Elevated Expression of Matrix Metalloproteinase-9 and Inflammatory Cytokines in Keratoconus Patients Is Inhibited by Cyclosporine A


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PURPOSE. The present study was designed to understand the role of inflammatory cytokines secreted by corneal epithelial cells in keratoconus (KC) and the response to treatment with cyclosporine A (CyA).

METHODS. The study involved 129 Indian KC patients clinically graded according to Amsler-Krumeich classification and 20 healthy, nonectactic subjects as controls. Tear levels of matrix metalloproteinase-9 (MMP9), interleukin-6 (IL6), and tumor necrosis factor-α (TNFα) were measured using ELISA kits. Gene expression was measured by qPCR in corneal epithelial cells obtained by debridement from subjects undergoing ocular surface surgeries. In addition, epithelial cells were simulated with TNFα and treated with CyA to study its role on MMP9 expression. Finally, 20 KC patients (27 eyes) with inflammatory symptoms were treated with topical CyA application.

RESULTS. We observed that MMP9, TNFα, and IL6 levels were strongly upregulated at the mRNA level in KC patient epithelia. Similarly, tears collected from KC patients exhibited high levels of MMP9 and IL6 protein. Cyclosporine A treatment significantly reduced the mRNA expression levels of IL6 and TNFα in both short- and long-term treatments; however, it reduced MMP9 levels only in long-term treatment in cultured corneal epithelial cells. Subsequent treatment of KC patients with CyA for approximately 6 months reduced tear MMP9 levels and led to local reduction in corneal curvatures as determined by corneal topography maps.

CONCLUSIONS. The data indicate that corneal epithelium contributes to elevated MMP9 and inflammatory cytokine expression in tears of KC patients. Cyclosporine A treatment reduced MMP9 and inflammatory cytokine levels in an in vitro inflammation model system. In KC patients, CyA treatment reduced MMP9 levels measured in tears with concomitant arrest of disease progression. Therefore, CyA might be a novel treatment strategy in KC patients but requires additional evaluation in larger cohorts. (ClinicalTrials.gov number, NCT01746823.)

Keywords: keratoconus, cyclosporine A, MMP9, TNFα, IL6, ectasia, cornea, epithelial cells

Keratoconus (KC) is a common dystrophy of the cornea causing stromal thinning and astigmatism resulting in loss of visual acuity. The etiology of KC is poorly understood, and factors driving ectasia still remain unclear. However, atopy, eye rubbing, inflammatory factors, and hard contact lenses have been associated with KC. Previous reports suggest that progression of KC could be related to allergy or oxidative stress. Recent clinical reports on KC have suggested that its pathogenesis involves inflammatory mediators and matrix degrading proteins. However, currently, it is not clear whether these proteins are the major factors driving ectasia. Furthermore, there are no drugs available to halt the progression of the disease; thereby, this necessitates surgical intervention such as corneal crosslinking and keratoplasty.

Several studies investigating biochemical and pathologic changes in structural and cellular levels of cornea have been reported, but specific molecular mechanisms involved in KC pathogenesis are not fully elucidated. Although the structural integrity of the cornea is disrupted in KC, there are few differences in the type or content of collagen architec-
Role of Cyclosporine A in Keratoconus

It has been reported that a variety of epithelial and stromal proteins related to structure and function are deregulated in KC compared with controls and could be a response to ectatic stress.15,19,20 Tears of KC patients exhibit higher levels of proinflammatory cytokines, cell adhesion molecules, and matrix metalloproteinases (MMPs) compared with controls.10,11,21 Enzymes of the MMP family, which mediate degradation of extracellular matrix proteins typically in response to stress or injury,22 matrix metalloproteinases play several important roles in normal and pathological processes, including embryogenesis, inflammation, wound healing, development of arthritis, central nervous system (CNS) recovery and regeneration, pulmonary diseases, cardiovascular diseases, and cancer. Matrix metalloproteinases cause degradation of various types of collagen and are upregulated during matrix remodeling,23,24 which is a characteristic feature of KC.

Matrix metalloproteinase-9 (MMP9) or gelatinase B is the primary matrix-degrading enzyme produced by human corneal epithelium.26 Matrix metalloproteinase-9 activity is regulated by a variety of inflammatory cytokines.27 Regulation of MMP9 by IL6, has been implicated in degradation of type 1 and 4 collagen fibers in cancer and corneal disorders.28 To better understand the molecular regulation of MMP9 and its role in pathogenesis of KC in a cohort of Indian patients, we evaluated the levels of proinflammatory factors (IL6 and TNFα) and matrix remodeling protein (MMP9) in their corneal epithelium and tear samples. We further investigated the effects of Cyclosporine A (CyA) in modulating transcript levels of MMP9, IL6, and TNFα in their corneal epithelium.26 Matrix metalloproteinase-9 (MMP9) or gelatinase B is the primary matrix-degrading enzyme produced by human corneal epithelium.26 Matrix metalloproteinase-9 activity is regulated by a variety of inflammatory cytokines.27 Regulation of MMP9 by IL6, has been implicated in degradation of type 1 and 4 collagen fibers in cancer and corneal disorders.28 To better understand the molecular regulation of MMP9 and its role in pathogenesis of KC in a cohort of Indian patients, we evaluated the levels of proinflammatory factors (IL6 and TNFα) and matrix remodeling protein (MMP9) in their corneal epithelium and tear samples. We further investigated the effects of Cyclosporine A (CyA) in modulating transcript levels of MMP9 and inflammatory cytokines using human corneal epithelial cells followed by a proof-of-concept study in KC patients.

Methods

Study Design and Endpoints

This study followed the tenets of the Declaration of Helsinki and was approved by the Narayana Nethralaya (Bangalore, India) institutional review board. It was carried out as per Indian Council of Medical Research (ICMR) and institutional ethics guidelines for obtaining patient samples after prior informed written consent. For minor patients, written informed consent was obtained from their guardians or next-of-kin. The study has been registered at www.ClinicalTrials.gov (Identifier: NCT01746823).

The study is composed of three stages. In the first, gene expression in corneal epithelial cells was compared between KC patient and nonectatic control corneas. In the second arm, levels of MMP9, IL6, and TNFα were measured in the tears of KC patients and healthy subjects. The third arm comprised a single-center, open-label, unmasked, and nonrandomized treatment to evaluate the effect of daily instillation of topical 0.05% CyA eye drops in KC patients over a period of 6 months.

For study recruitment, 600 patients who reported to the tertiary care ophthalmic clinic at Narayana Nethralaya underwent an eligibility screening between April 2013 and July 2013. A total of 129 KC patients were included in the study along with 20 healthy controls. Of these, 94 KC patients and all 20 controls were included in the tear analysis by ELISA for MMP9, IL6, and TNFα levels. Additionally, 12 KC patients and 10 subjects with no corneal ectasia, undergoing surgery for unrelated conditions were used as controls for the corneal epithelial samples. In the CyA (Restasis; Allergan, Inc., Irvine, CA, USA) treatment group, a total of 23 patients (35 eyes) were included; of which three patients (6 eyes) dropped out during the study. In remaining cohort, seven patients (14 eyes) had tears collected before and approximately 6 months after treatment (paired treatment group). Posttreatment tears for all the treated patients were compared with untreated KC cohort of 94 patients (unpaired treatment group). A CONSORT flow chart has been provided as Supplementary Figure S1.

Inclusion criteria for cases were all KC patients of Indian origin, with or without any association with ocular or systemic allergy. Patients using contact lenses, using any type of anti-inflammatory ocular or systemic medications (e.g., antiallergic, anti-inflammatory drugs), or who had undergone any surgical intervention (e.g., penetrating keratoplasty/corneal collagen cross-linking, cataract surgery, etc.) for either of the eyes were excluded from the study. Patients with recent (less than 3 months) systemic/ocular allergy or infection history were also excluded. All subjects also underwent a dry eye evaluation using Schirmer’s test and corneal staining. Subjects with concurrent symptoms of dry eye were excluded. Patients with systemic inflammatory or autoimmune diseases were also excluded. The study cohort had a mean age of 24 ± 8.4 years with a male to female ratio of 1.22.

To assign subjects to the treatment cohort, the KC patients were requested to answer a questionnaire on eye rubbing, irritation, and pain in their eyes. Patients who complained of at least two of these three symptoms were invited to participate in an open-label study on potential immunomodulatory benefits of CyA for management of KC. Cyclosporine A is already approved for treatment of dry eye patients for inflammation or irritation.31 Patients who were recruited after written informed consent applied 1 to 2 drops of CyA, twice daily for approximately 6 months. Each patient in this cohort was followed up after 1, 3, and 6 months. The withdrawal criteria were worsening or acceleration of corneal curvature as determined by corneal topography measurements during follow up; any adverse reactions to the drug or sudden onset of any confounding ocular condition/infection or systemic illness.

The primary endpoints were analysis of MMP9 levels in tears post treatment. Secondary endpoints were corneal topography measurements post treatment. Safety endpoints in the treatment cohort included monitoring of inflammation, unintended reactions, or loss of visual acuity (determined by biomicroscopy and visual acuity measurements). None of the patients experienced any adverse reaction to the treatment and three patients dropped out.

Patient Diagnosis and Grading

Corneal topographic and tomographic techniques were used for diagnosis and grading of the KC patients.2-5 The topography of all the patients was acquired with Pentacam (OCULUS Optikgeräte GmbH, Wetzlar, Germany) upon first presentation at the clinic, before and after administration of topical CyA drops, and at consecutive follow-ups. The Amsler-Krumeich classification was used in this study, which defines KC grade from biomicroscopy data, mean central keratometry reading, spherical and cylindrical refraction change, and corneal thickness.5,6 This study did not involve any drug-based tests or analysis.

Patient Tear Collection

Tear samples were collected using capillary micropipette tubes as described earlier.11 The tears were collected from the exterior one-third of the lower fornix, taking care not to touch the conjunctiva or produce any reflex tearing. The tears were extracted from the micropipette tubes into sterile microfuge tubes (Axxygen, Tewksbury, MA, USA) followed by storage at −80°C, until analysis.

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Role of Cyclosporine A in Keratoconus

Patient-Derived Corneal Epithelial Cells

Corneal epithelial cells were collected after obtaining written informed consent. It was collected from epithelial debridement during ocular surface surgeries involving corneal collagen crosslinking or topo-guided photorefractive keratectomy (TOPO-PRK) in KC patients (n = 12; 5 in grade I, 5 in grade II, 2 in grade III, and 2 in grade IV). Control corneal epithelial cells (n = 10) were collected from subjects undergoing PRK who did not demonstrate clinical signs of KC. Epithelial tissue was homogenized in TRIzol reagent and RNA was extracted for gene expression analysis.

ELISA Assay

Matrix metalloproteinase-9 (BioTrak assay; GE Healthcare, Pittsburgh, PA, USA), IL6 and TNFα ELISA assay kits (Quantikine Assay; R&D Systems, Minneapolis, MN, USA) were used to analyze tear samples from patients and control tear samples as per manufacturer’s instructions.

Telomerase-Immortalized Human Corneal Epithelial Cells (hTCEpi)

Telomerase-immortalized human corneal epithelial cells were a kind gift from Winston Kao, University of Cincinnati (Cincinnati, Ohio, USA). Cells were cultured on Primaria-treated culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA) in serum-free, calcium-free keratinocyte culture media (Derma-Life, Lifeline Cell Technology, Frederick, MD, USA) supplemented with 6 mM L-glutamine, 0.4% Extract P, 1.0 μM epinephrine, 0.5 ng/mL recombinant human (h)TGFβ 100 ng/mL hydrocortisone hemisuccinate, 5 μg/mL recombinant hInsulin, 5 μg/mL apo-transferrin, and maintained at 37°C in 5% CO2. Passage 18 hTCEpi was used in the experiment.

Drugs and Chemicals

Recombinant human TNFα (hTNFα 210-TA-010/CF; R&D Systems, Minneapolis, MN, USA) was used at 5 ng/mL in the absence or presence of 30 μg/mL and 50 μg/mL of CyA (C3662; Sigma-Aldrich Corp., St. Louis, MO, USA). Dimethyl sulfoxide (DMSO; D2438; Sigma-Aldrich Corp.) was added to control wells. Telomerase-immortalized human corneal epithelial cells were cultured for 3 days to 100% confluence, and pretreated with CyA for 1 hour before hTNFα was added for 2 hours (short-term treatment). For the long-term treatment, both CyA (10 μg/mL) and hTNFα (1 ng/mL) were added at the same time for 15 minutes every 24 hours for 7 days. Recombinant human IL6 (hBA-184, Santa Cruz Biotechnology, Dallas, TX, USA) was used at 10 ng/mL for the high-dose, short-term treatment and 2 ng/mL for the low-dose, chronic treatment.

Isolation of RNA, cDNA Synthesis and Real-Time PCR

Cells were washed twice with PBS(+); thereafter, 350 μL of RLT buffer with 1% β-mercaptoethanol was added directly to each 35-mm culture dish and scraped with a rubber policeman. Cells were transferred to a QIAshredder spin column (Qiagen, Valencia, CA, USA) and centrifuged for 2 minutes. Total RNA was purified from supernatant following manufacturer’s instructions for RNeasy Mini Kit (Qiagen). Ribonucleic acid yield was estimated using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). Ribonucleic acid was extracted from patient epithelia using the TRIzol reagent according to the manufacturer’s instructions (Invitro-}


gen, Carlsbad, CA, USA) followed by purification through RNeasy mini kit. Complementary DNA was synthesized using Superscript III (Life Technologies, Carlsbad, CA, USA). Quantitative real-time PCR was performed as previously reported. The PCR cycle included preincubation at 95°C for 5 minutes, 45 amplification cycles at 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 30 seconds using a CFX Connect real-time PCR detection system (Bio-Rad, Philadelphia, PA, USA). Primer sequences are described in Supplementary Table S1.

Statistical Analysis

All statistical analysis was performed with MedCalc v12.5.0 (MedCalc, Inc., Ostend, Belgium) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). If the test was statistically significant, post hoc comparison of the group means was performed. The Friedman test for comparison of group means was used. The mean value of the individual groups was reported as mean ± SEM.

RESULTS

KC Corneal Epithelium Shows Increased Levels of Inflammatory Cytokines and MMP9 Gene Expression

Although levels of cytokines, enzymes, and other proteins in the tears are readily measurable, it is not always clear whether the corneal cells crosstalk with the tear proteins and serve as the source of the deregulated proteins. Therefore, to determine if indeed MMP9, IL6, and TNFα are activated at the level of mRNA expression in the cornea epithelial cells from KC patients, we performed quantitative real-time PCR from the same patients undergoing surgical treatment procedures such as PRK, Topo-PRK, corneal crosslinking, and so on. The data presented in Figure 1 demonstrates that the expression levels of MMP9, IL6, and TNFα transcripts are indeed higher in the corneal epithelium of KC patients compared with the control cohort. Matrix metalloproteinase-9 levels were 5.16 ± 0.95 compared with controls 2.47 ± 0.89 (P = 0.01). Similarly, both IL6 and TNFα transcript levels were elevated in KC corneas at 4.05 ± 0.73 and 1.88 ± 0.59 compared with their respective controls at 2.65 ± 0.89 and 0.53 ± 0.14 (P = 0.03 and P = 0.04, respectively). The data from this cohort suggests that KC patients’ epithelial cells are in active inflammatory state as observed by the elevated levels of proinflammatory cytokines.

Tears From KC Patients Demonstrate High Levels of MMP9 Protein

Since we observed the increased gene expression levels of MMP9, IL6, and TNFα in KC patient epithelia, we then evaluated the protein levels of these genes in the tears of the patients by ELISA. Our cohort of patients included 94 patients with different grades of KC and 20 controls. MMP9 levels in tears were highly elevated at 45.56 ± 0.67 ng/mL compared with controls 3.66 ± 1.56 ng/mL (P < 0.001). Although, the MMP9 levels across different grades of KC patients demonstrated an increasing trend, they were not significantly different (Fig. 2B). Interestingly, if the KC grades 1 and 2 are grouped together and compared with KC grades 3 and 4 combined, we observed a statistically significant difference (P < 0.05). We also measured IL6 and TNF9 levels in the tears of the same patients (Fig. 2C). Interleukin-6 levels were increased to 0.92 ± 0.07 compared with controls 0.77 ± 0.11, although the differences were not statistically significant (P = 0.6). However, IL6 levels when compared within the different
grades demonstrated a grade dependent increasing trend, which was statistically significant ($P = 0.002$). Surprisingly, the levels of TNF$\alpha$ did not appear significantly different between two groups in the tears ($P = 0.87$) as shown in Figure 2E. Furthermore, between the different grades of KC, there was a discordant trend of TNF$\alpha$ levels in the higher grades (Fig. 2F), and the data was not statistically significant ($P = 0.87$). Therefore, in summary, tears of this cohort of KC patients demonstrated an acute increase in MMP9 levels over controls. While IL6 levels are also increased, TNF$\alpha$ levels did not show any significant associations with different grades of KC.

**MMP9 Expression Is Regulated by Inflammatory Signaling in Human Corneal Epithelial Cells and Inhibited by CyA**

To investigate the effects of CyA on inflammatory cytokines and extracellular matrix (ECM) proteins in the corneal epithelial cells, we used the telomerase transformed human corneal epithelial cells (hTCEpi) established previously.$^{35}$ The cells were treated with a single dose of recombinant hTNF$\alpha$ for 2 hours alone or with CyA. Cyclosporine A is the active compound in the commercially available formulation drug Restasis (Allergan, Inc.), which is approved for treating inflammatory dry eye disease. Figure 3A demonstrates that the treatments with CyA or hTNF$\alpha$ did not alter the morphology of the cells. Gene expression analysis was performed for MMP9, IL6, TNF$\alpha$, Collagen 1A1 (ColIA1), and Collagen 4A1 (ColIVA1). Previous reports suggest that collagen expression may differ in some KC corneas,$^{15,16}$ which could be associated with the disease pathology. As expected, short-term treatment of hTNF$\alpha$ at a dosage of 5 ng/mL increased MMP9, IL6, and TNF$\alpha$ transcripts significantly, whereas it did not affect mRNA levels of either form of collagen. However, when treated with two independent doses of CyA (30 $\mu$g/mL and 50 $\mu$g/mL), we observed a significant reduction in IL6 and TNF$\alpha$ mRNA levels indicating a strong anti-inflammatory effect of the drug.

Also, short-term treatment of CyA alone did not affect the basal levels of IL6 and TNF$\alpha$. While CyA appeared to have a minimal effect on MMP9 mRNA levels, the combination of TNF$\alpha$ with CyA appears to have an amplified effect on MMP9 mRNA. However, all these treatments have very little effects on ColIA1 and ColIVA1 transcripts. These experiments suggest that there is a definitive effect of CyA treatment on inhibition of inflammatory signaling in corneal epithelial cells. Additionally, short-term treatment with IL6 (10 ng/mL) increased the levels of IL6 (Supplementary Fig. S2B), TNF$\alpha$ (Supplementary Fig. S2C), and MMP9 (Supplementary Fig. S2D), but CyA treatment (30 $\mu$g/mL and 50 $\mu$g/mL) had an additive effect on the expression levels of these genes similar to the TNF$\alpha$. Interleukin-6 treatment also slightly increased CollA1 and CollIVA1 (Supplementary Figs. S2E, S2F).

**Long-Term Inhibition of Inflammatory Signaling By CyA Reduces MMP9 Expression Levels**

The data in Figures 1 through 3 demonstrate that corneal epithelial gene expression as well as tear protein levels of MMP9 and IL6 are associated with KC. Since the previous set of experiments demonstrates that gene expression of IL6 and TNF$\alpha$ in response to high dose (TNF$\alpha$, 5 ng/mL) of inflammatory stimulus is abolished by CyA treatment, we extended the study to low-dose of inflammatory stimulus and long-term treatment of CyA. To simulate the chronic inflammatory stimuli, we treated the corneal epithelial cells with 1 ng/mL hTNF$\alpha$ for 15 minutes every 24 hours for 7 days. The media was changed 15 minutes post treatment each day. The same treatment was performed using either CyA (Restasis, 0.05% CyA) at a dosage of 10 $\mu$g/mL or CyA and hTNF$\alpha$ together for 15 minutes. After 7 days, the cells were harvested 6 hours after the final dose and analyzed for gene expression of IL6, TNF$\alpha$, and MMP9. Cyclosporine A treatment reduced the transcript levels of IL6 (Fig. 4A) and TNF$\alpha$ (Fig. 4B) as was previously observed for the single; high-dose TNF$\alpha$ treatment.
Role of Cyclosporine A in Keratoconus

Figure 2. Tear protein levels of MMP9 and IL6 correlate with KC and progression of the disease. ELISA of MMP9, IL6, and TNFα in tear samples of controls and patients with different grades of KC. (A) Expression of MMP9 in normal versus KC tear sample (p < 0.001). (B) Expression of MMP9 in different grades of KC (p < 0.001). (C) Expression of IL6 in normal versus KC tear samples (p = 0.8). (D) Expression of IL6 in different grades of KC (p = 0.002). (E) Expression of TNFα in normal versus KC tear samples (p = 0.87). (F) Expression of TNFα in different grades of KC (p = 0.87).
FIGURE 3. Corneal epithelial cells respond to acute inflammatory stimuli that are inhibited by CyA. Levels of gene expression after inflammatory stimuli (short-term treatment with 5 ng/mL hTNFα) in hTCEpi corneal epithelial cells and effect of CyA (30 and 50 μg/mL) were evaluated. (A) Photomicrographs of hTCEpi cells cultured with the indicated treatment conditions demonstrating cuboidal epithelial morphology. Expression of IL6 (B), TNFα (C), MMP9 (D), CollIA1 (E), and CollIVA1 (F) transcripts with the treatments as indicated.
Matrix metalloproteinase-9 transcript levels were significantly increased after long-term hTNFα treatment, which were significantly decreased following CyA treatment (Fig. 4C). Furthermore, treatment of these cells with 2 ng/mL IL6 for 15 minutes every 24 hours for 7 days did not affect TNFα (Supplementary Fig. S3A) levels. But CyA treatment at a dosage of 10 μg/mL for 15 minutes every 24 hours for 7 days reduced the basal levels of TNFα. The same IL6 treatments led to an

FIGURE 4. Chronic inflammatory stimulus mediated upregulation of MMP9 and inflammatory gene expression is inhibited by CyA. Long-term treatment of hTCEpi cells with low doses hTNFα (1 ng/mL) and CyA (10 μg/mL) decreased the expression of IL6 (A), TNFα (B), MMP9 (C), ColIA1 (D), and ColIVA1 (E) transcripts with the treatments as indicated.
increase in IL6 (Supplementary Fig. S3B) and MMP9 levels (Supplementary Fig. S3C). However, CyA treatment (10 µg/mL) significantly reduced the expression levels of these genes and antagonized the effect of IL6 (Supplementary Figs. S3B, S3C).

Interestingly, we observed a reduction in CollA1 transcript levels while CollA1 remained unchanged upon the chronic hTNFα treatment. However, CyA treatment alone reduced the CollA1 transcript levels while increasing CollIVA1 transcript levels. In both cases, treatment of CyA appears to mildly rescue the effects of hTNFα. Interleukin-6 treatment did not induce significant changes in collagen transcripts, but CyA treatment slightly reduced CollA1 levels (Supplementary Fig. S3D) and increased the CollIVA1 expression (Supplementary Fig. S3E). These experiments suggest that chronic inflammation can lead to upregulation of MMP9 gene expression, and CyA may be useful in attenuating the levels of MMP9 in the cornea and may have some beneficial effects in KC eyes.

Treatment of Keratoconus Patients With CyA Leads to Reduction in Tear MMP9 Levels

The previous experiments demonstrate that MMP9 levels are critically elevated in KC corneas and hence its inhibition by CyA (Restasis) might be a novel treatment modality. Since CyA is approved for treatment of dry eye, a small cohort of KC patients were treated with topical CyA with prior informed consent. The CyA treatment is currently a well established treatment modality for dry eye of inflammatory etiology as a potent immunomodulator.36 Therefore, on the basis of a questionnaire detailing patient complaints of irritation, eye rubbing, or ocular surface pain (typical of inflammatory etiology), KC subjects were assigned to a treatment cohort for CyA. A total of 20 patients (27 eyes) were treated with a dose of 0.05% CyA (Restasis), 1 to 2 drops, two times daily, for approximately 6 months. Within this cohort of CyA-treated patients, tears were collected both before and after the end of treatment period for a total of 14 eyes (paired group). To analyze the MMP9 levels and topographic data in the remainder of the CyA-treated group, we used the data from the previous cohort in Figure 2 as the no-treatment group (unpaired group). The data in Figures 5A and 5B demonstrate that CyA-treated cohort demonstrated a reduction in tear MMP9 levels compared with the negative controls. In the paired treatment group (tears available for both before and after CyA treatment for the same individual), there was a significant reduction in MMP9 levels. On evaluation of the topography of the subjects from this cohort of CyA treatment, we observed local changes in the corneas upon simulated keratometry at the end of last follow-up. Figures 6A through 6F show axial curvature maps of the anterior corneal surface before and after application of CyA in left and right eyes of three patients, respectively. The areas where significant local flattening of the corneal curvature is observed are marked in the figure with red circles. The levels of MMP9 in tears of the patients collected at the time of corneal topography scans were also indicated. In Patient 1, there are pockets where axial curvature decreased, for example, 54.7 diopters (D; axis 270° inferior) reduced to 53.2 D (Fig. 6A). Corresponding tear MMP9 levels reduced from 48.5 to 27.4 ng/mL in the same patient’s eye. In Figure 6D, the right eye of the same subject (patient 1) as in Figure 6A is presented and showed some reduction in curvature, for example, 52.3 D (axis 270° inferior) reduced to 51.2 D while tear MMP9 reduced from 38 to 33.5 ng/mL. Similarly, 49.8 D (axis 300° inferior-nasal) reduced to 48.5 D. In Figure 5B (patient 2), 56.2 D (axis 210 inferior-nasal) reduced to 54.9 D. The tear MMP9 level reduced from 43 to 28.5 ng/mL in the same eye. Similarly in Figure 6E (patient 2), the inferior cornea shows some decrease in curvature as well. Corresponding tear MMP9 levels reduced from 42 to 34.5 ng/mL in the same patient eye. Patient 3 (Figs. 6C, 6F) showed similar remodeling and reduction in MMP9 as patient 1 and 2 while tear MMP9 levels also reduced as indicated. This data raises the possibility that corneal remodeling may be partly due to the reduction of MMP9 levels observed in these KC patients after CyA treatment. However, this local flattening was not evident in all patients, but disease progression was not observed over the 6-month period (Supplementary Fig. S4) as demonstrated by mean keratometry and corneal thickness values (Supplementary Table S2).

**DISCUSSION**

Numerous clinical studies in recent years on KC support the idea that its pathogenesis involves an inflammatory component.4,21,37 The tear may be a vehicle of some of the pathogenic protagonists of KC, such as IL6, TNFα, or MMP9 that provoke progressive ectasia, but the source of these proteins remains unknown.4,11 Atopy, eye-rubbing, contact lens wear, oxidative stress,3–5 and genetic factors38 have been suggested to cause the disease. Balasubramanian et al.39 made an interesting observation that 1 minute of eye rubbing could increase inflammatory cytokine and MMP levels in tears. This observation further supports a causative role for cytokines and MMP9 in KC progression. However, the increase in MMP9 levels observed in tears of KC patients compared with healthy controls is much higher (Fig. 2) compared with the amount induced by short-term eye rubbing.39 Since KC is characterized by the thinning of the corneal stroma, one of the potential
Figure 6. Cyclosporine A treatment shows reduced corneal curvatures in patients. The figures show the axial curvature maps of the anterior corneal surface before and after application of CyA in the left and right eyes of three patients, respectively, as indicated. These subjects were treated with CyA (Restasis) topically over a period of 6 months. The corresponding tear MMP9 levels are indicated under each map. The red circles indicate examples of areas where the changes in curvature are observed before and after treatment. (A–C) Left eyes of each patient. (D–F) Right eyes of each patient.
sources contributing to the disease pathology could be inflammatory and matrix remodeling proteins secreted by corneal epithelial cells. In support of this hypothesis, we found that the expression levels of MMP9, IL6, and TNFα transcripts increased in KC patient corneal epithelia compared with controls. Our data demonstrate for the first time that the inflammatory cytokines and MMP9 may be secreted by the corneal epithelium in KC patients. Furthermore, MMP9 levels strongly correlated with the disease in tears of KC patients. Interleukin-6 and TNFα were also found to be elevated in the tears in the KC cohort compared with age-matched controls. These results indicate that the concentration of MMP9 and IL6
in tears may be associated with the progression of KC in the Indian cohort; however, a similar association with TNFα in tears with the progression of ectasia was not found in our study (Fig 2F). These data suggest an interesting paradigm in inflammatory signaling wherein we observe a concordant increase in levels of MMP9 and IL6 with the severity in the clinical grades of KC, but not with the acute phase inflammatory agent TNFα. It is well known that both TNFα and IL6 can induce MMP9 expression at the transcriptional level.24,40 The correlation of MMP9 and IL6 secretion has been documented in renal manifestations of primary Sjögren’s Syndrome, an inflammatory condition that also has ocular manifestations. Interleukin-6 has also been shown to regulate MMP9, MMP2, and TIMP1 (tissue inhibitor of MMP1) in lymphomas.25 Metalloproteinase-9 activity regulation by IL6 has been implicated in degradation of type 1 and 4 collagen fibers in cancer26 and in several corneal disorders.27 Therefore, the present data suggest that an IL6-dependent inflammatory response driving MMP9 secretion may lead to the stromal thinning in KC.

Previous studies in tears from Spanish populations have demonstrated high levels of IL6 and TNFα to be associated with both clinical4,5 and subclinical KC11; MMP9 levels were associated only with clinical KC in these studies.4,5,11 However, in our study cohort consisting of Indian population, we observe a different relationship between IL6 and TNFα levels in the tears. While our experimental cohort of 94 patients is higher than the previous reports, the presence of high IL6 but low TNFα could be explained in two ways. First, the data can simply be a function of the peculiarities of the inflammatory profile in Indian patients. Second, it could be regulatory signaling between IL6 and TNFα. It has been demonstrated previously that IL6 can reduce circulating TNFα levels by having opposing effects on granulomas,42 and can attenuate the acute phase inflammatory response,43 which may be a natural mechanism in vivo. Interleukin-6, however, is associated with chronic inflammatory conditions and cancer promoting inflammation.34,44,45 Therefore, the association of IL6 with increasing grades of KC and correlation with MMP9 may be reflective of a chronic inflammatory situation, which leads to ectatic pathology. We note that while the mRNA levels of the inflammatory cytokines in KC corneas were higher compared with the control group, this was not reflected directly in the tears. This could be due to posttranscriptional regulation of cytokine production, or the interplay of other factors that regulate the secreted levels of these factors.

We further examined the mechanism of MMP9 induction by inflammatory stimuli in a human corneal epithelial cell model in vitro. Since our data supports the hypothesis that inflammation may have a critical role in progression of KC, we used the well documented inflammatory pathway activating factor recombinant hTNFα to stimulate inflammatory cytokine expression and CyA to inhibit the inflammatory response. Cyclosporine A (Restasis) was selected as a drug of choice since it is US Food and Drug Administration approved for dry eye disease.30,46 Especially in dry eye disease where inflammation and increased cytokine expression are part of the etiology, CyA functions as a potent immune-modulator that benefits the patients.50 Furthermore, CyA has been previously documented to reduce IL6 levels in a systemic chronic inflammatory disease such as rheumatoid arthritis.57 We observed that CyA was able to suppress the hTNFα-stimulated inflammatory cytokine gene expression (IL6 and TNFα), both in the high-dose, short-term and low-dose, long-term treatments. Interestingly, the high-dose 2 hour hTNFα treatment did not show any significant induction in MMP9 expression, but the combined treatment of CyA and hTNFα-induced MMP9 levels; this could be a stress response at the MMP9 promoter due to the drug treatments. Similar results were observed with short-term, high-dose (10 ng/mL) IL6 treatment in the same cells (Supplementary Fig. S2). However, in the hTNFα low-dose treatment for 7 days, MMP9 was significantly increased similar to IL6 and TNFα transcripts. Long-term treatment of CyA (low dose, 0.1 ng/mL) decreased these transcripts in the corneal epithelial cells (Fig. 4). On the contrary, treatment with CyA significantly increased ColIVα1 expression while slightly reducing Collα1 levels. A similar trend was also observed for long-term, low-dose (2 ng/mL) IL6 treatment (Supplementary Fig. S3). In this set of experiments, similar to Figure 4, we observed a reduction of MMP9 expression levels by CyA treatment that antagonized the effect of IL6. This data is consistent with previous report where CyA treatment reduced the levels of MMP and collagen I transcripts in dermal fibroblasts.49 The observed increase in ColIVα1 levels could be due to differential transcriptional regulation of the transcript in response to the inflammatory signaling cascades inhibited by CyA as described previously.49 Increase in ColIVα1 has been associated with corneal remodeling during wound healing and with cellular adhesion and migration.50-52 Therefore, it is possible, that the increase in ColIVα1 transcript upon CyA treatment is indicative of an active wound healing and remodeling process in the corneal cells that may be beneficial to KC patients. Human TNFα treatment results in a reduction of ColIVα1 levels consistent with previous report in cardiac fibroblasts, where it has been shown that TNFα reduces collagen expression and increases MMP expression and activity.40 These data demonstrate that corneal epithelial cells stimulated with chronic inflammatory stimuli not only induce expression of inflammatory cytokines such as IL6 and TNFα but also the matrix degrading enzymes like MMP9 while inhibiting collagen expression.

Since MMP9 expression is so strongly associated with KC (Figs. 1, 2) and long-term treatment with CyA appears to reduce both inflammatory stimulation and MMP9 levels in vitro, it may therefore be a viable option for treating KC patients. As proof-of-concept for the same, we performed a pilot study of 0.05% CyA treatment in a small cohort of 20 patients (27 eyes) with KC. Patients were followed for approximately 6 months with regular use of CyA eye drops. For a portion of the patients where tear samples were available both pre and post treatment (7 patients, 14 eyes), we observed that MMP9 values reduced significantly (Fig. 5A). Even in the unpaired, matched cyclosporine treated versus untreated groups, we observed a reduction of MMP9 levels in tears (Fig. 5B). The clinical definition of progressive KC is suggested as an increase in keratometry by at least 1 D in 6 months.13,14 In this cohort of patients, we observed that none of the subjects displayed significant progression by 1 D during and after stopping use of CyA. Furthermore, the corneal topography data in Figure 6 from patients treated with CyA and followed up over 6 months demonstrates significant local topographical changes in the cornea measured by keratometry that corresponds to a drop in tear MMP9 level. Although in the current study overall change in keratometry may not be very significant, the local flattening of the cornea and reduction of tear MMP9 levels are encouraging. It is therefore possible that the reduced progression of disease in these patients is due to a reduction of MMP9 levels.

The key limitations of this study are the small cohort size, the short treatment duration and the lack of an active comparator. Although local changes in corneal curvature of CyA-treated patients were observed, longer follow-up may be needed to fully validate the hypothesis that reduced levels of MMP9 may indeed offer a novel therapeutic approach. Furthermore, the sample size of the treatment cohort in our study may not be representative of all patients; hence, a larger
cohort study with longer follow-up may be necessary. Additionally, other inflammatory factors as well as collagenases need to be evaluated to understand the interplay of these proteins in the pathogenesis of keratoconus.

In conclusion, this study supports the hypothesis that elevation of MMP9 by inflammatory signalling may be a critical driver of KC disease pathogenesis and their reduction may attenuate disease pathogenesis in KC subjects as predicted by in vitro experiments. The present data for the first time suggests that corneal epithelial cells contribute to the inflammatory status in the KC patients, and that CyA treatment provides a new modality to reduce inflammatory cytokines and MMP9 levels to possibly arrest the progression of KC.

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