Tear Film Cytokine Profile of Patients With the Boston Keratoprosthesis Type 1: Comparing Patients With and Without Glaucoma

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Purpose. Inflammatory cytokines are involved in glaucoma pathogenesis. The purpose is to compare cytokine levels in the tear film of Boston keratoprosthesis (KPro) patients with and without glaucoma, relative to controls, and correlate levels with clinical parameters.

Methods. This cross-sectional study enrolled 58 eyes (58 patients): 41 KPro eyes with glaucoma, 7 KPro eyes without glaucoma, and 10 healthy controls. Twenty-seven cytokines were measured by multiplex bead immunoassay. Intraocular pressure (IOP), cup-to-disk ratio (CDR), retinal nerve fiber layer, visual acuity, topical medications, and angle closure were assessed in all KPro eyes. Cytokine levels between groups were analyzed by nonparametric tests, and correlations with clinical parameters by Spearman's test.

Results. Levels of TNF-α, IL-1β, FGF-basic, and IFN-γ were significantly higher in KPro with glaucoma compared to KPro without ($P = 0.020$; 0.008; 0.043; 0.018, respectively). KPro groups had similar characteristics and topical antibiotic/steroid regimen. Levels of IL-1Ra, IL-15, VEGF, and RANTES were significantly higher in KPro with glaucoma compared to controls ($P < 0.001$; $= 0.034$; < 0.001; $= 0.001$, respectively). IL-1β and IFN-γ levels were positively correlated with CDR ($r = 0.309$, $P = 0.039$ and $r = 0.452$, $P = 0.006$, respectively) and IOP ($r = 0.292$, $P = 0.047$ and $r = 0.368$, $P = 0.023$, respectively). TNF-α and FGF-basic levels were positively correlated with CDR ($r = 0.348$, $P = 0.022$ and $r = 0.344$, $P = 0.021$, respectively).

Conclusions. TNF-α, IL-1β, FGF-basic, IFN-γ are elevated in tears of KPro patients with glaucoma and correlate with CDR and IOP. These results show, for the first time in humans, concordance with documented elevations of TNF-α and IL-1β in the murine KPro model. Ocular surface inflammation may reflect inflammatory processes of KPro glaucoma.

Keywords: Boston keratoprosthesis, glaucoma, inflammation, tear fluid, cytokines

The Boston keratoprosthesis type 1 (KPro) is the most frequently used artificial cornea worldwide, and it rapidly restores vision in patients with corneal blindness.[1,2] The most common KPro design consists of front and back plates made of polymethyl methacrylate (PMMA), with a donor cornea placed in between the plates.[3] Despite many advances in implant design and management of postoperative complications, glaucoma is the most important threat to vision after KPro implantation. Glaucoma prevalence is reported to be 36% to 76% before KPro, and identified de novo in 8% to 75% of eyes after KPro.[4–6] Unfortunately, monitoring and controlling glaucoma is extremely challenging in KPro eyes.[7] Intraocular pressure (IOP) measurements are approximated by digital palpation by experienced practitioners,[1,8,9] and optic nerve and visual field evaluations can be hindered by concomitant media opacities after KPro.

The pathogenesis of glaucoma after KPro surgery is multifactorial. Postoperative IOP elevation is one of the main risk factors of glaucoma after KPro.[10] The presence of peripheral anterior synechiae has been reported by anterior segment optical coherence tomography (AS-OCT) and proposed to cause progressive angle closure and obstruction of aqueous flow.[5,11–13] Recent animal studies have suggested that inflammation might be one of the main

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IOP-independent causes, induced by the KPro device protruding in the anterior chamber, and diffusing to the retina, causing inevitable retinal ganglion cell (RGC) apoptosis.[14–17] Cytokines mediating immune and inflammatory responses play an important role in the process of glaucomatous optic neuropathy[18] and may play an important role in KPro-associated glaucoma, but this has not been thoroughly investigated. Increased expression of proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β have been shown in a miniature KPro murine model.[15,19] Our team also showed that soluble TNF-α is a major culprit in RGC death in glaucoma and that TNF-α inhibition is beneficial.[20] However, neither the exact mechanism of KPro glaucoma nor the role of cytokines has been elucidated yet in humans.

Lack of biointegration between the KPro front plate polymethyl methacrylate (PMMA) material and the corneal graft allows for some communication between the ocular surface and intraocular media and warrants lifelong antibiotic prophylaxis to avoid endophthalmitis.[21–23] Tear fluid is readily accessible in patients, and tear cytokines have been found to correlate with intraocular aqueous inflammation and clinical parameters in ophthalmological diseases, such as open angle glaucoma and bullous keratopathy.[23–27] Cytokines have become reliable biomarkers of ocular surface diseases.[28,29] Primary open and closed angle glaucoma have been associated with elevated levels of cytokines in tears and aqueous humor involved in inflammation, namely IL-1β, IL-6, IL-8, interferon (IFN)-γ, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, TNF-α, and vascular endothelial growth factor (VEGF), which in turn have been associated with increased IOP and suspected to have a role in RGC death and the pathogenesis of glaucoma.[30–42] Nonetheless, no studies have yet investigated the composition of ocular fluids, including tears, of KPro patients with glaucoma, possibly because of the KPro patient population not being as large as other ophthalmic patient populations, as well as the technical challenge of small tear volumes in general.

The purpose of this study was to compare prospectively the tear cytokine profile of KPro patients with and without glaucoma, relative to healthy controls, and to correlate these cytokine biomarkers with clinical glaucoma parameters. We hypothesized that inflammatory cytokine concentrations would be elevated in KPro eyes with glaucoma, with the goal of being used as biomarkers for rapid diagnosis and potential treatments in the near future. To the best of our knowledge, this is the first study in humans investigating the association of glaucoma after KPro implantation and the cytokine concentrations in ocular fluid (tear), shedding light on potential biomarkers and treatment pathways.

Methods

Participants

This prospective study received ethical approval from the institutional review board of the Centre hospitalier de l'Université de Montréal (CHUM; CE20.004) and adhered to the tenets of the Declaration of Helsinki. The study was registered at www.ClinicalTrials.gov (NCT04339907). Written informed consent was obtained from all participants.

Patients having undergone KPro surgery performed by a single surgeon (M. H.-D.) between 2008 and 2019 were recruited between May 2020 and September 2020 when they presented in a consecutive fashion for visits at the Centre hospitalier de l'Université de Montréal (average age 67.2 years). Patients with KPro were contacted from our surgeon's database and requested to come in for their regular follow-up visits. In addition, they were invited to participate in this study. Of 55 patients called, 52 came for their visit. Of 48 patients invited to this study because they met inclusion criteria, all patients agreed. 3 patients declined visit because they lived in rural areas and did not have access to transportation to our hospital, and no patient declined the study. The study also enrolled healthy controls (average age 68.0 years). We attempted successfully to match the KPro patients with glaucoma, without glaucoma, and the controls with regards to sex and age. KPro patients with glaucoma were defined as such if they had a clear history of glaucoma, if they were taking IOP-lowering medications, or if they had undergone previous glaucoma surgery. All of the patients who received a KPro since 2008 at our institution, who never developed glaucoma, and who met the inclusion criteria were recruited as nonglaucoma KPro patients. All KPro patients typically had the same topical steroids regimen. After KPro surgery, patients were prescribed topical prednisolone acetate 1% (Sandoz, Boucherville, Canada) and topical moxifloxacin 0.5% (Vigamox; Alcon, Mississauga, Canada) ophthalmic drops four times daily. Prednisolone was subsequently tapered to once daily, to be used indefinitely.

Inclusion criteria included age of 18 years old or above, KPro surgery having occurred at least one year ago, eyes with a KPro and a stable postoperative period of at least 6 months, and non-phthisic eyes with best-corrected visual acuity (BCVA) better than or equal to light perception. A stable postoperative period was defined by absence of other surgery, additional drops or new complications in the last six months. Exclusion criteria included any intraocular surgery in the last 12 months, a history of systemic inflammatory, immunological, or cardiovascular diseases (other than hypertension and hyperlipidemia), as well as a history of uveitis, phthisis bulbi, and extrusion or explant of KPro. Eyes with active inflammation of the cornea and anterior chamber, and a preoperative diagnosis of ocular cicatricial pemphigoid, Stevens-Johnson syndrome, and keratoconjunctivitis were excluded; previous studies showed that proinflammatory tear cytokine levels are elevated in these ocular surface diseases.[43–45] No patient had moderate or severe dry eye diseases needing dry eye treatment.

Clinical Examination

Routine examinations, including slit-lamp evaluation, BCVA, IOP, visual field, spectral domain optical coherence tomography (OCT) and AS-OCT were performed in KPro patients on the same day when tears were collected. The Snellen chart was used to measure BCVA, which was converted into the logarithm of minimum angle of resolution (LogMAR). Extrapolated values of 2.0, 2.3, 2.6 represented BCVA of counting fingers, hand motion, and light perception, respectively.[46,47] IOP was measured by digital palpation by a single experienced clinician (M.H.-D.) by using scleral indentation felt by the finger and then assessing the IOP. The mean of an estimated range was recorded, as commonly performed in KPro eyes.[1,7,9] Visual field was assessed using the Humphrey Visual Field Analyzer (Carl Zeiss, Dublin, CA, USA) using the 24-2 program, or by Automated Goldmann Octopus 900 (Haag-Streit AG, Koeniz, Switzerland) if known history of poor visual field. Visual field indices were obtained from the Humphrey Visual Field when reliable, and reliability criteria

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were established as less than 20% fixation losses, less than 33% false-negative error, and less than 33% false-positive error, as recommended by Humphrey Instruments.[48] The retinal nerve fiber layer (RNFL) thickness and optic nerve excavation (cup-to-disk ratio [CDR]) were assessed by OCT with the Cirrus HD-OCT 5000 (Carl Zeiss, Dublin, CA, USA) when deemed reliable if no membranes or opacities interfered and if signal strength was equal or above 6/10, as recommended by Zeiss.[49] AS-OCT of the anterior chamber was performed to observe the iridocorneal angle with the anterior chamber lens adapter (Carl Zeiss) used in the wide-angle mode, when possible if no opacities interfered.[50] The four quadrants (inferior, superior, nasal, and temporal) were imaged in primary gaze position. The AS-OCT definition of angle closure is contact between the iris and structures anterior to the scleral spur, using the identification of the scleral spur as a landmark for the angle measurement.[12,50–52] Visual field, OCT, and AS-OCT scans were analyzed by an experienced glaucoma specialist (Y.A.). Gonioscopy is impossible in KPro patients because of peripheral cornea opacification, retroprosthetic membrane and KPro front plate.[12]

Tear Collection

Tear collection was conducted at the beginning of the scheduled clinic visits, in the afternoon (between 1 and 4 PM) to control for diurnal variation,[53] and before instillation of any eye drops, as described previously.[23,24,26,27,54] Briefly, 60 µL of sterile saline solution were instilled into the inferior fornix by using a sterile micropipette. Afterwards, subjects were asked to look left, right, up, and down, three times without blinking, to mix the tear fluid content. Next, the diluted tears with sterile saline solution were collected from the inferior fornix using the micropipette and transferred to an Eppendorf tube, kept on ice during collection. Samples were centrifuged at 10,000*g* at 4˚C for 15 minutes to eliminate mucoid and cellular debris, as per immunoassay manufacturer's recommendations. Soluble fractions were stored at −150˚C in individual sterile screw-cap polypropylene tubes until analysis.

Tear Cytokine Protein Levels

We selected a panel of inflammatory cytokines involved in immune and inflammatory responses and which have been investigated and observed in glaucoma in several prior reports.[32–36,38–42] The cytokine levels of IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, interferon gamma-induced protein (IP)-10, MCP-1, MIP-1α, MIP-1β, platelet-derived growth factor (PDGF)-AB/BB, regulated on activation, normal T cell expressed and secreted (RANTES), TNF-α, VEGF, fibroblast growth factor (FGF)-basic, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IFN-γ were measured using a 27-plex Luminex magnetic bead-based multiplex immunoassay (Milliplex Human Cytokine Panel A [HCYTA-60K-27]; EMD Millipore, Merck, MA, USA) at Princess Margaret Genomics Centre, Toronto, Canada (www.pmgenomics.ca). This assay has been validated for human tears in several prior reports.[28,55–60] The assay was performed according to the manufacturer's instructions, in a masked fashion. Briefly, 25 µL of tear fluid were dispensed into defined wells of the 96-well plate. Sample and antibody-coated capture beads were mixed and incubated at 4 °C for 18 hours (protected from light). Washed beads were further incubated with biotin-labeled anti-human cytokine antibodies, followed by streptavidin-phycoerythrin incubation. Samples were analyzed using a Luminex 200 instrument (Luminex Corporation, Austin, TX, USA). Each sample was run as a single measurement because of the limited quantity of collected tear fluid. The median fluorescent intensity data was analyzed using a five-parameter logistic curve-fitting method. Standard curves of known concentrations of recombinant human cytokines were used to convert fluorescence units to concentrations (pg/mL). The percentage of samples with detected cytokines was calculated.[24]

Statistical Analysis

Statistical analyses were performed using SPSS version 25.0 (IBM, Chicago, IL, USA) and Prism version 8.2.1 (GraphPad, San Diego, CA, USA). Statistical significance was defined as $P < 0.05$, and data were presented as the mean ± standard deviation (SD). Normality assumption was assessed by the Shapiro-Wilk test. To compare categorical variables, the χ^2 test was used. To compare continuous variables, a two-tailed Student's *t*-test for independent samples or the Mann-Whitney *U* test were used, as appropriate. The ANOVA and Kruskal-Wallis test were used to compare more than two groups, as appropriate. To assess homogeneity of variances, the Brown-Forsythe test was used. Welch's *t*-test was used when there was significant heterogeneity of variance. The Dunnett T3 multiple comparison test, recommended for groups with n < 50, was used to correct for multiple comparisons using statistical hypothesis testing. In cases where the amount of the studied molecule was under the limit of detection, the number 0 was assigned for the concentration of the analyte.[61] Molecules that were detected in less than 60% of the samples in the groups were not further statistically compared. Spearman's correlation analysis was used to correlate cytokine levels and numerical clinical parameters. For the strength of the correlation coefficient, 0.20 to 0.39 was regarded as weak, 0.40 to 0.59 as moderate, and 0.60 to 0.79 as strong.[62]

Results

Patients Demographics

Tear fluid samples were obtained from a total of 58 patients: 41 KPro with glaucoma, 7 KPro without glaucoma, and 10 healthy controls. Average age of the cohort was 67.2 years old, with 26 (54.2%) being of male sex. The mean time from KPro surgery to tear fluid sampling was 7.9 ± 3.5 years. Of the 156 eyes of 135 KPro patients operated for KPro surgery at our center between 2008 and 2019, the maximum number of KPro patients without any glaucoma (n = 7) was recruited from this population for this study. All of the KPro patients were aphakic. None of the subjects had active inflammation of the cornea or anterior chamber based on slit-lamp examination. Baseline characteristics of study participants are listed in Table 1. Age (range, 29.1–90.9 years old) and sex distribution was similar between the three groups ($P > 0.05$).

Clinical Parameters

In KPro eyes, clinical parameters were compared between those with and without glaucoma (Table 2). IOP was significantly higher in the KPro group with glaucoma compared

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Table 1. Baseline Characteristics of Study Patients

	KPro Without Glaucoma	KPro With Glaucoma	Control	*P* Value
Eyes, n	7	41	10	
Male sex, n (%)	5 (71)	21 (51)	6 (60)	0.577
Age (y), mean ± SD	69.2 ± 16.3	66.8 ± 13.6	68.0 ± 14.9	0.806

Statistical significance between groups was determined using either the Kruskal-Wallis test or the χ^2 test for continuous or categorical variables, respectively.

KPro, Boston keratoprosthesis.

Table 2. Clinical Parameters of Study KPro Eyes

	KPro Without Glaucoma	KPro With Glaucoma	*P* Value
Years from KPro until sampling, mean ± SD	9.6 ± 1.9	7.6 ± 3.7	0.267
Eyes with prior corneal graft, n (%)	4 (57)	20 (49)	0.683
Number of prior corneal grafts, mean ± SD	0.71 ± 0.76	1.1 ± 1.4	0.473
Preoperative diagnosis, n (%)			
Corneal endothelial disorders	1 (14)	8 (20)	0.743
Trauma	1 (14)	3 (7)	0.538
Corneal scar	4 (57)	15 (37)	0.304
Aniridic keratopathy	1 (14)	13 (32)	0.349
Other congenital anterior segment disorders	0	2 (5)	0.551
Eyes using drops, n (%)			
Lubricant	5 (72)	19 (46)	0.220
NSAID	0	2 (5)	0.551
Quinolone antibiotics	7 (100)	37 (90)	0.388
Steroid	7 (100)	40 (98)	0.676
Eyes with glaucoma surgery, n (%)			
Cyclophotocoagulation	0	19 (46)	**0.033**
Glaucoma drainage device	0	23 (56)	**0.010**
Trabeculectomy	0	5 (12)	1.000
Number of glaucoma drops, mean ± SD	0	3.0 ± 1.3	**0.00003**
Eyes using glaucoma drops, n (%)			
Prostaglandin analog	0	32 (78)	**0.00005**
Beta blocker	0	31 (76)	**0.0001**
Carbonic anhydrase inhibitor	0	23 (56)	**0.006**
Cholinergic agonist	0	3 (7)	0.460
Alpha agonist	0	22 (54)	**0.008**
BCVA, LogMAR, mean ± SD	1.39 ± 0.78	1.17 ± 0.80	0.509
IOP, mmHg, mean ± SD	11 ± 5	16 ± 5	**0.015**
Cup-to-disk ratio, mean ± SD	0.44 ± 0.25	0.65 ± 0.20	**0.015**
Humphrey visual field indexes, mean ± SD[*]			
MD, db	−10.91 ± 4.56	−15.25 ± 10.59	0.520
PSD, db	5.43 ± 2.29	5.86 ± 2.96	0.828
VFI, %	78.67 ± 14.01	67.17 ± 36.45	0.519
RNFL thickness, µm[†]	78.0 ± 2.83	72.19 ± 15.42	0.608
Closed angle, n (%)[‡]	6 (100)	29 (88)	0.368
Open angle, n (%)[‡]	0	4 (12)	

Statistical significance between groups was determined for continuous variables using either the Student *t*-test or Mann-Whitney U test, or for categorical variables using the χ^2 test. Statistically significant differences are indicated in bold font. BCVA, best-corrected visual acuity; IOP, intraocular pressure; KPro, Boston keratoprosthesis; MD, mean deviation; NSAID, nonsteroidal anti-inflammatory drug; PSD, pattern standard deviation; RNFL, retinal nerve fiber layer; VFI, visual field index.

[*] Measurements were available for 21% of eyes.

[†] Measurements were available for 48% of eyes.

[‡] Measurements were available for 81% of eyes.

to the one without glaucoma (16 mm Hg vs. 11 mm Hg, $P = 0.015$). Optic nerve excavation measured by the CDR was also significantly increased in the KPro group with glaucoma compared to the one without glaucoma (0.65 vs. 0.44, $P = 0.015$). Both groups were similar in terms of time elapsed between KPro surgery and sampling, number of eyes with prior corneal graft, number of prior corneal grafts, and preoperative diagnosis. KPro eyes with glaucoma had a history of prior glaucoma surgery: 46% of eyes had undergone cyclophotocoagulation, 56% had undergone glaucoma drainage device implantation, and 12% had undergone trabeculectomy. The use of drops including lubricant, nonsteroidal anti-inflammatory drugs (NSAID), quinolone antibiotics, and steroid drops was similar between groups, because KPro patients are typically on the same topical antibiotic and steroid regimen for life (all, $P > 0.05$). The differences between groups in the use of topical drops were related to the glaucoma medications.

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Table 3. Cytokine Concentrations in Tear Fluid of KPro and Control Eyes

Cytokine (Measured % Samples Per Group)	KPro Without Glaucoma	KPro With Glaucoma	Control	*P* Value*	*P* Value†	*P* Value‡
Eotaxin (100, 98, 100)	15.99 ± 3.67	19.85 ± 8.74	15.61 ± 4.67	0.169	0.997	0.127
FGF-basic (100, 100, 100)	35.52 ± 10.47	59.40 ± 46.41	61.21 ± 28.86	**0.018**	0.067	0.998
G-CSF (100, 90, 90)	154.99 ± 163.44	193.31 ± 440.56	80.15 ± 91.93	0.691	0.519	0.402
GM-CSF (0, 0, 0)	0	0	0	—	—	—
IFN-γ (86, 78, 100)	2.09 ± 1.48	6.61 ± 9.47	3.13 ± 2.36	**0.043**	0.635	0.166
IL-1β (100, 98, 100)	6.43 ± 2.37	12.97 ± 11.72	11.74 ± 5.85	**0.008**	0.064	0.952
IL-1Ra (100, 100, 100)	4875.71 ± 4886.68	4058.59 ± 3169.37	1028.62 ± 892.84	0.682	0.159	**<0.001**
IL-2 (43, 54, 50)	0.42 ± 0.27	1.02 ± 1.43	0.22 ± 0.13	0.188	0.324	0.051
IL-4 (29, 32, 50)	0.50 ± 0.16	2.76 ± 3.88	0.81 ± 0.40	0.165	0.199	0.187
IL-5 (100, 100, 100)	2.13 ± 2.78	2.64 ± 3.32	1.68 ± 0.87	0.892	0.892	0.289
IL-6 (100, 100, 100)	17.64 ± 11.40	31.91 ± 83.04	12.78 ± 17.67	0.512	0.512	0.452
IL-7 (3, 14, 0)	7.91 ± 12.15	3.74 ± 3.92	0	—	—	—
IL-8 (100, 100, 100)	311.45 ± 386.17	486.42 ± 614.85	198.25 ± 260.28	0.560	0.560	0.084
IL-9 (29, 56, 70)	6.25 ± 0.96	16.46 ± 15.17	12.21 ± 5.16	**0.013**	0.063	0.591
IL-10 (100, 100, 100)	2.39 ± 1.47	19.26 ± 45.26	13.58 ± 13.71	0.064	0.084	0.869
IL-12p70 (0, 14, 0)	0	10.34 ± 12.74	0	—	—	—
IL-13 (43, 61, 70)	2.10 ± 2.14	14.62 ± 19.71	9.14 ± 6.45	**0.016**	0.089	0.563
IL-15 (100, 100, 100)	5.37 ± 3.91	7.40 ± 4.53	4.58 ± 2.40	0.434	0.645	**0.034**
IL17A (86, 93, 90)	2.99 ± 1.68	6.48 ± 8.17	5.74 ± 2.87	0.069	0.100	0.956
IP-10 (100, 100, 100)	7802.66 ± 6542.06	9404.64 ± 6324.43	5362.31 ± 4312.56	0.069	0.071	0.651
MCP-1 (100, 100, 100)	274.92 ± 338.03	236.77 ± 366.75	81.81 ± 105.78	0.792	0.341	0.070
MIP-1α (14, 24, 20)	15.08 ± 0.1	37.86 ± 26.13	7.81 ± 4.79	>0.999	>0.999	0.190
MIP-1β (86, 90, 100)	8.83 ± 13.62	10.18 ± 15.35	10.13 ± 14.74	0.995	0.995	0.995
PDGF-AB/BB (0, 2, 0)	0	236.96 ± 176.36	0	—	—	—
TNF-α (100, 95, 100)	2.75 ± 0.95	5.75 ± 6.19	4.42 ± 3.23	**0.020**	0.371	0.729
VEGF (100, 100, 100)	189.44 ± 166.74	225.49 ± 211.47	54.14 ± 30.91	0.940	0.195	**<0.001**
RANTES (100, 100, 100)	7.86 ± 4.00	9.14 ± 7.98	4.07 ± 1.42	0.522	0.092	**0.001**

Cytokine concentrations (measured in a percentage (%) of samples in each group named consecutively) are expressed in pg/mL as mean ± SD. Concentrations under the limit of detection were assigned the number 0. Statistically significant differences between groups are highlighted in bold.

G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IP, interferon gamma-induced protein; KPro, Boston keratoprosthesis; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cells expressed and secreted.

* Compared between KPro eyes with glaucoma and without glaucoma, Brown-Forsythe and Welch ANOVA test with multiple comparisons.
† Compared between KPro eyes without glaucoma and control group, Brown-Forsythe and Welch ANOVA test with multiple comparisons.
‡ Compared between KPro eyes with glaucoma and control group, Brown-Forsythe and Welch ANOVA test with multiple comparisons.

The KPro glaucoma group used on average 3.0 ± 1.3 glaucoma drops, distributed among five classes including prostaglandin analog, beta blocker, carbonic anhydrase inhibitor, alpha agonist, and cholinergic agonist. No glaucoma drops were used in the KPro without glaucoma group, by definition. Both groups had similar remaining ophthalmological parameters reported ($P > 0.05$).

Cytokine Levels in Tear Fluid

A total of 27 cytokines were analyzed in tear fluid from KPro patients and controls. Mean cytokine concentrations for each group with related *P* values for all comparisons between groups are listed in Table 3. The concentrations of four cytokines (TNF-α, IL-1β, FGF-basic, IFN-γ) were significantly higher in the KPro with glaucoma group compared to KPro without glaucoma ($P = 0.020$; 0.008; 0.043; 0.018, respectively). Of these, IFN-γ showed the highest increase in concentration (6.61 vs. 2.09 pg/mL, thus 3.2-fold) in KPro patients with glaucoma relative to KPro patients without glaucoma. Also, the concentrations of four other cytokines (IL-1Ra, IL-15, VEGF, RANTES) were significantly higher in the KPro with glaucoma group compared to healthy controls (P <0.001; =0.034; <0.001; =0.001, respectively). Of these, VEGF showed the highest increase in concentration (225.49 vs. 54.14 pg/mL, 4.2-fold). There was no statistically significant difference in cytokine concentrations between KPro without glaucoma and controls (all, $P > 0.05$), suggesting strongly that our positive cytokines play a role in glaucoma. In total, 19 cytokines were not associated with an increase or decrease in our study and appear to be noncontributory to the development of glaucoma.

Furthermore, subgroup analyses were performed to compare cytokine concentration data between different factors among glaucomatous KPro eyes. Cytokine levels were not significantly different between the five preoperative KPro diagnosis categories (all $P > 0.05$; for details see Supplementary Table S1). Cytokine data comparing eyes with and without use of topical glaucoma medications was also analyzed. With regards to the cytokines that were previously identified to be of interest (TNF-α, IL-1β, IFN-γ, FGF-basic, IL-1Ra, IL-15, VEGF, and RANTES), no significant differences were shown between eyes with and without use of topical prostaglandin analog, beta blocker, carbonic anhydrase inhibitor, cholinergic agonist, alpha agonist, and steroid (for details, see Supplementary Tables S2 to S7, respectively). The only statistically significant differences in cytokines levels for those comparisons were G-CSF elevated in eyes with prostaglandin analog and beta blocker drops, MCP-1 elevated in eyes with beta blocker

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Table 4. Correlation Analysis Between Levels of Four Selected Cytokines and Glaucoma Parameters in KPro Eyes

	FGF-Basic		IFN-γ		IL-1β		TNF-α	
Glaucoma Parameter	***r***	***P* Value**	***r***	***P* Value**	***r***	***P* Value**	***r***	***P* Value**
Cup-to-disk ratio	0.344	0.021	0.452	0.006	0.309	0.039	0.348	0.022
Intraocular pressure			0.368	0.023	0.292	0.047		

Data are expressed as correlation coefficient r and *P*-value.
Only significant correlations with clinical parameters are shown.

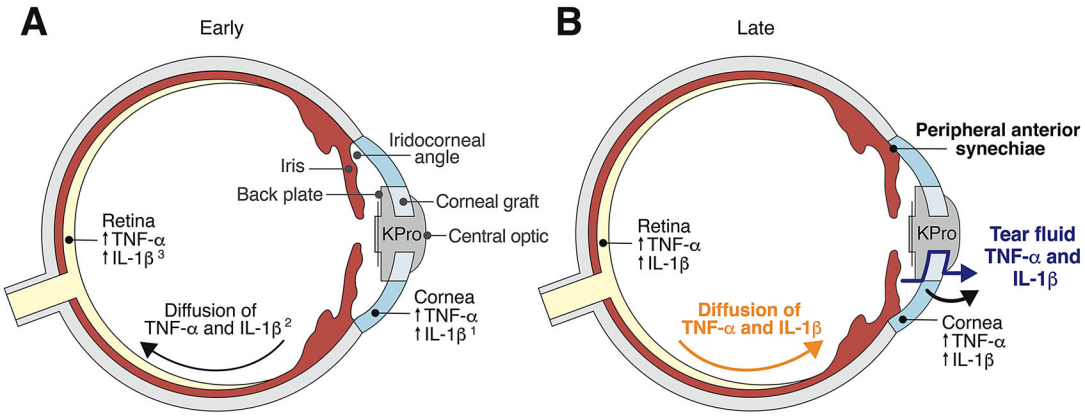

Figure. Proposed diagrammatic illustration of glaucomatous inflammation in an aphakic eye implanted with a Boston keratoprosthesis type 1 (KPro). (**A**) The KPro has been shown by Crnej et al.[15] to induce overexpression of TNF-α and IL-1β in the cornea (1). TNF-α and IL-1β diffuse from the anterior segment to the posterior segment (2), as suggested by Chen et al.[16] TNF-α and IL-1β are overexpressed in the retina by infiltrating monocytes/macrophages (3), resulting in ganglion cell apoptosis and glaucomatous damage.[19] (**B**) Later, the cornea and engrafted monocytes/macrophages may continue to overexpress TNF-α and IL-1β, which could diffuse to the anterior chamber. The inflammation could also provoke peripheral anterior synechiae formation, obstructing the iridocorneal angle. With lack of biointegration between the KPro device and the corneal graft, TNF-α and IL-1β could leak out from the anterior chamber into the tear fluid on the ocular surface.

drops, and IP-10 elevated in eyes with cholinergic agonist drops (Supplementary Tables S2, S3, and S5, respectively). Moreover, cytokine data comparing eyes with and without history of incisional glaucoma surgery, which included glaucoma drainage devices and trabeculectomy, did not show significant differences for any of the cytokines of interest; only IL-10 was significantly lower in eyes with prior incisional glaucoma surgery (Supplementary Table S8). In addition, when comparing KPro eyes with open angle to those with closed angle, levels of G-CSF ($P = 0.029$) and RANTES ($P = 0.027$) were significantly lower in eyes with angle closure (Supplementary Table S9). Overall, data for TNF-α, IL-1β, IFN-γ, FGF-basic, IL-1Ra, IL-15, VEGF, and RANTES did not demonstrate significant associations with the confounding factors of ocular drops, preoperative diagnosis, and history of glaucoma surgery studied herein.

Correlation Between Cytokine Levels and Clinical Parameters in KPro Eyes

We then hypothesized that the inflammatory cytokines may also be associated to the clinical parameters of glaucoma in KPro eyes. The four cytokines that were significantly elevated in more than 60% of samples and between KPro eyes with and without glaucoma were integrated in correlation analyses. These included FGF-basic, IFN-γ, IL-1β, and TNF-α. Correlation analysis was performed between cytokine levels and CDR, IOP, number of glaucoma drops, BCVA, visual field indices (mean deviation, pattern standard deviation, visual field index), RNFL, number of failed grafts, and time to sampling. CDR and IOP were the two parameters related to glaucoma that yielded significant correlations with tear cytokine concentrations. The other clinical measures were not correlated with cytokine elevations. Significant correlations are listed in Table 4 (see Supplementary Figure S1 for scatter plots of correlations). The concentrations of IL-1β and IFN-γ levels were significantly positively correlated with CDR (Figure; $r = 0.309$, $P = 0.039$ and $r = 0.452$, $P = 0.006$, respectively) and IOP ($r = 0.292$, $P = 0.047$ and $r = 0.368$, $P = 0.023$, respectively). The concentrations of TNF-α and FGF-basic levels were significantly positively correlated with CDR (Figure; $r = 0.348$, $P = 0.022$ and $r = 0.344$, $P = 0.021$, respectively).

Discussion

Glaucoma development and progression is one of the most frequent and devastating complications for the vision of KPro patients. The prevalence of glaucoma is particularly high after KPro surgery, but monitoring tools and IOP-lowering medications and surgeries remain insufficient to control glaucoma progression that occurs in the majority of KPro patients.[5,7] Increased IOP after KPro surgery is a known risk factor for postoperative glaucoma, but in cases without IOP elevation, other mechanisms have been suggested. Neuroinflammation has been proposed to cause glaucoma by RGC apoptosis, either directly or by the intermediate of infiltrating immune cells in KPro

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eyes.[15,19] There is evidence of TNF-α, IL-1β and other inflammatory cytokines causing RGC apoptosis and being involved in glaucoma pathogenesis in both humans and animal models.[18,20,31,63–65] In this regard, there is increasing need to understand specifically why glaucoma develops and progresses so dramatically.

The inflammatory process can be reliably monitored in ocular fluids such as aqueous humor and the tear film in a variety of ophthalmic pathologies by quantifying cytokine concentrations.[28,29,37] Several studies have successfully examined in glaucoma and other ocular diseases the composition of tear fluid, which is the most readily accessible ocular fluid, by using multiplex bead immunoassays to quantify multiple protein targets despite the concomitant use of ocular drop.[23–27] Indeed, tear cytokines have been shown to be representative of aqueous humor cytokines, for example IL-4 and IL-8 in bullous keratopathy and ciliary neurotrophic factor (CNTF) in open-angle glaucoma.[24,25] Collection of tear fluid is less invasive compared to collection of aqueous humor, which can be performed in KPro patients only if they present for intraocular surgery in the operating room. Otherwise, aqueous humor collection done in those patients without any concurrent surgical necessity or indication could lead to consequences such as endophthalmitis. For these reasons, analyzing tear fluid is a valuable way to investigate glaucoma pathogenesis in KPro patients. Tear matrix metalloproteinases levels have been associated with underlying ocular surface pathology in tear fluid of KPro patients.[23] Although one study has reported elevated systemic blood levels of TNF-α in KPro patients,[66] there is no report in the literature, to the best of our knowledge, on inflammatory cytokine composition of any ocular fluid in KPro patients, with or without glaucoma. The study presented herein is clinically significant because identifying inflammatory cytokines of KPro glaucoma would inform the possibility to predict glaucoma progression from tear cytokine levels and suggest pathways for treatment interventions.

In the current study, we compared the differences in inflammatory cytokine concentrations in KPro eyes with and without glaucoma because we hypothesized that inflammation contributes to glaucoma development and progression after KPro. Our cohort of KPro patients is one of the largest in North America, thus our population is particularly unique and valuable. We chose the 27 cytokines investigated in this study because they are the ones mediating immune and inflammatory responses mostly involved and observed in glaucoma studies. We simultaneously determined the concentration of 27 cytokines using multiplex immunoassay, thus eliminating inter-assay variability. We successfully sex- and age-matched KPro patients and controls. The two KPro patient groups were also matched for baseline characteristics, pre-KPro diagnoses, and topical antibiotic and steroid regimen. We observed that KPro eyes with glaucoma had different concentrations of specific cytokines in tears. The cytokine profile of KPro patients with glaucoma revealed a significant increase in TNF-α, IL-1β, IFN-ɣ, FGF-basic, IL-1Ra, IL-15, VEGF, and RANTES, without any observed associations with confounding factors, which included the use of glaucoma eye drops, preoperative diagnosis, and previous glaucoma surgeries. We demonstrated that the tear levels of specific cytokines, IL-1β, TNF-α, IFN-ɣ, and FGF-basic, were positively correlated with CDR and IOP in KPro eyes. We did not expect particularly strong correlations given the heterogeneous postoperative course of KPro patients having different time periods since surgery and since glaucoma diagnosis, which are important variables related to cytokine concentrations. IOP measurements by digital palpation and rates of CDR progression may have also been different. Nonetheless, the observed correlations that were in general weak are statistically and clinically significant. Interestingly, cytokine variations in KPro eyes without glaucoma were not statistically different than those of healthy controls, suggesting that the KPro device alone does not contribute to chronic ocular surface inflammation many years after KPro implantation. The observed differences in cytokine levels between KPro eyes with and without glaucoma are, hence, statistically associated to glaucoma in KPro eyes.

In glaucoma, several inflammatory cytokines have been found to be associated with increased IOP, RGC apoptosis, and visual field defects, suggesting a plausible role in its pathogenesis. In open-angle glaucoma, increased levels of IL-1β, IL-6, IL-8, IL-10, MCP-1, MIP-1β, FGF-basic, VEGF, IL-12, IL-15, IFN-ɣ and TNF-α compared to controls have been observed in the aqueous humor and associated with increased RGC death and IOP rise.[30–33,35,37,38,42] In comparison, patients with chronic angle closure glaucoma have shown increased levels of IP-10 and VEGF, and lower levels of MCP-1, TNF-α, and GM-CSF.[40,41] In our study, IOP elevation was associated with increased concentrations of IFN-ɣ and IL-1β in tear fluid, also significantly correlated with CDR. Although some cytokines and chemokines have been correlated to visual field loss,[37] we were not able to highlight correlations between cytokine concentrations and visual field indices. KPro eyes with angle closure displayed lower concentrations of G-CSF and RANTES compared to those with open angle. In fact, although RANTES has not been associated to angle closure in previous reports, G-CSF has been observed to be elevated in acute primary angle-closure compared to cataracts[67] and to fellow eyes with primary angle closure suspicion.[68] In addition to being known to be associated with elevated IOP, our team has shown that TNF-α is involved in RGC death in glaucoma and its inhibition can prevent RGC apoptosis.[20,69] Although TNF-α levels did not correlate with IOP elevation in our study, they did correlate with optic nerve excavation (CDR), with a significant twofold increase for KPro eyes with glaucoma compared to those without. In addition, the fact that TNF-α correlates with CDR but not IOP may support the IOP-independent pathway theory of glaucoma.[17] Because our results relate to tear fluid composition, we intend to investigate further the specific cytokines secreted in the aqueous humor of KPro eyes.

Inflammatory cytokines involved in glaucoma and various ocular diseases are often intrinsically linked with common cellular pathways. The current study showed that TNF-α, IL-1β, FGF-basic, and IFN-ɣ were elevated in KPro tears with glaucoma compared to those without glaucoma. In many neurodegenerative disorders, monocytes, macrophages and microglia involved in retinal damage secrete TNF-α, IL-1β, and IL-6, which overlap in their signaling pathways namely through signal transducer and activator of transcription (STAT) 3 protein.[70] TNF-α acts in an upstream fashion of IL-1β, as supported by TNF-α inhibition in alkali burn mice leading to IL-1β suppression.[14,70] TNF-α and IL-1β were found in both aqueous humor and tears in noninfectious anterior uveitis, where TNF-α amplified inflammation through a positive feedback loop.[71,72] IFN-γ has cellular pathways that interact with those of IL-6 and STAT3, through STAT1, suppressor of cytokine signaling (SOCS) 1 and SOCS3.[73,74] IFN-γ promotes proinflammatory

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responses in age-related macular degeneration by activating proinflammatory cytokines and recruiting immune cells such as macrophages and T cells.[75] Moreover, differences in the expression of growth factors like FGF-basic and VEGF are found in distinct ocular pathologies, such as neovascular glaucoma.[33,76,77] FGF-basic and CTNF have been shown to play an important role in macrophage production after optic nerve injury.[78] Other mechanisms for the significant differences observed herein may involve systemic inflammation. However, subjects in our study did not have any apparent systemic inflammatory disease nor diabetes. We compared the cytokine levels between preoperative diagnoses and confirmed that there were no significant differences between them. Further studies will be necessary to elucidate interactions between these cytokines.

In a mouse model of KPro implantation, it has been shown that mRNA expression of TNF-α and IL-1β is elevated in both the cornea and the retina of implanted eyes.[15,19] In our study, we identified significant elevation of TNF-α and IL-1β levels in tear fluid on the surface of the eyes of glaucoma KPro patients, as well as correlations between TNF-α and CDR, and between IL-1β, CDR, and IOP. We postulate the following mechanism (Figure). KPro implantation and allogeneic immunity of the corneal graft used for KPro assembly act as an acute insult to the cornea.[15] Early after KPro implantation, infiltrating immune cells in the cornea, as well as corneal epithelial cells and keratocytes, secrete TNF-α and IL-1β up to eight weeks after.[15] These cytokines diffuse from the anterior segment to the posterior segment and become overexpressed in the retina, provoking monocyte infiltration.[19] These monocytes/macrophages, as well as other immune cells in the retina, may in turn overexpress TNF-α and IL-1β, activating significant apoptosis signals, such as caspase 3 and endonuclease G, resulting in optic nerve degeneration.[14] If the damage to RGC is significant, it may later result in severe visual field defects and glaucoma.[79,80] Only a minority of patients are indeed spared from glaucoma after KPro surgery, as shown by our center's previous reports and by the small number of eligible patients to recruit for this group in our study.[6,81] Later, once the eye has healed and scarred, glaucoma mechanisms may be different. Our results provide valuable information regarding the chronic inflammation present. Monocytes/macrophages engrafted permanently in the retina continue overexpressing TNF-α and IL-1β for years after surgery, perpetuating retinal damage in glaucomatous eyes.[16,82,83] We postulate that the source of inflammation in glaucoma is intraocular, as suggested by prior reports.[16,17,82,83] The cornea may also continue secreting cytokines later on.[15] We consider that the immune cells residing in the retina mediate the correlations between tear cytokines, IOP elevation, and optic nerve excavation. We speculate that TNF-α and IL-1β diffuse from the posterior segment to the anterior segment, where they can make their way to the tear fluid through the optic-corneal button interface where biointegration is impossible (Figure).[21–23] Similarly, elevated VEGF concentrations in the aqueous humor of patients with diabetic retinopathy suggest diffusion from the retina to the anterior chamber.[84] In addition, inflammatory cytokines may act on corneal epithelial cells and keratocytes, which would then produce cytokines themselves.[15] Cytokines may also diffuse through the cornea with altered endothelium and epithelium barrier integrity and disrupted intercellular junctions under inflammatory conditions.[85,86] KPro implantation is most often performed in aphakic eyes. Hence, these commonly unicameral eyes would allow for even easier diffusion of mediators. As inflammation diffuses between intraocular compartments, formation of peripheral anterior synechiae can occur, closing the iridocorneal angle in a majority of patients, and also, sometimes, provoking elevation of IOP.[87] Taken together, the results of this study support previous reports of the contribution of TNF-α and IL-1β to retinal damage in glaucoma after KPro implantation. We conclude that glaucoma in KPro eyes, and not the KPro device alone, contributes to the observed ocular surface inflammation in KPro-implanted eyes.

Investigating the tear film cytokine profiles of KPro patients with and without glaucoma provides a precious opportunity to further our understanding of rapid glaucoma progression in KPro patients. Nevertheless, this study had some limitations. First, the heterogeneity of postoperative course between subjects, including glaucoma surgeries and cyclophotocoagulation interventions, might have caused some confounding, although preoperative characteristics were similar between groups and patients with systemic inflammatory diseases were excluded. Second, cytokine levels have been shown to be similar between eyes with and without use of topical steroids, quinolones, or NSAID drops in prior studies.[27,45,88–90] Reports have highlighted that cytokine concentrations are poorly affected by topical treatment and that glaucoma is truly an inflammatory pathology.[33,91] Nonetheless, the preservatives contained in glaucoma eye drops may cause ocular surface and anterior chamber inflammation and affect cytokine levels.[92–95] We could not control for glaucoma medication usage in glaucoma KPro patients because it was not possible to perform a previous washout of these drops due to ethical reasons, since patients were diagnosed with advanced glaucoma and required ongoing treatment. Furthermore, a washout period of one month, for example, would not guarantee the absence of cytokine concentration artefacts. Moreover, in agreement with our study, other research groups have not implemented a topical glaucoma treatment washout before tear sampling.[33,36] Third, a larger sample size for the groups of KPro patients without glaucoma and controls would be necessary to detect significant cytokine differences between these groups; still we attempted to recruit a maximum number of patients who had a KPro, who never developed glaucoma, and who met inclusion criteria. Fourth, we performed all routine clinical examinations for all patients included in this study to provide ancillary data to cytokine analysis, but OCT and visual field tests were only deemed reliable for some of the KPro patients. These tests are known to be more challenging to perform in KPro patients.[7] Reasons include that a large proportion of KPro patients at our center suffer from aniridia and often nystagmus as well. Most importantly, patients who underwent operation for KPro many years before tear sampling had more time for glaucoma progression and retroprosthetic membrane and media opacity formation. Hence, KPro patients often had more advanced glaucoma, as well as more membranes and opacities, and could not reliably undergo Humphrey 24-2 perimetry and OCT. This induced a bias, where reliable visual field and OCT data were strictly available for eyes with overall less-severe glaucoma or with no interfering membranes. Together with the small sample size ($n = 7$), OCT and visual field data could, hence, not be statistically differentiated from those of KPro eyes without glaucoma. Absence of glaucoma in the latter group was also validated by the average RNFL thickness being within KPro normal range of values of 71 to 101 µm[96] and by symmetry

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in OCT scans, considering that asymmetry is a hallmark of early glaucoma. Last, longitudinal studies with tear collection performed at the same postoperative times would be required to establish tear biomarkers related to glaucoma progression in KPro patients. Despite these limitations, our study was able to discern several important and significant data. In addition to pointing to several new cytokine and glaucoma correlations in KPro patients, we also identified correlations between cytokine levels and IOP and CDR which were significantly positive and clinically relevant.

In conclusion, we showed that TNF-α, IL-1β, FGF-basic and IFN-ɣ levels were elevated only in KPro eyes with glaucoma, and not in KPro eyes without glaucoma which had cytokine variations similar to those of normal eyes. These elevated cytokine levels correlated with IOP elevation and optic nerve excavation. Ocular surface inflammatory mediators may represent intraocular mechanisms of glaucoma damage. Tear fluid profile of KPro patients with glaucoma corroborates for the first time the reported elevation of TNF-α and IL-1β in preclinical models. This study provides novel insights into the mechanisms by which KPro surgery may lead to chronic inflammation and glaucoma in humans. Although cytokine levels may be variable in tear film, tear fluid is readily accessible compared to aqueous humor, and its cytokines are objectively quantifiable compared to IOP measured in KPro eyes. For these reasons, tear fluid cytokine analysis may become a useful tool in the future for glaucoma risk stratification of KPro patients, once validated in further studies. This new knowledge provides us with important and easily accessible biomarkers to follow disease progression in a more personalized fashion. Also, these data are crucial for suggesting disease pathways that may be amenable to the development of targeted therapies aimed at saving eyes from devastating consequences of glaucoma progression after KPro.

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