Cytokine and chemokine levels in tears and in corneal fibroblast cultures before and after excimer laser treatment

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PURPOSE: To measure multiple cytokine and chemokine production in tears of myopic patients before and after laser in situ keratomileusis (LASIK) and in human corneal fibroblast (HCF) cultures before and after excimer laser treatment.

SETTING: Department of Neuroscience, Ophthalmology Unit, University of Padua, Italy and Vissum-Instituto de Oftalmológico de Alicante, Alicante, Spain.

METHODS: Tear samples were obtained from 15 myopic patients before LASIK and 1 and 24 hours after LASIK. Quiescent HCF cultures were treated using the same laser energy. Culture medium was collected before treatment and after 1 and 24 hours. Cytokine concentrations were determined using multiplexed bead analysis.

RESULTS: Compared with baseline values, interleukin (IL)-12 tear levels were significantly increased 1 hour after surgery and eotaxin levels were significantly increased at 24 hours (both $P<.05$). Culture medium of HCF contained high levels of IL-6, IL-8, and monocyte chemotactic protein (MCP)-1 and low levels of IL-1, eotaxin, and regulated on activation, normal T expressed, and secreted (RANTES) cytokine. One hour after treatment, levels of all cytokines were significantly reduced. At 24 hours, IL-1, IL-6, IL-8, and MCP-1 levels were significantly increased compared with values at baseline and at 1 hour while RANTES cytokine and eotaxin levels had returned to baseline levels.

CONCLUSIONS: In vivo and in vitro studies showed that after excimer laser treatment, cytokines are released to modulate the wound-healing process; however, they can potentially induce inflammation. However, these types of in vitro studies, although useful for evaluating changes in cytokine profiles before and after treatment, only partially reproduce in vivo corneal behavior.

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Laser in situ keratomileusis (LASIK) is a relatively safe surgical procedure that involves creating a thin corneal flap with a microkeratome followed by excimer laser ablation of the stroma. This surgical procedure is considered relatively safe and is followed by low or mild inflammation in the 24 hours after surgery.[1] The cellular, molecular, and neural regulatory phenomena associated with postoperative inflammation and wound healing are likely to be involved in the adverse effects of LASIK such as flap melt, epithelial ingrowth, and regression. For these reasons, corticosteroid or nonsteroidal antiinflammatory agents are always used to minimize inflammation in the postoperative period.

The typical incisional wound of LASIK has been studied extensively.[2–5] However, the possible inflammatory mediators released in a lamellar intrastromal wound are not well known. Stimulated keratocytes can produce several chemokines that might initiate potentially severe corneal inflammation,[6–9] leading to corneal haze and other unsatisfactory sequelae.[1] Keratocyte activation induced by LASIK is of short duration compared with that reported after photorefractive keratectomy (PRK).[3] In a recent study, regardless of the method of flap formation, all corneas showed early transformed morphology of the keratocytes located below the flap.[10]

Several studies have focused on tear proteins such as cytokines, chemokines, and growth factors that are known to modulate wound healing, apoptosis, cell cycling and migration on the ocular surface in physiological, postsurgical, and pathological conditions.[11–17] In

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most of the studies, the limited tear-sample size restricted the analysis to the measurement of 1 or a few proteins. The multiplex bead assay is a highly sensitive quantitative analysis of a wide range of factors that offers several advantages for detecting cytokines in small sample volumes.[18] This technology has been used to study the diurnal rhythm of tear cytokines[19] and the effects of eye closure and tear reflex on their balance[20,21] and to analyze tear samples of patients with ocular allergy diseases.[22]

The aim of the present study was to measure a wide pattern of cytokines and chemokines in tears to evaluate whether a specific cytokine pattern is induced by LASIK in the early postoperative period. Proinflammatory cytokine, T helper (Th) 1-type and Th2-type cytokines, and chemokine production were measured in tears of myopic patients before and after LASIK. In addition, the same cytokine and chemokine pattern was measured in tissue culture medium of human corneal fibroblasts treated in vitro with excimer laser energy to determine whether the cytokine pattern is similar to that in tears.

PATIENTS AND METHODS

Patients

This study comprised myopic patients scheduled to have LASIK at Vissum-Instituto de Oftalmológico de Alicante, Spain. The study was approved by the local institutional review board.

Before LASIK, patients had a full ophthalmologic examination including manifest and cycloplegic refractions, determination of uncorrected and best corrected visual acuities, elevation computerized videokeratography, slitlamp biomicroscopy, Goldmann applanation tonometry, indirect ophthalmoscopy, and ultrasonic pachymetry.

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Surgical Technique

The same surgeon (J.A.) performed all LASIK procedures using the same standard surgical technique. The LASIK was performed with the Technolas 217-Z laser system using the PlanoScan algorithm (Bausch & Lomb). The target was full correction in all eyes. Antiseptic prophylaxis was performed by applying 1 drop of povidone–iodine 5% solution to the conjunctiva immediately before surgery. Lamellar keratotomy was performed using the Hansatome microkeratome (Bausch & Lomb).

After primary LASIK, no topical eyedrops were used for the first 24 hours. Because it is not ethical to leave patients without treatment, tear samples were collected only within the first 24 hours. After the examination at 1 day, topical tobramycin and dexamethasone eyedrops (TobraDex) were used every 6 hours for 1 week. Routine follow-up examinations were at 1 week and 1 and 3 months.

Tear-Sample Collection

Tear samples were collected from the treated eyes before LASIK and 1 and 24 hours after surgery. Using a glass capillary Pasteur micropipette, 20 to 50 μL of tears were gently collected in 5 minutes from the external canthus, avoiding the tear reflex as much as possible. These samples were placed in Eppendorf tubes and stored at −80°C for subsequent determination of cytokines and chemokines.

Cytokine Measurement

Twenty microliters of each tear sample was diluted with an equal volume of phosphate-buffered saline, 1% bovine serum albumin, and 0.05% Tween-20 (total volume 50 μL) and analyzed for the presence of interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, eotaxin, monocyte chemotactic protein (MCP)-1, and regulated on activation, normal T expressed, and secreted (RANTES) cytokine using multiplex bead analysis, which allows analysis of multiple molecules from each sample as previously described.[18,22] Briefly, samples were incubated with anticytokine antibody–coated capture beads for 2 hours at 20°C. Washed beads were further incubated with biotin-labeled anticytokine antibodies for 1 hour followed by streptavidin–phycoerythrin for 30 minutes (Upstate Biotechnology). Samples were analyzed using a Luminex 100 system. Standard curves of known concentrations of recombinant human cytokines were used to convert fluorescence units to cytokine concentration (pg/mL). Minimum detection levels (pg/mL) for each cytokine were as follows: 10 (IL-1β), 20 (IL-2), 5 (IL-4), 2 (IL-5), 1 (IL-6), 10 (IL-8), 2 (IL-10), 10 (IL-12), 2 (IL-13), 10 (TNF-α), 5 (IFN-γ), 2 (eotaxin), 10 (MCP-1), and 10 (RANTES).

A subjective clinical score (0 to 10) based on the severity of symptoms (pain, burning, foreign-body sensation) and signs (redness, corneal haze, lid swelling) was given 1 and 24 hours after LASIK.

Human Corneal Fibroblasts

Two human corneas were obtained from the local eye bank (Banca degli Occhi del Veneto, Mestre, Italy). The donors were 36-year-old and 51-year-old healthy white men whose corneas were considered suitable for transplant by the eye bank. The central 6.0 mm diameter portion was

excised, and the epithelial and endothelial cell layers were removed with a scalpel. The remaining stroma was cut in small pieces, seeded in 4-well plates (NUNC) containing 0.5 mL of Ham F12 medium (Sigma) supplemented with 20% fetal calf serum and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM/L L-glutamine), and incubated at 37°C in 5% carbon dioxide in a humidified atmosphere. Keratocytes were allowed to migrate from the explants. After reaching confluence, cells were subcultured with 0.05% trypsin and replated into 4-well plates (>95% vitality). The purity of the cell cultures was judged based on the typical morphology of corneal fibroblasts and their reactivity with antibodies to vimentin by immunofluorescence. No contamination with corneal epithelial cells was detected. Under these culture conditions, no cornea-derived dendritic cells were present. All experiments were performed using fifth- to eighth-passage corneal fibroblasts. All media and supplements were obtained from Sigma-Aldrich.

Cells were left to overgrow in the 4-well plates (>95% vitality) for 14 days; the tissue culture medium was changed twice a week. Twenty-four hours after the last replacement of culture medium, cells were treated with an excimer laser. Laser treatment was performed in 3 wells for each of the 2 corneal fibroblast cultures using the same laser energy. After 1 hour of treatment, the medium was removed, centrifuged, and stored at −20°C and then replaced with fresh medium for an additional 23 hours. Twenty-four hours after treatment, the culture medium was collected, centrifuged, and stored for further cytokine measurement. A cell count was performed in 1 of the 4-well plates for each culture before treatment and in the 3 treated wells 24 hours after treatment.

Cytokine and chemokine measurement was performed as described for tears without medium dilution. Data were expressed as mean values/10^6 cells.

Statistical Analysis

All cytokine and chemokine data sets were analyzed for normal distribution using the Kolmogorov-Smirnov normality test. Because the majority of the data sets were not normally distributed, the Friedman ranking test with the Dunn posttest was performed using GraphPad Prism for Mac (version 4.00, GraphPad Software). Correlations were calculated using the Spearman correlation coefficient (r). The minimal level of confidence at which the results were judged significant was $P < .05$.

RESULTS

The study comprised 15 patients with a mean spherical equivalent refraction of −4.7 diopters (D) ± 2.4 (SD) (range −1.0 to −10.0 D). Of the patients, 11 were women and 4 were men with a mean age of 31 ± 7 years.

Tear Cytokines and Chemokines Before and After Surgery

The only chemokine found consistently in tears before LASIK was IL-8. Compared with baseline levels, the MCP-1, RANTES, IL-8, and eotaxin tear levels were increased 24 hours after surgery in 5 patients, 6 patients, 8 patients, and 9 patients, respectively (Figure 1). Only eotaxin tear levels were statistically significantly higher ($P < .05$).

Of the Th1-type and Th2-type cytokines, the IL-4, IL-5, and IFN-γ tear levels were below detection limits of the assay in 11 patients. None of the cytokine tear levels was modified by LASIK treatment.

Of the inflammatory cytokines (IL-1β, IL-6, IL-12, TNF-α), IL-12 tear levels were increased in 10 patients at 1 hour after surgery ($P < .05$) (Figure 2); the TNF-α and IL-1β levels were increased in 7 patients and 6 patients, respectively. The IL-6 tear levels were increased at 24 hours in 9 patients, although the increase was not statistically significant.

The IL-6 mean tear levels were significantly correlated with the mean symptom score 1 hour after surgery. No correlations were found between the mean cytokine levels and the myopic defect corrected by LASIK.

Human Corneal Fibroblast In Vitro Study

None of the Th1- (IL-2, IFN-γ) or Th2-type (IL-4, IL-5, IL-10, IL-13) cytokines, IL-12 or TNF-α was detected in culture medium before or after in vitro photoablation.

Culture medium of HCF at baseline contained high levels of IL-6, IL-8, and MCP-1 (Figure 3), and lower but still detectable levels of IL-1β, RANTES and eotaxin (Figure 4). One hour after laser treatment, because many of the cells were destroyed, levels of all detected cytokines in culture medium were reduced. At 24 hours, cytokine levels were significantly increased compared with baseline and 1 hour (IL-6 and MCP-1) or compared with 1 hour (IL-1 and IL-8) (both $P < .05$), whereas RANTES and eotaxin had returned to baseline levels (Figures 3 and 4).

One hour after treatment, IL-1, eotaxin, and RANTES were not detectable in culture medium. At 24 hours, only IL-1 was significantly increased compared with 1 hour ($P < .05$).

DISCUSSION

Although the inflammatory and healing response is less after LASIK than after PRK, all refractive surgery procedures induce the activation of corneal cells and the release of several cytokines to modulate the corneal inflammatory and healing processes. The morphologic changes induced by myopic LASIK in human corneal stroma have been extensively characterized by confocal microscopy and histological studies.[2–4,10,23–24] Analysis of extracellular matrix proteins and cytokines in tear fluid after PRK showed increased levels of tenascin, TNF-α, and several growth factors,[12–15,25–26] suggesting that growth-modulating cytokines may be involved in healing processes. Laser in situ

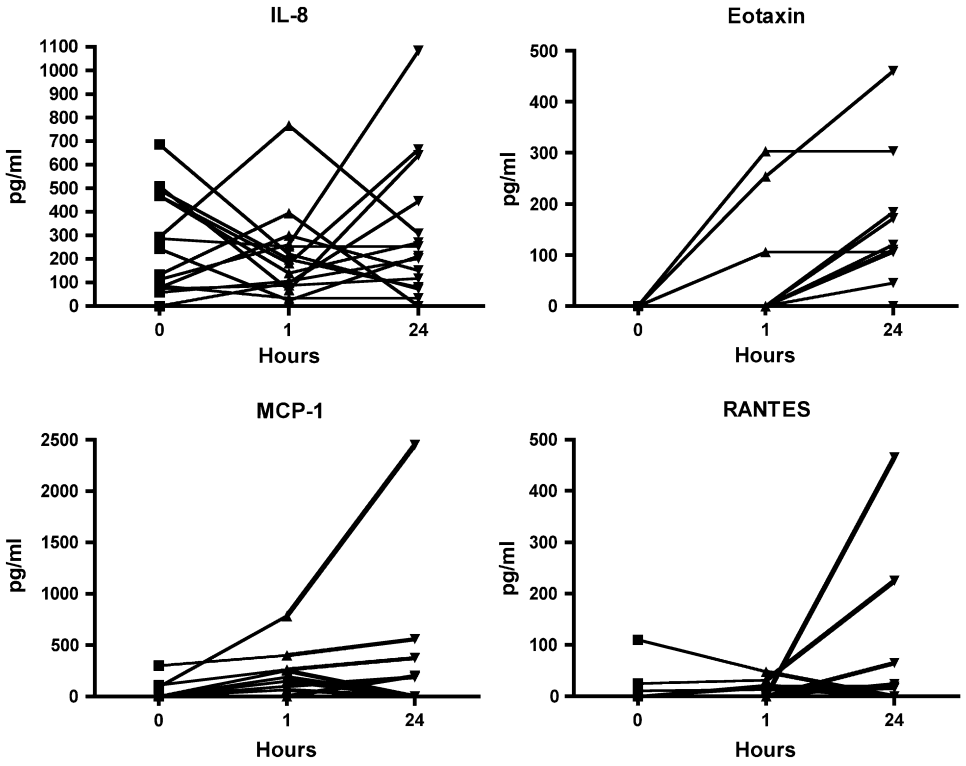

Figure 1. Tear chemokines before and after LASIK. (IL = interleukin; MCP = monocyte chemotactic protein; RANTES = regulated on activation, normal T expressed and secreted cytokine).

keratomileusis and laser-assisted subepithelial keratectomy seem to be less traumatic than PRK because a lower amount of tear transforming growth factor (TGF-β) is released and expressed in the early postoperative days than in PRK,[27–29] indicating that different techniques stimulate different corneal cell activation. However, the pattern of cytokine release in the early period after LASIK is not well delineated.

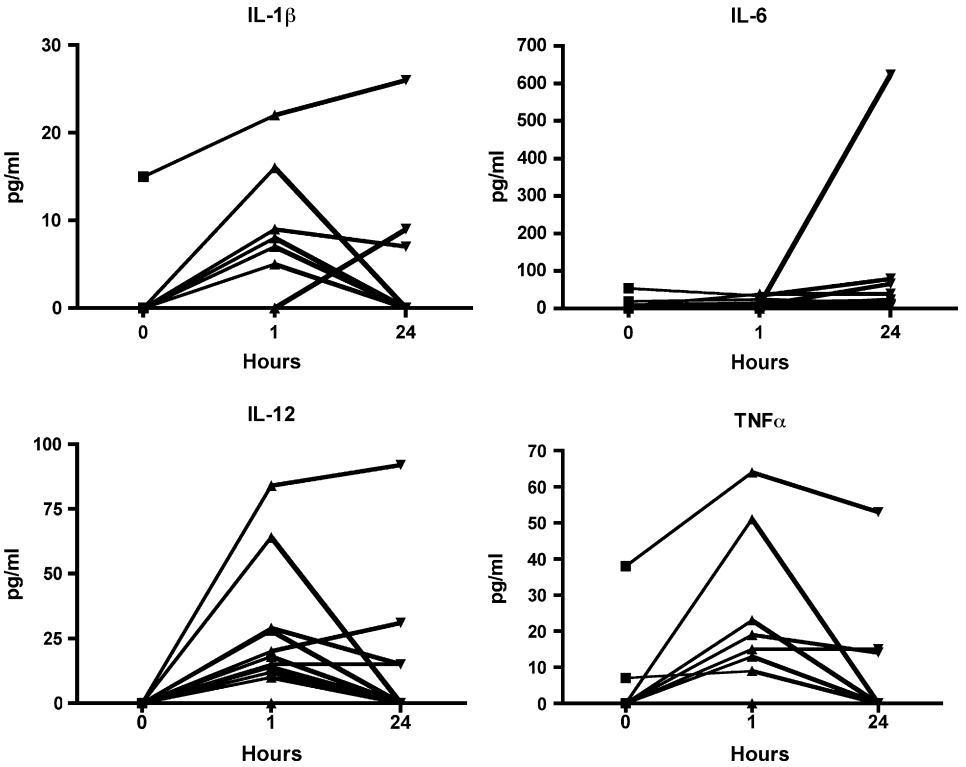

Figure 2. Tear proinflammatory cytokines before and after LASIK (IL = interleukin; TNF = tumor necrosis factor).

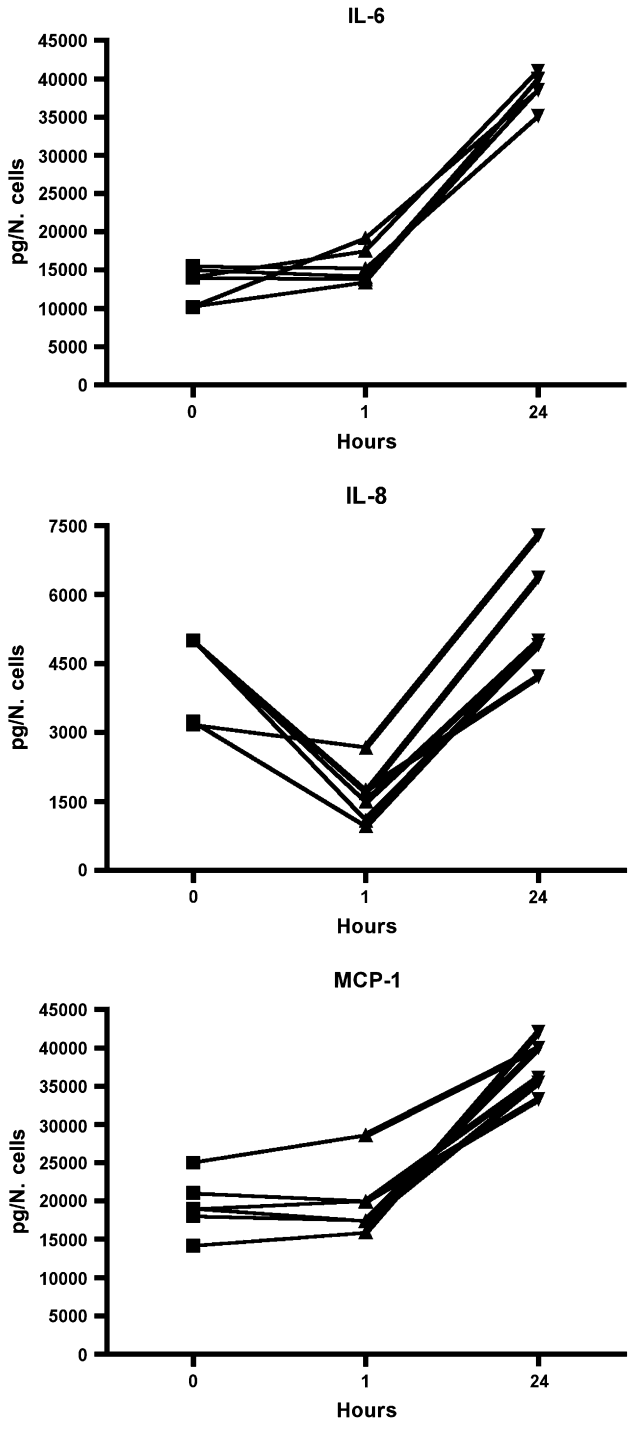

Figure 3. Cytokines and chemokines before and after laser treatment of corneal fibroblasts in vitro. Values are expressed as picograms per number of cells (IL = interleukin; MCP = monocyte chemotactic protein).

The multiplex bead immunoassay technology quantifies, in small volumes, a wide range of cytokines and chemokines.[18] This technology has been used to study the diurnal rhythm of tear cytokines, the effects of eye closure and tear reflex, and cytokine pattern release in allergic conjunctivitis.[19–22]

In the present study, we used this method to measure multiple cytokine and chemokine production in tears after LASIK and in an in vitro model of wound healing. In nonstimulated tears before surgery, a condition that can be considered equivalent to normal because no pharmacological or surgical treatment has been performed, IL-8 was the only cytokine consistently present in all patients, while the levels of Th1-type and Th2-type cytokines were low or under detection limits. This is in agreement with results in previous studies of cytokines in normal tears.[17,22]

Tear IL-12, although at low levels, was increased 1 hour after surgery in 10 of 15 patients ($P < .05$), probably as a result of corneal dendritic cell stimulation. In fact, it is now known that nonmature dendritic cells may also be present in the central corneal stroma in nontraumatized corneas.[30,31] Moreover, IL-12 was not detectable in culture medium of corneal fibroblasts before or after treatment, suggesting that levels found in tears may not be released by keratocytes.

Eotaxin, a chemokine involved in the recruitment of eosinophils, monocytes, and mast cells, was increased in tears 24 hours after surgery in 9 the 15 patients in our study ($P < .05$). Eotaxin has been shown to be produced by keratocytes and conjunctival fibroblasts, but not by corneal and epithelial cells.[32,33] In the in vitro model, eotaxin was detectable at baseline and 24 hours after treatment, when corneal fibroblasts were growing during the healing process. The other chemokines (MCP-1, RANTES, and IL-8) were also present in tears at baseline and increased after surgery in about half the patients. In the in vitro model, both MCP-1 and IL-8 were significantly increased 24 hours after laser treatment, confirming that stimulated corneal fibroblasts produce these factors after injury.[6,7] It is also evident that in the first hour after treatment, traumatized HCF cultures produce less cytokines and chemokines and that this production is increased only after 24 hours, when the cell number has increased, the genes are up-regulated, and cytokine expression is increased.[7] However, HCF in vitro response represents only a model to study the in vivo behavior of keratocytes during the wound-healing process after excimer laser treatment. In addition, the cytokine profile detected in culture may only partially reflect the cytokine profile produced in vivo by keratocytes, as shown by the tear cytokine level. Cultured stromal fibroblasts were shown to be more similar to wound-healing fibroblasts than to the keratocytes in the unwounded cornea in vivo.[34,35]

Several chemokines are involved in the recruitment and activation of inflammatory cells in the corneal wound-healing process.[9,36] Most studies report tissue

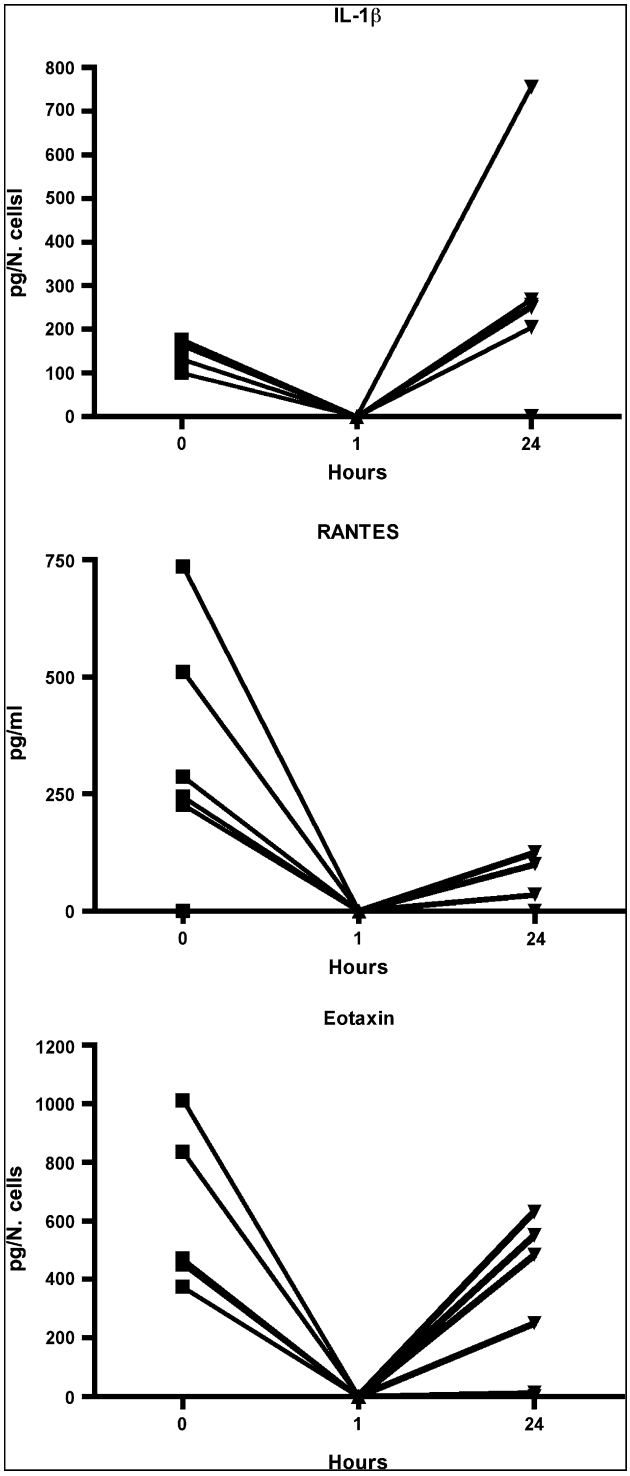

Figure 4. Cytokines and chemokines before and after laser treatment of corneal fibroblasts in vitro. Values are expressed as picograms per number of cells (IL = interleukin; RANTES = regulated on activation, normal T expressed, and secreted).

or in vitro expression of chemokines and chemokine receptors without quantitative analyses in human tears after surgery.[6–9] The CXC chemokine, IL-8 (CXCL8), is the most potent chemoattractant for neutrophils, while MCP-1 (CCL2) and eotaxin (CCL11), both members of the CC chemokine family, are chemoattractant for monocytes and T cells. Eotaxin acts preferentially on eosinophils via CCR3, which is highly expressed on these cells.[37] Interleukin-8, produced by keratocytes and neutrophils, was shown to contribute to the development of diffuse lamellar keratitis in an animal model.[38] It is possible that overexpression of these chemokines are responsible for noninfective LASIK complications. However, the role of these chemokines in the postoperative LASIK period must be further evaluated.

The other cytokine found in tears and in the in vitro model was IL-6, a multipotent proinflammatory cytokine reported to be increased in other active ocular surface inflammatory conditions and in corneal and conjunctival fibroblast culture media.[22,39] It is notable that the symptom score after surgery was correlated only with IL-6 tear levels, indicating that this cytokine is directly involved in the development of postsurgical inflammation and in the wound-healing process. Conversely, IL-1β, a cytokine reported to play a major role in corneal wound healing,[9] was not particularly increased in tears or in culture medium. In fact, IL-1β release is related mostly to corneal epithelial cell healing after a corneal epithelial defect, a situation that minimally occurs after LASIK, indicating that the low levels of IL-1β released after LASIK may be related to the low inflammatory response after this procedure.

In conclusion, after LASIK, low levels of cytokines and chemokines can be found in tears. After destroying keratocytes and extracellular matrix, photorefractive procedures activate stromal corneal fibroblasts to produce cytokines and chemokines that may modulate wound healing. Some of these cytokines, if overexpressed, may induce severe inflammation and thus corneal complications. Although new laser techniques and procedures are less traumatic and considered safer for patients, immediate postoperative modulation of key proinflammatory cytokines and chemokines and prophylactic antiinflammatory therapy are always advisable.

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