg/ml; IL-18: 3.34 pg/ml; IFNγ: 1.6 pg/ml; CXCL8: 0.5 pg/ml; CXCL10: 6.0 pg/ml; CCL5: 25 pg/ml.

Statistical methods

Cytokine concentrations and their ratios to that of IL-10 were calculated from tear samples of patients with auto- and allotransplantation shown on scatter plots. Empirical trends were calculated from the data of the controls throughout the follow-up period using locally weighted regressions of outcome versus day of follow-up. The resulting Lowess curves, along with line charts based on the data from Patients A and B, were superimposed on the plots.

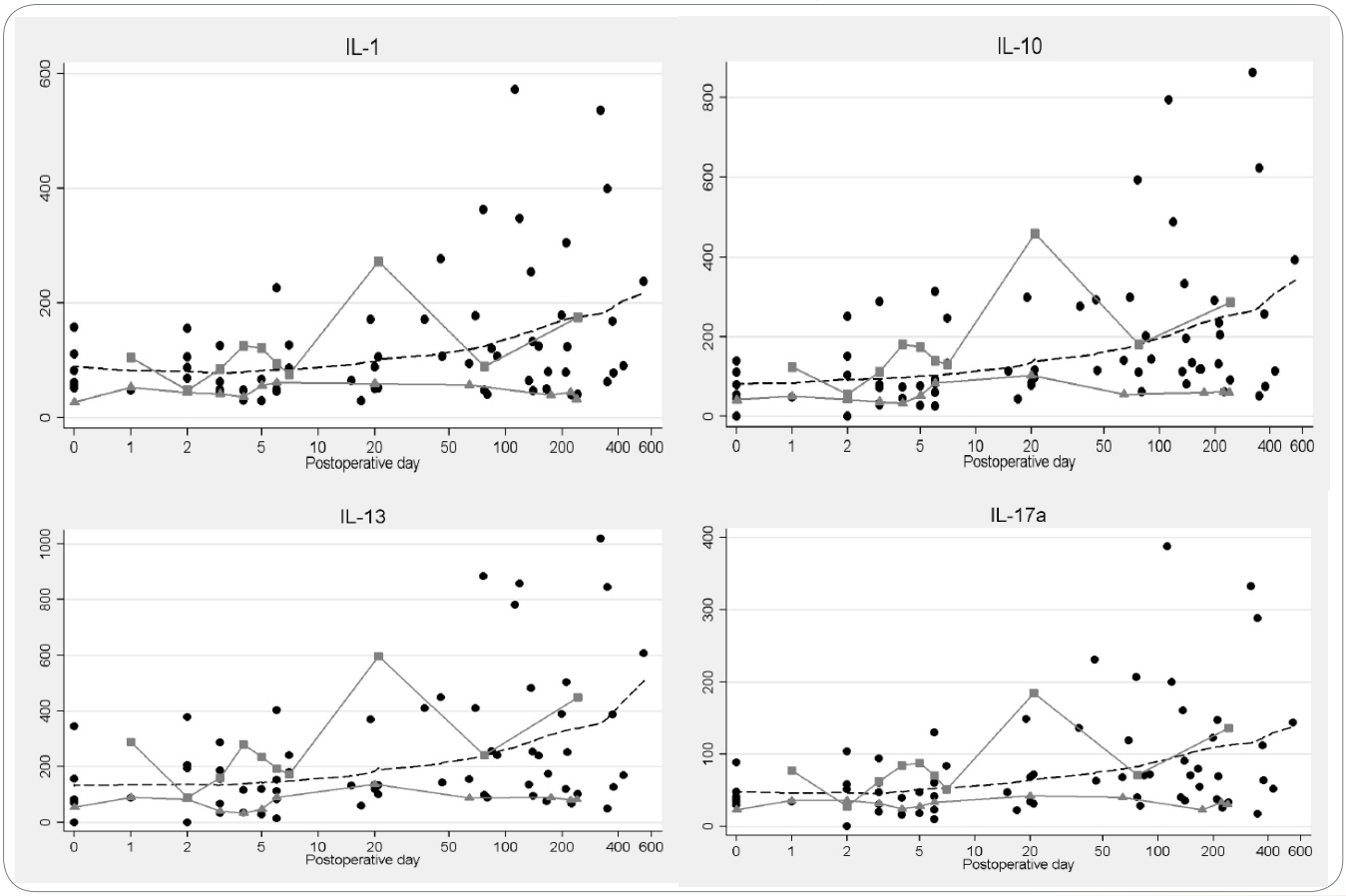
Results

All the grafts were monitored on a regular basis. Except for one autograft, all the other grafts remained clear. The reason for the irreversible partial autograft failure in Patient B was a decreased preoperative endothelial cell count, the partial graft opacification not being due to any immunological process. All cytokines could be detected in all patients during the whole follow-up period. During the very early phase, there was no evidence for difference in any of the cytokine levels between allogeneic or autologous recipients and complicated or uncomplicated autografted cases.

IL-1β, IL-10, IL-13, IL-17 and IFNγ all shared a similar profile during the observation period. The level of these cytokines was similar and consistently low in tears of the patient with uncomplicated autograft; thus their concentrations were definitely under the empirical trends of the controls (Figure 1). The above cytokines continued to rise in the tears of the patient with partial autograft failure (Patient B), and stayed mainly above the empirical trendline of the controls. Autograft dysfunction induced altered cytokine release. In the late postoperative period, the secreted cytokines could have mediated not only the allograft response, but taken part in the corneal non-immune failure and in the pathomechanism of corneal decompensation.

The largest difference between complicated and uncomplicated autokeratoplasty was observed for IP-10/CXCL10 (see Figure 1). Interestingly, the concentration of CXCL10 was markedly high in tears of the patient with uncomplicated, clear autograft (Patient A) in the early postoperative period and remained high throughout the follow-up period. CXCL10 concentration in tears of Patient A with clear autograft was continuously above the empirical trendline of the controls and definitely under that in tears of Patient B with complicated, partial decompensated autograft.

Concentrations of IL-6, CXCL8 and CCL5 varied widely, with the uncomplicated AKP mainly having lower levels and the complicated AKP mainly higher levels of these cytokines as compared to the controls-based empirical trend. IL-18 and TNF-α did not show a difference during the observation period following human corneal autokeratoplasty as compared to allokeratoplasty. IL-18 and TNF-α levels appeared similar in the AKPs with/without complication suggesting that the partial autograft dysfunction was not responsible for the altered cytokine release, although their concentrations were overall widely variable.



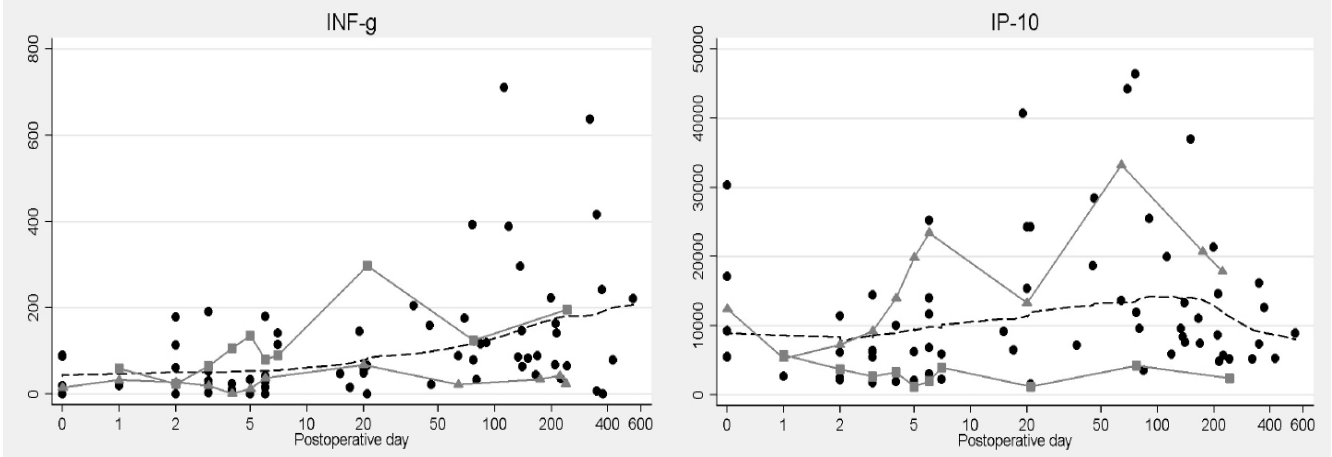


Figure 1: Tear levels (pg/ml) of IL-1β, IL-10, IL-13, IL-17, IFNγ and IP-10/CXCL10 in the controls (each measurements = •; empirical trend = ---), Patient A with uncomplicated autokeratoplasty and clear graft= ▲, and Patient B with complicated autokeratoplasty and partial autograft dysfunction=▪.

The patient with partial autograft dysfunction (Patient B) had obviously elevated levels of pro-inflammatory mediators including IL-1β, IL-17, and the anti-inflammatory cytokines IL-10, IL-13 and IFNγ when compared to the allografts. In tears of the autografted patient with uncomplicated cornea (Patient A) the cytokine levels remained low except for CXCL10. According to these findings the ratio of the tear levels of CXCL10/IL-10 were markedly low for the Patient A with an uncomplicated, clear autograft (Figure 2).

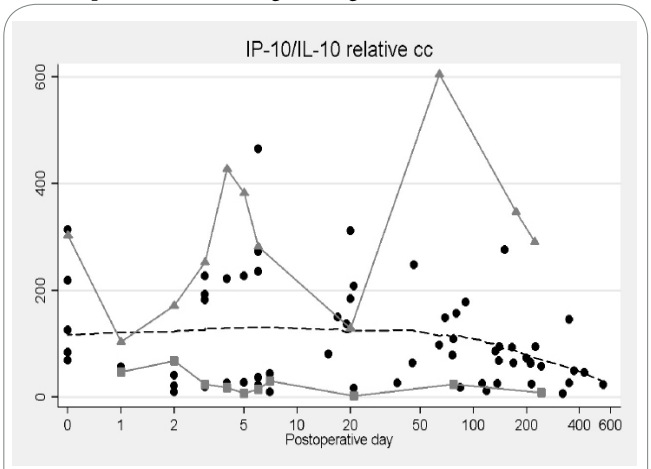


Figure 2: Ratio of the tear levels (pg/ml) of IP-10/IL-10 in the controls (each measurements = •; empirical trend = ---), Patient A with uncomplicated autokeratoplasty and clear graft=▲, and Patient B with complicated autokeratoplasty and partial autograft dysfunction=▪.

Discussion

Corneal graft failure remains being a major threat to a successful transplant survival [10, 22]. Determining the complex mechanisms of graft failure is a priority in ocular immunology research [16, 23-24]. Corneal autotransplantation avoids the risk of allograft rejection [1, 9-13]. Tear samples offer a unique opportunity to analyze both inflammatory and anti-inflammatory mediators of the auto- and allogeneic response in contact with donor tissue.

This study is the first to measure such mediators in human tears after AKP with respect to graft dysfunction over time. Several interesting observations arise, in particular regarding the remarkable difference in tear CXCL10 levels between autokeratoplasty with and without graft dysfunction, as well as the lack of the marked difference in IL-6, CXCL8, CCL5, IL-18 and TNF-α response in tears between auto- and allotransplantation. For the first time we have shown that in case of uncomplicated autokeratoplasty the tear concentration of CXCL10 is consequently high and a similar profile of IL-1β, IL-10, IL-13, IL-17 and IFNγ were observed, which were otherwise low postoperatively.

The level of IFNγ in the tears of patient with uncomplicated autograft were consequently lower than in controls, consistent with the results of Panda et al. and Maier et al., reflecting the role of IFNγ in the activation of the immune system in allografts [16, 25-26]. Interestingly, in rat models, corneal expression of IFNγ and IL-13 has been shown at the onset of corneal allograft rejection and was not observed in syngeneic corneas at any point of time [19, 21, 27-28]. IL-13 and IFNγ must have another role from the one in immunological rejection, since they are both elevated after autokeratoplasty with decompensation, remaining unclear whether this is the cause or the consequence of the partial endothelial dysfunction. Increased intraocular levels of IL-10 and IFNγ[29] as well as IL-6 have been observed during rejection [15, 28]. As IFNγ is a strong inflammatory cytokine inducing Th1 immune responses, increased levels after allokeratoplasty compared to autokeratoplasty do not seem surprising.

The pro-inflammatory cytokine IL-1β takes part in the process of foreign antigen presentation [25, 28], and together with IL-6 and TNF-α they participate in corneal wound healing and regeneration. Wounding and operation of the cornea is associated with loss of stromal keratocytes due to their apoptosis. TNF-α, which otherwise is a component of the normal tear fluid [30], has been implicated in corneal allograft rejection [16, 25, 28, 31-32]. Members of the TNF family, such as FasL and TNF, can inhibit allograft rejection after PKP. Corneal epithelial and endothelial cells are susceptible to TNF-induced apoptosis [32], which might take place in the rejection process and in endothelial cell loss. TNF-α plays a privotal role in the induction of anterior chamber-associated immune deviation (ACAID) and is a principal inducer of IL-10 biosynthesis [32]. We did not observe any difference in the levels of IL-18 and TNF-α between allo- and autokeratoplasty which led us to conclude that the role of those cytokines during non-immune graft decompensation is less important. It is not clear, however, whether TNF-α and IL-18 play any

role in non-immune graft edema. Even in the case of an autograft, release of pro-inflammatory cytokines may lead to graft failure. IL-17 has been found to influence the secretion of Th1-derived IL-12 by antigen presenting cells (APCs), thereby restraining Th1 development [24], being implicated in the pathomechanism of the rejection process [28].

The pleiotropic cytokine IL-6 is expected to be a nonspecific marker of inflammation [15]. Very high levels of IL-6 have been found in aqueous humor of humans during graft rejection [14, 28], but also in other anterior segment diseases. It is therefore not surprising that the tears of the patient with complicated autokeratoplasty had the highest IL-6 levels, followed by the allografts and the uncomplicated autokeratoplasty, although the differences were not really pronounced. In rats, corneal expression of IL-6 and IL-10 were prolonged in allografts, and were observed only for a short period of time postoperatively in autografts [21]. Several studies reveal the immunosuppressive properties of IL-10 including the development of ACAID. IL-10 has the potential of reducing the incidence of corneal graft rejection and prolonging corneal allograft survival in animal models, but the mechanism by which IL-10 is exerting this effect is unknown [33]. Systemic gene transfer of IL-10 has been able to prolong allogeneic rat corneal graft survival [34]. IL-10 most likely influences the afferent arm of the allograft response, and possibly within the intraocular environment itself, by affecting APC maturation, migration or function, and decreasing local production of IFNγ [35]. Elevated levels of IL-10 during corneal immune reactions may reflect a down regulation of the delayed-type hypersensitivity, and this can be the result of donor specific activation of ACAID, preventing damage during immune reactions [29]. Besides the anti-inflammatory effects of IL-10, this cytokine can promote cytotoxic T cell activity *in vitro* [36], and prolonged administration of IL-10 may have a detrimental effect on graft survival [37]. Overall, this reinforces our findings that in the tears of the patient with non-immune decompensation of the autograft the level of IL-10 is higher than in uncomplicated allografts, the lowest level being measured in the case of uncomplicated autograft.

Chemokines are involved in homeostatic non-inflammatory processes and are believed to contribute to the immune privilege and the alloimmune response to corneal transplants [20, 38]. We have measured three chemokines in this study: IFNγ-inducible protein (IP-10/CXCL10), regulated on activation normal T-cell expressed and secreted (RANTES/CCL5) and CXCL8. Increased expression of CXCL8 has been associated with endothelial dysfunction in pseudophakic bullous keratopathy [39]. The role of IL-8/CXCL8 in allograft rejection has also been shown [28]. In a previous study, high level of CXCL10 was found in the aqueous humor of patients during endothelial rejection [14] and high corneal expression was associated with potent alloreactivity [20]. This is in line with the fact, that CXCL10 up-regulates the production of Th1 cytokines and down-regulates the production of Th2 cytokines. Surprisingly, we found markedly elevated levels of CXCL10 in the patient tears following uncomplicated autokeratoplasty compared to the control allografted sample and the decompensated graft. These increased levels seem to stand in contrast to the functions of CXCL10, however, this chemokine has a role in autoimmunity and a dual effect in diabetes, being involved both in initiation and maintenance of the autoimmune process, as well as abrogation of autoimmunity [40-41]. CXCL10 can antagonize angiogenesis [42] depending on its receptor binding. Indeed, cytokines can sometimes exert paradoxical effects, suggesting that further investigation is required to determine the precise role of CXCL10 in tears after auto- and allokeratoplasty. Experimental data show that CXCL10 plays a key role in early injury after organ transplantation [17, 20]. High pretransplant serum levels of CXCL10 predicted the risk for development of acute rejection and chronic allograft vasculopathy: patients with serum CXCL10 levels greater than 150 pg/ml showed a nearly 2-fold greater frequency of rejection [43]. The concentration of CXCL10 in tears was markedly higher than in serum after organ transplantation, suggesting a local production of this chemokine. Although no direct proof is available, maintenance of corneal graft transparency by CXCL10 can be deduced from the result of this study. Despite straightforward evidence obtained in basic studies and experimental animal models of keratoplasty, the results provided by this clinical study remain controversial and in some cases difficult to interpret. Primary graft dysfunction after human lung transplantation was associated with elevated levels of pro-inflammatory mediators, like CXCL10 and IL-1β, variable IFNγ and TNF-α, decreased IL-13 in sera and was linked to chronic allograft rejection [17, 44]. The reason for the discrepancies between our findings and those of other transplantation models can be that solid vascularized human organ transplantations and animal models are not comparable to the avascularised corneal tissue transplantations. Obviously, there are other factors influencing graft survival that have not been discovered yet.

CCL5, a late chemokine, is primarily involved in corneal transplant immunity by mediating recruitment of alloreactive T cells to the anterior segment microenvironment [20, 38]. Increased expression of CCL5 has been observed during rejection in the murine model [19-20], but not in human studies on aqueous humor [14]. In agreement with these findings, in our study, the concentration of CCL5 in tears of patient with clear autograft was the lowest.

Our study has several limitations. Only descriptive statistical analysis could be made because of the only case of complicated and of uncomplicated autokeratoplasty. Therefore, negative results should be interpreted with caution. Moreover, the two cases and the control group had differences in clinical variables, including diagnoses and postoperative treatment although we tried to select the most proper controls. The source of the examined cytokines in the tears of this study could not be identified. Despite these limitations it is important to emphasize that our results highlight the fact that many cytokines take not only place in the induction phase of alloimmunity but also in the complex mechanism of autograft failure caused by endothelial dysfunction. By comparison, any cytokine change after autokeratoplasty is unaffected by the transplantation-related immunological reactions. Our observation is in line with the prior demonstration of Liu et al. of a significantly altered cytokine microenvironment in the allogeneic as compared to the syngeneic setting of corneal transplantation [45]. However, it is difficult to conclude from our data that only low level of CXCL10 is responsible for the decompensation as there are no data in the literature confirming this question. Not only Th2 (IL-10, IL-13, IL-6) cytokines but also IL-17 can be involved in the non-immune decompensation of the transplanted autograft. The consequences of the decreased corneal endothelial cell count caused by the previous operation of the donor eye in case of autokeratoplasty could be various: release of different cytokines by the damaged endothelial cells stimulating cytokine production of other cell populations and influencing the immunological features of the tear film. Our findings suggest the importance of these data for directing future clinical and basic studies, including investigations of the clinical utility of these biomarkers in predicting the rejection process.

Conclusion

Our study is the first to measure mediators in tears after autokeratoplasty with respect to graft dysfunction over time. We provide new evidence on the tear levels of multiple cytokines and chemokines after corneal auto- and allotransplantation. In case of autokeratoplasty with primary graft dysfunction, an increase in the tear levels of IL-1β, IL-10, IL-13, IL-17, IFNγ, IL-6, CXCL8 and CCL5 was observed, while they remained similar to those seen in allografts after complicated autokeratoplasty. Reasons other than the allogeneic response, such as primary graft failure due to insufficient donor endothelial cell count, tissue injury or healing process itself are probably involved in the long-term kinetics of the immunological response.

Competing Interests

The authors declare that they have no competing interest.

Author's contributions

Dorottya Pásztor, Bence Kolozsvári and Péter Gogolák finished the experiments and drafted the manuscript. Mariann Fodor and Bence Kolozsvári collected the clinical information and samples. Péter Gogolák and Éva Rajnavölgyi helped to analyze the data. Autokeratoplasties were performed by András Berta. András Berta and Mariann Fodor designed the study and helped to revise the manuscript. All authors read and approved the final manuscript.

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