Functional Activity of Matrix Metalloproteinases 2 and 9 in Tears of Patients With Glaucoma

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PURPOSE. To evaluate the differential expression of tear matrix metalloproteinases (MMP) 2 and 9 in of patients with various forms of glaucoma.

METHODS. Tear samples were collected with a Schirmer's strip from 148 eyes of 113 patients (medically naïve patients with primary open-angle [POAG] or angle closure glaucoma [PACG] and those with pseudoexfoliation syndrome [PXF] or glaucoma [PXG]). These were compared to patients undergoing cataract surgery (controls) for this cross-sectional study. Functional activities of tear MMP-9 and MMP-2 were analyzed by gelatin zymography. Tenon's capsules ($n = 15$) were harvested from the inferior quadrant in those undergoing cataract surgery and protein expression of MMP-9 was analyzed by immunohistochemistry (IHC). Hydrogen peroxide (H_2O_2) stress-induced effects on in vitro activities of MMP-9 in human trabecular meshwork (HTM) cells were analyzed.

RESULTS. The MMP-9 activity in tears was increased significantly in POAG, ($n = 27$), PACG ($n = 24$), and PXF ($n = 40$) eyes compared to controls ($n = 35$), and was increased significantly in eyes with glaucoma compared to moderate/severe glaucoma ($P < 0.001$). The MMP-9 expression was significantly lower in PXG ($n = 22$) eyes. Immunohistochemistry of Tenon's capsule revealed increased expression of MMP-9 in primary glaucoma eyes. Increased MMP-9 activity was seen in in vitro by gelatin zymography and was confirmed by Western and immunofluorescent assay on HTM upon 800 and 1000 μM H_2O_2-induced stress for 2 to 3 hours with approximately 80% cell death.

CONCLUSIONS. Increased tear MMP-9 activity in early glaucoma and pseudoexfoliation syndrome suggesting activation of extracellular matrix (ECM) degradation can be used as a tear-based predictive biomarker. Decreased expression in advanced stages suggests exhaustion of the degradation response.

Keywords: matrix metalloproteinases, glaucoma, tear film, ocular surface

While the etiopathogenesis of different forms of glaucoma, such as primary open-angle glaucoma (POAG), primary angle closure glaucoma (PACG), pseudoexfoliation syndrome (PXF), and pseudoexfoliation glaucoma (PXG), are varied, increased extracellular matrix (ECM) degradation and matrix metalloproteinase (MMP) activation in anterior segment tissues and aqueous is known in established glaucoma.[1–4] Though advances in technology have helped improve diagnostics in the last decade, current glaucoma diagnostic modalities available for detecting structural or functional damage are not only cumbersome and time-consuming, but also expensive and not feasible in areas with poor access to health care. There is a compelling need for better and easy diagnostic tests for effective glaucoma screening in rural or low resource settings. MMPs have been implicated in glaucoma pathogenesis and are identified as potential markers for various ocular diseases, including glaucoma.

Proteins, such as MMPs, are involved in ECM turnover, improving aqueous humor outflow and trabecular meshwork (TM) functions.[2,3] Increased levels of MMPs (namely MMP-1 and MMP-9) in aqueous and plasma have been reported to be involved in earlier studies in different forms of primary glaucoma and experimental glaucoma models.[1] Understandably, altered functions of MMPs in the eye would lead to abnormal ECM degradation and turnover, leading to TM dysfunction and outflow resistance.[2–5] Yet, aqueous sampling in all patients as a screening test may not be a feasible option due to its invasive nature. Tear film cytokines, which are regulated by tissue MMPs, are known to be differentially expressed in patients with glaucoma, which may be due to glaucoma pathogenesis or those that are altered by use of antiglaucoma drugs.[6] These cytokines have been reported to be expressed in the human tears and Tenon's capsule in the human eye, suggesting a role in ECM turnover in the ocular surface paralleling their role in ECM turnover in the TM and aqueous.[7,8] Yet, the differential MMP levels at the ocular surface in different forms and severities of glaucoma have not been explored to our knowledge. Considering the fact that tear sampling is a fast and easy standard procedure in ophthalmologic examinations, assessing the tear film could be a potentially powerful noninvasive tool in glaucoma screening. Such tear-based analysis would give us a noninvasive method of

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evaluating the molecular events at the ocular surface in glaucoma. This study is aimed to determine functionally active MMP-9 and MMP-2 levels in tears and ocular surface tissue of patients with different forms of glaucoma naive to any form of medical therapy and to evaluate their role in in vitro TM cell death.

MATERIALS AND METHODS

This cross-sectional study included glaucoma patients attending the glaucoma service at LV Prasad Eye Institute, Bhubaneswar and included only those naïve to any form of prior antiglaucoma medical treatment. The study was approved by the institutional review board (IRB) and adhered to the tenets of the Declaration of Helsinki. An informed written consent was collected from each subject before any ocular examination or procedure as institutional protocol. All newly diagnosed patients with glaucoma visiting the glaucoma service underwent detailed evaluation, including slit-lamp evaluation, Goldmann applanation tonometry at baseline, 4 mirror gonioscopy +90 diopter (D) fundus biomicroscopy, tear breakup time, Schirmer's 1 test and Humphrey visual fields.

Patient Characterization

POAG and PACG were diagnosed in adults >40 years old with IOP > 21 mm Hg on any visit, open or closed angles with optic nerve and visual field damage consistent with glaucomatous optic neuropathy. Inclusion criteria for pseudoexfoliation syndrome were newly diagnosed patients with evident classical dandruff or flaky exfoliation deposits on the pupil, lens or other ocular structures, and radial pigment over the lens surface with normal IOP. Pseudoexfoliation glaucoma was diagnosed in the presence of raised IOP and glaucomatous optic neuropathy with consistent visual field changes in addition to the above features. Glaucoma was defined as those with glaucomatous optic neuropathy evidenced by cupping, rim thinning, notch or retinal nerve fiber layer defects with corresponding visual field defects with no prior use of antiglaucoma drops (naive to medical therapy including any systemic drugs for glaucoma, 2 patients were on systemic antihypertensives at the time of presentation and sample collection). The patients were classified into mild, moderate, and severe glaucoma groups based on mean deviation (MD; early MD > −6 dB), moderate (MD −6 to −12 dB), and severe glaucoma (MD < −12 dB) at baseline according to Hoddap–Parrish–Anderson criteria. Controls were screened from patients attending the outpatient services for routine checkup or for cataract surgery with normal IOP with no evidence of optic nerve damage or other ocular pathology.

Patients with uveitis, neovascular glaucoma, past laser procedures, or antiglaucoma medical treatment, conjunctivitis, allergic blepharitis, or dry eye (Schirmer's 1 < 15 mm) were excluded (Supplementary Table S1) after detailed slit-lamp evaluation to also rule out meibomian gland dysfunction or rounding of duct orifices. Patients with any other autoimmune or neurodegenerative disorder and diabetes mellitus were excluded. Patients with any ocular surface pathology, history of contact lens wear, and absolute glaucoma also were excluded from this study.

Sample Collection and Processing

Using a TearFlo (Accutome, Malvern, PA, USA) Schirmer filter strip, tears were collected from the outer canthus of POAG (n = 27), PACG (n = 24), PXF (n = 40), and PXG (n = 22) patients, and preoperatively from 35 controls who underwent routine

cataract surgery. For tear sample collection, patients were asked to sit in an upright position after which tear strips were placed in the inferior fornix for 5 minutes under aseptic conditions without topical anesthetic. The entire tear strip was collected from each patient and was immediately treated with 200 μL of extraction buffer containing 0.5 M NaCl and 0.5% tween 20 followed by incubation at ambient temperature for 3 hours on a rocker (Tarsons Products, West Bengal, India). They then were centrifuged (Eppendorf, Hamburg, Germany) at approximately 10,000 rpm for 1 to 2 minutes and stored at −80°C for subsequent analysis. Total protein in each sample was analyzed by Bradford-assay (Bio-Rad Protein Assay Dye Reagent-1X; Bio-Rad Laboratories, Hercules, CA, USA). A Biospectrometer (Eppendorf) was used to obtain absorbance at 595 nm and readings were reported in μg/μL. MMP levels in tears were analyzed using gelatin zymography.

Tenon's capsule sample sized 2 × 2 mm was harvested from the inferior quadrant of cases and controls requiring cataract surgery which was done after tear sample collection under topical anesthesia application. All tissues were procured under full aseptic precautions before any surgical incision or initiation of topical glaucoma medication. The site of sampling was washed with antibiotics at the end of the sampling and the samples were stored under 10% formalin for immunohistochemical analysis.

Gelatin Zymography Analysis for MMPs

Gelatinolytic activity of MMP-9 and MMP-2 was examined by substrate gelatin zymography. Equal amounts of proteins obtained from tear samples of patients and controls were separated on 10% SDS-PAGE gels containing 0.1% gelatin. The gels were washed twice with an interval of 1 hour in 2.5% Triton X-100 washing buffer and then incubated in incubation buffer containing 50 mM Tris-HCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$ and 200 mM NaCl, pH 7.5 at 37°C for 18 to 20 hours. Gels were stained with Coomassie solution (0.05% Coomassie brilliant blue R-250, in 40% methanol and 10% acetic acid) and partially destained with destaining solution (20% methanol and 10% acetic acid) to visualize the clear zone of gelatin lysis against the blue background stain indicating the presence of MMPs. The zymographic gels were imaged and lysis zones in every lane analyzed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health [NIH], Bethesda, MD, USA) to obtain band intensity with metalloproteinase 2 and 9 activity expressed in arbitrary units (AU).

Immunohistochemistry Assay

Immunohistochemistry was done on Tenon's capsule harvested from the inferior quadrant surgery (before any incision or topical medication) from patients undergoing either glaucoma or cataract surgery and analyzed for MMPs expressions. Tissues were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in graded alcohol and embedded in paraffin. Paraffin blocks were cut in 6-μm sections and were deparaffinized at 55°C for 30 minutes. Slides were dipped twice in xylene solution for 5 minutes each and subsequently dipped in gradient alcohol solution (90%, 70%, and 50%) for 3 minutes each. After washing the slides in running tap water, antigen unmasking was done in 10 mM sodium citrate buffer with pH 6.0. Sections were washed in wash buffer (1% PBS, 0.1% Tween 20) and subsequently blocked in 3% hydrogen peroxide for 6 to 8 minutes at room temperature following incubation with mouse anti-MMP-9 antibody (1:100, MAB13458; Merck Millipore, Billerica, MA, USA) at room temperature for 1.5 hours. After washing with PBST, sections were incubated with biotinylated secondary goat

TABLE 1. Baseline Characteristics of Patients With Different Types of Glaucoma and Controls Whose Tear MMP Levels Were Assessed

Variables	PXG, $n = 22$, Mean ± SD	PXF, $n = 40$, Mean ± SD	POAG, $n = 27$, Mean ± SD	PACG, $n = 24$, Mean ± SD	Controls, $n = 35$, Mean ± SD	P Value
Age, y	68 ± 8.8	67 ± 7.3	63 ± 7.9	67 ± 11.1	63 ± 8.3	0.8
Male:female, %	90:10	68:32	71:29	54:46	72:28	0.9
IOP, mm Hg	24 ± 12.9	30 ± 5.1	24 ± 6.6	23 ± 14.2	12 ± 2.1	<0.001*
PSD	6.9 ± 3.1	7 ± 5.4	5 ± 3.2	6 ± 3.4	10 ± 2.1	<0.001*
VFI	44 ± 32.7	81 ± 17.5	74 ± 27.4	58 ± 29.2	98 ± 7.9	<0.001*

PSD, pattern standard deviation; VFI, visual field index; IOP, intraocular pressure.
* ANOVA.

anti-mouse antibody (Dako) for 30 minutes. Immune reactions were visualized with incubation in 3,3′diaminobenzidine tetrahydrochloride (DAB) for 8 minutes in dark following the manufacturer's protocol (Dako; LSAB2 System-HRP). Sections were counterstained with hematoxylin and mounted in DPX mounting medium (Fisher Scientific Ltd, India). Slides were examined examination under a bright field (Leica DM 2000) microscope with ×20 and ×40 magnification and images were analyzed by LAS V4.3.Ink software. Three independent images of each patient of ×20 were analyzed under a bright field microscope and the total number of MMP-9–positive cells that were having strong staining was calculated manually.

In Vitro MMP Activity in Human Trabecular Meshwork (HTM) Cells

HTM cells (established cell lines obtained as a gift from Council of Scientific Industrial Research [CSIR], New Delhi, India) were maintained in Dulbecco's modified eagle medium (DMEM) with low glucose (1 gm glucose per liter of media with sodium bicarbonate and pyruvate) supplemented in 10% fetal bovine serum (FBS; PAN-Biotech, Aidenbach, Germany) placed in a 5% CO_2 incubator (Eppendorf) at $37°$C. Cells were treated with different concentrations of hydrogen peroxide (H_2O_2); 100, 200, 400, 600, 800, and 1000 μM for 3 hours to check the effect on cell viability and MMPs expression. Cell viability was checked by MTT (3-[4,5-dimethyl thizol-2-yl]-2,5-diphenyl tetrazolium bromide, tetrazole) assay.

Total proteins were extracted from the samples using protein extraction buffer (150 mM NaCl, 1% Triton X-100, 10% glycerol, 50 mM Tris-HCl with 100 μM phenylmethylsulfonyl fluoride [PMSF] and 100 μM sodium othovanadate) and resolved in 10% SDS-PAGE (Bio-Rad Laboratories). Proteins were transferred to nitrocellulose membrane (Immobilon-N; Merck Millipore) using a mini trans-blot electrophoretic transfer system (Bio-Rad Laboratories) and probed with the following primary antibodies: anti-rabbit MMP 9 (AV33090; Sigma-Aldrich Corp., St. Louis, MO, USA) at 1:400 and anti-GAPDH (ABM22C5, abgeneX at 1:2000, loading control). HRP conjugated anti-rabbit secondary antibody (621140380011730, at 1:3000; Abcam, Cambridge, United Kingdom) was used and bands were visualized by using Enhanced Chemiluminesence (ECL) from Bio-Rad Laboratories. The blots were analyzed using gel-Doc system (Bio-Rad Laboratories) analysis.

Immunofluorescence

HTM cells were plated on sterile coverslips coated with 0.1% gelatin in six-well dishes at 1×10^4 cells per well. Stress was induced with different concentration of H_2O_2 after treating the cells with 800 μM, 1000 μM concentrations for 3 hours. Subsequently, cells were fixed in freshly prepared 4% formalin for 15 minutes at room temperature. After washing with 1× PBS, cells were permeabilized in 0.2% Triton X-100 for 10

minutes at room temperature. Nonspecific sites were blocked using blocking solution (5% FBS) for 20 minutes at room temperature with gentle rocking. Cells were incubated for overnight at 4°C in 1:500 mouse anti-MMP-9 antibody (MAB13458; Merck Millipore). After rinsing with 1× PBS, the cells were incubated in fluorescein isothiocyanate (FITC)-labeled anti-mouse antibody (ab6785 at 1:2000; Abcam) with 2 μg/ml Hoechst for 2 hours at 4°C for nuclear staining. After washing, the coverslips were mounted in DPEX and examined using an Olympus (Tokyo, Japan) fluorescence microscope (Model BX-61) with image-Pro Express software.

Gelatin zymography was performed as described previously for analyzing functional MMPs levels in HTM cells after exposure to H_2O_2.

The primary outcome was to evaluate the tear MMP expression in different forms of glaucoma and controls. Secondary outcomes included the MMP expression on the trabecular cells (TC) harvested from cases and controls and to evaluate the MMP expression after stress induction in HTM cell lines. Differences in MMP expression in different stages of the disease also were compared.

Statistical Methods

All analyses were done using StataCorp (College Station, TX, USA) software (Version 10). Data are presented as means ± SEM. All in vitro experiments were performed in triplicate and repeated three times. Variables between different stages of glaucoma or different groups were analyzed using the 1-way ANOVA test (or Kruskal-Wallis test for nonparametric parameters) with α error set at $P < 0.05$. Correlation of MMP activity with clinical variables, such as baseline IOP, mean deviation on visual field, and diagnosis, was done using Spearman correlation.

RESULTS

A total of 148 tears samples from 90 eyes of 113 cases and 35 controls were examined. This included POAG ($n = 27$), PACG ($n = 24$), PXF ($n = 40$), and PXG ($n = 22$) with no statistical difference in age of the patients between groups ($P = 0.8$). Of these, there was significant difference in mean deviation and visual field index between early glaucoma (10 POAG, 13 PACG, 21 PXF, 16 PXG) with moderate (9 POAG, 10 PACG, 11 PXF, and 3 PXG) and severe glaucoma (Table 1; $P < 0.001$.The mean baseline IOP differed significantly between cases and controls, with the maximum difference noted between PXG and controls (Table 1).

Gelatin Zymography and Immunohistochemistry Analysis

Increased activity of MMP-9 was observed in POAG cases, followed by cases with PACG and PXF compared to cataract. However, the MMP-9 activity was significantly reduced in PXG

TABLE 2. Expression of MMP-9 and MMP-2 in Tear Samples of Glaucoma Patients and Controls

MMPs	Control, Mean ± SEM, $n = 35$	POAG, Mean ± SEM, $n = 27$	PACG, Mean ± SEM, $n = 24$	PXF, Mean ± SEM, $n = 40$	PXG, Mean ± SEM, $n = 22$	Value
MMP-9	1678 ± 160.69	4260 ± 840.58	3701 ± 171.58	3495 ± 335.39	2022 ± 263.64	<0.001*
MMP-2	781 ± 111.33	1093 ± 160.07	527 ± 554.71	866 ± 135.49	820 ± 129.42	0.003*

* 1-way ANOVA with post hoc Bonferroni test.

($P = 0.03$) cases and was comparable to levels in controls. The MMP-2 levels were increased only in POAG cases with no significant differences in PACG, PXF, and PXG cases compared to controls (Table 2; Fig. 1). Supplementary Figures S1 to S4 show some representative gels of individual patients with activities of MMPs for PXF, POAG, PACG, control, PXG while Supplementary Figure S5 shows the tear elution at different time points showing maximum elution at 2 to 3 hours.

MMP-9 Activity in Tenon's Capsule

Increased immunostaining of MMP-9 was observed in the tissue of Tenon's capsule of the human eye in patients with PACG, PXF, and POAG compared to the control with light or absence of immunostaining (Fig. 2). This was reproducible in triplicates.

In Vitro MMPs Activity on HTM

Microscopic examination revealed 100 to 1000 μM H_2O_2 treatment in cultured HTM induced morphologic changes in a concentration dependent manner. Treatment for 2 to 3 hours with H_2O_2 (800 and 1000 μM; Fig. 3a) induced cell–cell adherence into nonrecognizable clusters, elongation of cells, and distorted cell morphology (Fig. 3b; Supplementary Fig. S6), whereas at lower concentration no significant changes ware observed. There was a significant decrease in cell viability upon treatment with H_2O_2 ($P < 0.001$; Table 3; Fig. 3a). Western blot analysis showed overexpression of MMP-9 3 hours after treatment with 800 and 1000 μM of H_2O_2 compared to untreated sample (Fig. 3c), which resulted in high activity of MMP-9 by gelatin zymography (Fig. 3d). Immunofluorescent studies showed increased expression of MMP-9 in HTM cells upon H_2O_2 treatment (Fig. 3e). MMP-9 is localized progressively toward the cell membrane that will be a prerequisite for secretion. We observed increased nuclear size with overexpression of MMP-9 in the presence of higher concentrations of H_2O_2, which may be due to cytotoxic effects of H_2O_2 (Fig. 3e).

We evaluated the correlation between the phenotypic characteristics and glaucoma severity at baseline in each group, and the MMP expression in the patients. The MMP-9
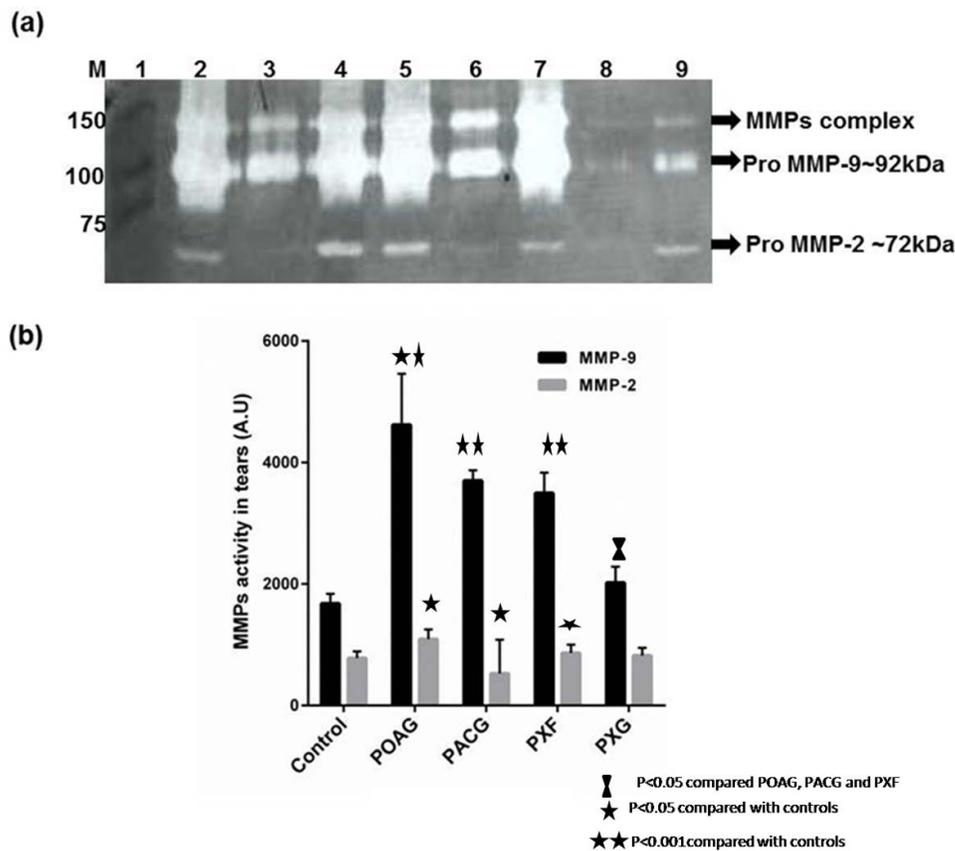

FIGURE 1. Expression of MMP-2 and MMP-9 in tear sample with glaucoma. (A) Representative image of expression of MMP-2 and MMP-9 was studied by gelatin zymography. *Lane 1*, protein molecular weight marker. *Lanes 2* and *3*, PXF. *Lanes 4* and *5*, POAG. *Lanes 6* and *7*, PACG. *Lanes 8* and *9*, Control (cataract). (B) Gelatin zymography showed overexpression of MMP-9 in POAG, PACG, and PXF patients compared to control. No significant change was observed in MMP-2 activity. *Error bars*: SEM.
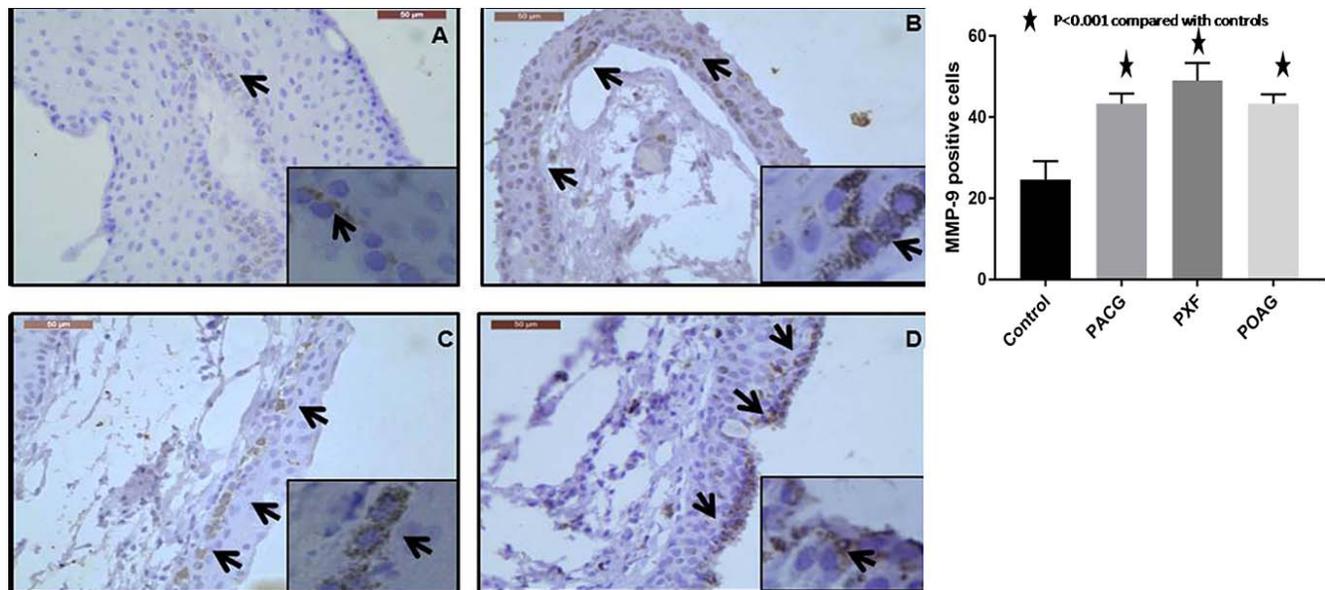
FIGURE 2. Expression of MMP-9 in Tenon's capsule of human eye. Immunohistochemical analysis showed overexpression of MMP-9 in Tenon's capsule of human eye. **(A)** Control, **(B)** PACG, **(C)** POAG, and **(D)** PXF. Bright field microscopic images at ×20 and ×40 (*inset*) magnification. *Scale bar*: 50 μM.

activity correlated with curled edges of exfoliation deposits on the lens surface ($r = 0.7$, $P = 0.02$) and pupillary ruff atrophy in PXG and PXF. It also correlated positively with severity in early glaucoma patients ($r = 0.6$, $P = 0.001$), while it correlated negatively with severity in eyes of patients with severe glaucoma (MD < -12 dB; $P < 0.0001$).

DISCUSSION

We observed increased MMP-9 expression in all forms of glaucoma, with a definite difference between early and severe forms of glaucoma and between pseudoexfoliation syndrome (increased MMP-9 activity) and pseudoexfoliative glaucoma (significantly reduced MMP-9 activity). This was associated with increased MMP-9 expression in Tenon's capsule and in patients with glaucoma compared to controls. There was no significant change in MMP-2 activity in the same patients in any group, which were comparable to expression in controls.

MMPs constitute part of a superfamily of metalloproteinases with conserved catalytic domain which become activated upon cleavage.[2,8] MMP-9 and MMP-2 belong to the family of gelatinases because of their unique ability to degrade gelatin and are distinct from other families of MMPs, such as collagenases (MMP-1, MMP-8, and MMP-13), which can degrade collagens I, II, and III; stromeolysin (MMP-3, MMP-10, and MMP-11), which cannot degrade collagen I but can degrade fibronectin; laminins; and proteoglycans.[2] These have a crucial role in ECM turnover and integrity in tissues and have been studied in various ocular diseases, including glaucoma.[1–4,9] They also are important for cytokine regulation, which is vital for a multitude of processes involved in cell survival and functions. This mandates tight regulation of MMP functions and levels at multiple levels, such as transcription activation, posttranscriptional or epigenetic regulations, and MMP clearance/inhibition by tissue inhibitor of MMPs (TIMPs).[8–12] In the eye, MMPs have a vital role in ECM degradation and remodeling in the TM, which maintains the outflow pathway and IOP homeostasis.[10–11,13,14] Raised IOP induces upregulation of MMPs in TM, which, in turn, increase ECM degradation thereby decreasing outflow resistance. Reversible increase in

outflow facility has been demonstrated in vitro by perfusing the human anterior segment organ culture system with recombinant MMP-2 and -9, which was reversed with inhibition of endogenous MMP activity.[15] Laser trabeculoplasty has been demonstrated to cause increased outflow facility, which is postulated to be caused by increasing MMP activity and ECM degradation along with other mechanisms.[8,11,12,14] Most of these earlier studies evaluated MMP expressions in cell culture and animal models, or in aqueous humor of human patients with glaucoma. This study found increased MMP-9 activity in tears and Tenon's capsule with no parallel increase in MMP-2 activity in any POAG, PACG, or PXG eyes suggesting alterations of MMP levels and function at the ocular surface in different form and severities of glaucoma.

Reduced levels of active MMP-2 and increased TIMP-2 concentrations with decreased proteolytic activity have been reported in patients with PXG and to a lesser extent in POAG eyes.[15] Yet, a parallel increase in total inactive protein level of MMP-2 has been reported, which may indicate an accumulation of the endogenous reserves of MMP-2 though the cause for such an observation is unknown. It also is known that increased protein levels are not accompanied by corresponding changes in their mRNA levels.[8,12,15] Kee et al.[16] reported similar MMP-2 activity in the aqueous humor, in chronic angle closure glaucoma and normal tension glaucoma patients compared to cataract patients, which they postulated to be caused by an alternated pathomechanism of glaucoma damage in these forms of glaucoma. The MMP activity also has been found to be significantly increased in patients with uveitis suggestive of activation of the inflammatory pathway in these patients.[17] So, while it is assumed that the MMPs predominantly exist in their latent state in most forms of human glaucoma, raised total protein levels and differences in MMP levels in different forms of glaucoma suggest an alternate theory behind these conflicting results. Our study found significant differences in early, moderate, and severe glaucoma, reflecting that these differences in results and MMP activity may be explained partly by different stages of the disease in different studies and in animal glaucoma models. Our results suggested an MMP-9 upregulation in early forms of glaucoma,
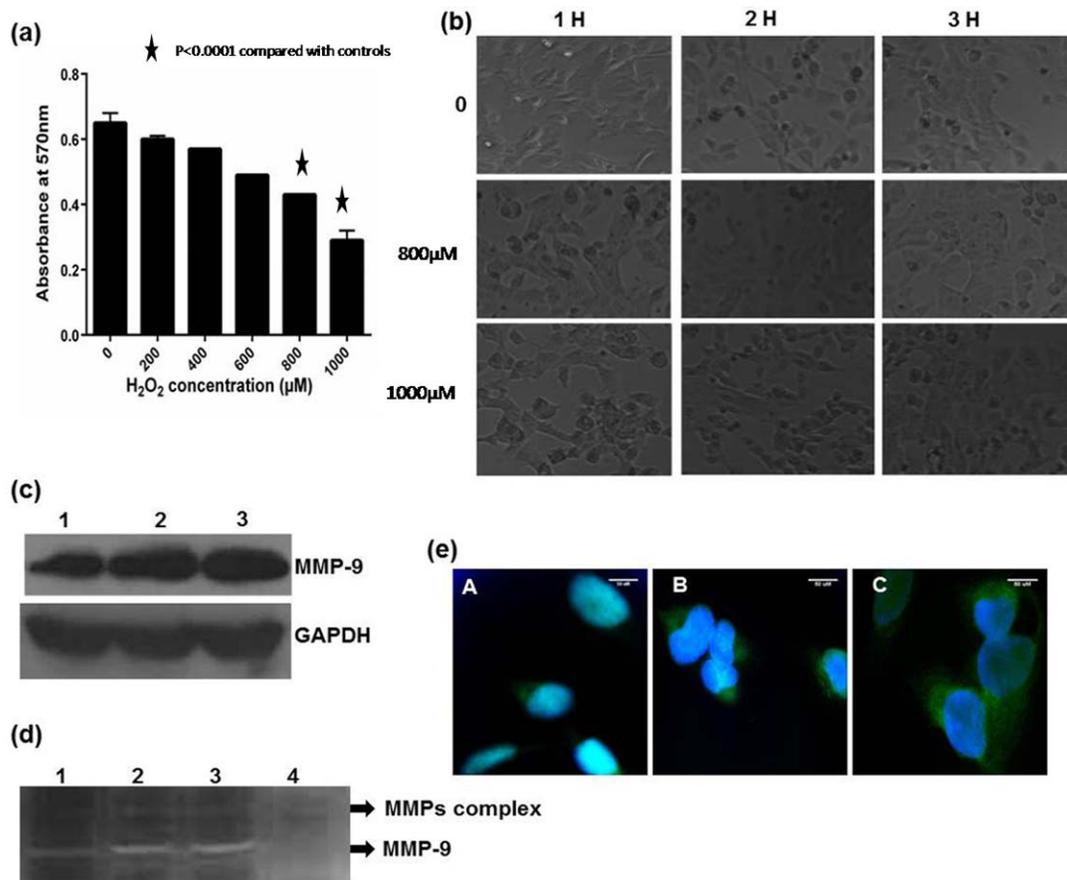
FIGURE 3. H_2O_2 treatment altered morphology in HTM cells with overexpression of MMP-9. (**A**) H_2O_2 treatment-induced cytotoxicity in HTM cells: Treatment with different concentrations of H_2O_2: 0, 200, 400, 600, 800, and 1000 μM of H_2O_2, in HTM cells examined by MTT assay. *Error bars*: SD. (**B**) Increasing concentration of H_2O_2 (800 and 1000 μM) induced different morphologic changes at different time intervals. *Row 1*, untreated cells. *Row 2*, 800 μM H_2O_2. *Row 3*, 1000 μM H_2O_2. Time interval after H_2O_2 treatment is shown on *top* of each column. Bright field (DIC) images at ×10 magnification. (**C**) H_2O_2 treatment induced overexpression of MMP-9 in HTM cells: HTM cells were treated with different concentration of H_2O_2 and MMP-9 expression level were analyzed by Western blotting. GAPDH was used as loading control. *Lane 1*, control. *Lane 2*, 800 μM H_2O_2. *Lane 3*, 1000 μM H_2O_2. (**D**) Overexpression of MMP-9 led to increased activity of MMPs: Gelatin zymographic assay was used to check the activity of MMP-9. *Lane 1*, control. *Lane 2*, [800 μM] H_2O_2. *Lane 3*, [1000 μM] H_2O_2. *Lane 4*, marker. (**E**) Immunofluorescent studies showed H_2O_2 treatment ([**A**] Control, [**B**] 800 μM, and [**C**] 1000 μM) induced expression of MMP-9 in HTM cells. (*blue*, nucleus stain; *green*, FITC conjugated MMP-9). Fluorescence microscopic images at ×100 magnification. *Scale bar*: 50 μm (×100).

which may be an attempt to increase ECM turnover and outflow facility, which is overwhelmed in later stages of the disease process as seen in severe glaucoma where the levels drops to levels comparable to controls. This may be the cause for reduced expression in PXG cases, most of which presented in later stages of the disease.

We observed increased MMP expression on in vitro experiments on HTM cell culture after stress induction with localization of MMP-9 near cell membrane. The role of MMPs and the inflammatory pathways in glaucoma pathogenesis has been known though the exact mechanism or regulatory process controlling MMP expression in different issues, especially the TM, in different stages, is unknown.[1–4,8] Activation of the inflammatory pathway molecules has been reported in PXF, which is known to behave differently compared to other primary glaucomas (where the pathogenesis is believed to be either IOP-induced, vascular, ischemic, or even anatomical as seen in PACG).[1,18] The glaucoma seen in PXG eyes is rapidly progressive and more resistant to conventional medical treatment with known associations with vaso-occlusive diseases.[18] While earlier studies have proven oxidative stress and activation of MMPs in different forms of glaucoma, these studies are based predominantly on

aqueous samples and TM.[1,10,11] Our results of increased MMP activity in early forms of POAG, PACG, and PXF with reduced expression of MMP-9 in PXG with similar results on HTM culture, reflected occurrence of parallel and similar molecular changes at the ocular surface and tears in eyes with glaucoma even without dry eye or those that are naïve to medical treatment. This can be characterized and analyzed further for

TABLE 3. Effect of H_2O_2 on Proliferation of In Vitro HTM Cells as Evidenced by MTT Assay Indicating Absorbance at 570 nm Indicating Proportion of Live Cells

H_2O_2 Concentration on HTM	Absorbance at 570 nm, Mean ± SD
0	0.65 ± 0.037
200 μM	0.60 ± 0.015
400 μM	0.57 ± 0.004
600 μM	0.49 ± 0.002
800 μM	0.43 ± 0.004
1000 μM	0.29 ± 0.032

Concentration of H_2O_2 in HTM compared to control. *P* value was 0.03 between control and 800 μM and 0.07 between 800 and 1000 μM.

use as a diagnostic assay for occurrence of glaucoma in PXF and noninvasive screening for glaucoma. Our results suggested an activation of the inflammatory pathway in early stages of the disease, which, however, is reversed in severe stages of the disease where the reduced expression may represent altered or aberrant regulation of MMPs and ECM turnover with predominant profibrotic process, or purely represent depletion of functional or viable cells with residual intact regulatory functions in advanced disease. Therefore, while upregulation of MMP activity can be a marker for early disease, reversal of this heightened activity can serve as a marker for disease progression.

Our results only showed increased MMP-9 activity in tears and Tenon's capsule of patients with different forms of glaucoma. Tear fluid assays can serve as a very novel diagnostic approach for ocular surface diseases owing to the low protein concentrations in tears compared to serum, which can reflect disease-correlated biomarker patterns and signature cytokine profiles. Studies have reported altered cytokine profile in eyes receiving antiglaucoma therapy.[5] Increased levels of MMP-3 and MMP-9 have been reported in tears of patients treated with prostaglandin analogues. Results have suggested that there may be active inflammation pathways related to the glaucoma disease or it may be induced by antiglaucoma therapy.[5,7] One pilot study evaluating the MMP-9 activity as a diagnostic assay found increased expression in tears of 80% of PXF cases and 20% controls.[19] This assay had a specificity and sensitivity of 80%, although the investigators included only PXF eyes and none with glaucoma. Similar results of tear cytokines signature profiles indicating disease have been reported in diabetic retinopathy and animal models of Sjögren's syndrome.[20,21] The MMPs also have been implicated in postoperative fibrosis after filtering surgeries and after antiglaucoma therapy.[6] It is unclear if the increased MMP activity in tears seen in our study represents actual MMP-9 upregulation in tears as a part of general inflammatory pathway activation in glaucomatous eyes or is a result of progressive leeching from the ocular surface tissues, like conjunctiva. While presence of inflammation was ruled out by clinical examination in our patients, the possibility of activation of inflammatory pathways at the ocular surface in patients naïve to medical therapy suggests the possibility of subclinical inflammation early in the disease process.[1–4,5] Monitoring of these events can help evaluate disease progression in future studies.

We did not evaluate aqueous levels in this study, which aimed to study differences in tear expression levels. Also, we did not evaluate Tenon's fibroblast cultures, which may have corroborated our observations of MMP-9 expression in surgical specimens. Our study was a cross-sectional study with no evaluation of long-term changes in MMP profile over time or glaucoma progression, which mandates future studies.

In summary, tear MMP9 profiles may act as useful biomarkers for screening of glaucoma, including POAG, PACG, and pseudoexfoliation syndrome. This may pave the way for easy and effective diagnostic tear-based assays to detect glaucoma cases at low cost settings in the future. Their use in prognosticating disease and detecting glaucoma progression with changing MMP profiles longitudinally needs further elaborative long-term studies.

Acknowledgments

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