Changes in Matrix Metalloproteinases in Diabetes Patients' Tears After Vitrectomy and the Relationship With Corneal Epithelial Disorder

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Purpose. Previous studies indicate involvement of matrix metalloproteinases (MMPs) in the pathogenesis of diabetic keratopathy. To evaluate MMP levels in the tears of patients with diabetes, we investigated changes in MMP levels during perioperative periods and clarify the relationship with corneal epithelial disorders following vitrectomy.

Methods. Matrix metalloproteinase levels in tears were measured by multiplex bead array in patients with or without diabetes who were scheduled for vitrectomy. Twenty-two patients with diabetes and proliferative diabetic retinopathy, and 20 patients with epiretinal membrane or macular hole (control group), were recruited. Changes in MMP levels during perioperative periods and the relationship with corneal epithelial disorders after vitrectomy were analyzed.

Results. The levels of MMP-2, -9, and -10 at 1 day after surgery in the diabetic group were significantly higher than in the control group. At 1 week after surgery, MMP-10 levels in the diabetic group were significantly higher than in the control group. After vitrectomy, corneal epithelial disorders occurred in six patients in the diabetic group but not in the control group. In the diabetic group, MMP-10 levels in tears of patients with corneal epithelial disorders were significantly higher than those in patients without corneal epithelial disorders.

Conclusions. The MMP concentration in tears of patients with diabetes was higher than in nondiabetic patients after vitrectomy. High MMP-10 levels were observed in patients with diabetes and corneal epithelial disorders after vitrectomy. Aberrant levels of MMP-10 may cause corneal epithelial disorder after vitrectomy.

Keywords: diabetic keratopathy, matrix metalloproteinase, MMP-10, tear

Diabetic retinopathy leads to severe vision loss in patients with diabetes mellitus (DM) and remains a major cause of blindness in the world.[1] Diabetes also affects other parts of the eye, such as the cornea, tear film, lens, iris, and optic nerve.[2] Corneal abnormalities are found in approximately 50% to 60% of patients with diabetes,[3,4] and keratopathy has been recognized as a major complication in patients with diabetes.[5] Diabetic keratopathy involves superficial punctate keratopathy, recurrent corneal epithelial erosion, and ulcers with epithelial detachment.[6] These corneal problems frequently develop after intraocular surgical procedures, such as cataract extraction or vitrectomy,[7–10] and can be difficult to treat because of delayed epithelial wound healing.

Several mechanisms for diabetic keratopathy have been proposed, including the accumulation of advanced glycation end products,[11,12] activation of the polyol pathway,[13–15] and increased osmotic stress. The changes in cell adhesion and tissue repair are apparently involved in the pathogenesis of diabetic corneal abnormalities, which are likely due to alterations in the adhesive molecules of the extracellular matrix and basement membrane.[16,17] Matrix metalloproteinases (MMPs) are proteolytic enzymes, which are collectively capable of degrading almost all the components of the extracellular matrix and basement membrane.[18–21] They are produced from epithelial cells, stroma, and inflammatory cells in the cornea; increased activities of MMPs are implicated in many critical physiological and pathologic processes, including development, wound healing, tissue remodeling, angiogenesis, and inflammation. Thus, it is necessary to understand and evaluate how MMPs contribute to the clinical feature of diabetic keratopathy.

Matrix metalloproteinase-10 is specifically upregulated in the human corneal epithelial layer and stroma of patients with diabetic retinopathy; diabetic corneas also have decreased expression of the major corneal epithelial basement membrane components laminin-10, nidogen-1, and the laminin-binding integrin $\alpha 3\beta 1$.[16,17,22,23] Recent in vitro analyses have demonstrated that the addition of recombinant MMP-10 protein attenuates the adhesion of cultured human corneal epithelial cells and decreases the expression of integrin $\alpha 3\beta 1$.[24] Moreover, the addition of MMP-10 delays corneal wound closure in a diabetic keratopathy rat model[25] and the knockdown of MMP-10 in the cultured model of human diabetic cornea improves corneal erosion,[26] indicating that aberrant levels of MMP-10 could damage corneal epithelium.

Many studies have indicated that MMPs in tears are involved in the pathogenesis of various ocular surface diseases, such as dry eye,[27] vernal keratoconjunctivitis,[28] conjunctivochalasis,[29]

ocular rosacea,[30] Sjögren's syndrome, and meibomian gland dysfunction.[31] Although the collection and evaluation of corneal tissue samples from patients would be clinically difficult owing to anatomic and ethical reasons, the analysis of tears could be a useful tool to understand the state of the ocular surface environment.[32]

Taken together, MMP-10 appears to play an important role in the pathogenesis of diabetic keratopathy including disorders of corneal epithelium following intraocular surgery. Tear analysis may be useful for monitoring the levels of MMPs and investigating their role in the pathogenesis of diabetic keratopathy. In this study, we analyzed the levels of MMPs in the tears of patients with diabetes, and investigated the changes in MMP levels during the perioperative periods as well as the relationship with corneal epithelial disorders following vitrectomy.

Methods

Patient Selection

This study was approved by the institutional review board of University of Fukui Hospital, Fukui, Japan. The protocol adhered to the tenets of the Declaration of Helsinki. All of the participants gave their informed consent to participate in this study.

In this clinical study, the levels of MMPs in tears were measured in patients with or without diabetes who were scheduled for vitrectomy. The patients were enrolled between November 8, 2013, and May 23, 2014, at the University of Fukui Hospital with the following inclusion criteria: patients aged 20 years or older and those who were scheduled for vitrectomy with or without type 2 DM. The patients with diabetes had proliferative diabetic retinopathy associated with vitreous hemorrhage and/or tractional retinal detachment. The control group patients without diabetes were selected from patients with epiretinal membrane or macular hole who were scheduled for vitrectomy.

The exclusion criteria included the presence or history of ocular surface diseases such as dry eye, allergic conjunctivitis including vernal conjunctivitis, or conjunctivochalasis, which could possibly interfere with the results; contact lens users; history of ocular surgery (except for phacoemulsification and intraocular lens implantation at least 1 year before study recruitment); and the presence of a corneal epithelial disorder before vitrectomy with fluorescein staining, such as superficial punctate keratopathy or corneal epithelial erosion.

Tear Sample Collection

The tears were collected from patients at 1 day before, 1 day after, 1 week after, and 8 weeks after surgery. The sampling procedure was performed essentially as described previously,[33] but with a few modifications. After checking that each eye was free from ocular surface diseases through slit-lamp microscopy, tear samples were nontraumatically collected from the inferior meniscus of patients by using a microcapillary tube (Drummond Scientific Company, Broomall, PA, USA). Tear samples were obtained by using capillary flow and without nasal stimulation. Care was taken to avoid touching the corneal and conjunctival surfaces. No ocular medication was instilled in the patients' eyes on the day of tear sample collection, and no anesthetic drops were instilled before tear collection. All tear samples were collected around 9 AM to avoid diurnal variations. Sampling was identically performed by the same clinician (TM). The collected tears were frozen at –80°C immediately and stored until analysis.

Tear Analyses With the Multiplex Bead Array

The concentration of each MMP (MMP-2, -9, and -10) in the tear samples was analyzed by using a MILLIPLEX MAP Human MMP Magnetic Bead Panel 2 kit (Merck Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Briefly, color-coded microspheres with two fluorescent dyes coated with specific capture antibodies were allowed to react with 25 μL of each diluted sample or with a standard solution at room temperature for 2 hours. The incubated beads were washed twice, and then a biotinylated detection antibody was introduced and incubated at room temperature for 1 hour. Streptavidin-phycoerythrin, which binds biotinylated detection antibodies, was used to detect the reaction mixture. The MMP levels were measured by a Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA, USA). The sample concentrations were calculated automatically by the Bio-Plex Manager Software (Bio-Rad Laboratories) by using a standard curve of known concentrations of recombinant human protein standards that had been supplied with the kits.

Surgical Procedures

All patients underwent a standardized transconjunctival 25-gauge pars plana vitrectomy as described previously[34] with the Constellation Vision System (Alcon Laboratories, Inc., Fort Worth, TX, USA). All 25-gauge vitrectomy procedures were performed by two experienced surgeons (YoT and TT) during the study periods. Four 25-gauge cannulas were inserted obliquely and transconjunctivally into the eye by using a trocar, with one cannula used for chandelier illumination. A wide angle viewing system, Resight 500 (Carl Zeiss Meditec AG, Jena, Germany), was used during the vitrectomy, and care was taken to avoid drying the corneal epithelium by coating the corneal surface with Viscoat Ophthalmic Viscoelastic Substance (Alcon Laboratories, Inc.) during surgery. A central core vitrectomy was performed, and detachment of the posterior hyaloid was induced with the aid of triamcinolone. Removal of the preretinal membrane, endophotocoagulation, and/or a fluid–20% SF_6 gas tamponade was performed as needed. Standard phacoemulsification and intraocular lens implantation were performed when needed before the vitrectomy. Surgery was completed by removing the cannulas without suturing the conjunctiva and sclera. When apparent leakage was observed from the wound, it was closed with a running 9-0 nylon suture, which was removed the next day. All patients received similar postoperative topical medications: 1.5% levofloxacin thrice a day for 2 months, 0.1% betamethasone sodium phosphate thrice a day for 1 month, and 0.1% bromfenac sodium hydrate twice a day for 3 months.

Examination of Corneal Epithelial Disorder

After vitrectomy, the corneal epithelium was stained with fluorescein dye, and then examined by slit-lamp microscopy. When we observed corneal epithelial erosion or severe superficial punctate keratopathy, which meant there was corneal staining with a score of 10 or more according to the National Eye Institute–recommended grading system,[35] we defined the ocular surface condition as corneal epithelial disorder after vitrectomy.

Statistical Analysis

Statistical analyses were performed by using JMP 10 (SAS Institute, Inc., Tokyo, Japan). Values are given as means ± standard deviation (SD). The Wilcoxon signed rank test was used to compare the pre- and postsurgical levels of MMPs in

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TABLE. Patient Demographic Data

Characteristic	Diabetic Group, $n = 22$	Control Group, $n = 20$	P Value
Sex, no. (%)			
Male	14 (64)	9 (45)	0.23
Female	8 (36)	11 (55)	
Age, y			
Mean (SD)	62.8 (12.6)	69.5 (9.6)	0.08
Range	32–82	42–85	
Side of surgical eye			
Right/left	10/12	11/9	0.54
Combined cataract surgery			
Yes/no	10/12	16/4	0.02
Surgical time, min			
Mean (SD)	62.8 (19.9)	70.5 (13.4)	0.14
Range	24–107	48–95	
Gas tamponade			
Yes/no	7/15	8/12	0.58
Hemoglobin A1c, %			
Mean (SD)	7.92 (1.68)	–	
Range	5.6–14.2	–	

tears from patients. Statistical comparisons between the diabetic group and control group were performed by using the χ^2 test and Mann-Whitney nonparametric test. Statistical comparisons of the tear MMP levels between the diabetic and control groups were performed by using the Mann-Whitney nonparametric test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

A total of 42 patients were enrolled in the study: 22 patients with diabetes for the diabetic group and 20 patients without diabetes for the control group. Sex, age, side of surgical eye, surgical time, or use of gas tamponade was not significantly different between both groups (Table). The rate of combined cataract surgery in the control group was higher than that in the diabetic group ($P = 0.02$). The differences in any MMP levels were not significant between the eyes that underwent vitrectomy alone and the eyes that were combined with cataract surgery at any time point in either the diabetic group or control group (data not shown).

We measured the levels of MMPs in the tears before and after vitrectomy with the multiplex bead array and compared them between the patients with diabetes and those without diabetes (control group) (Fig. 1). Preoperatively, there were no significant differences between the patients with diabetes and controls in the levels of MMPs. On the first postsurgical day, the levels of MMP-2 (Fig. 1A), MMP-9 (Fig. 1B), and MMP-10 (Fig. 1C) in both groups were significantly increased, and those in the DM group were approximately 2.5-, 2.0-, and 2.7-fold higher, respectively, than those of the control group in the levels of MMP-2 ($P = 0.008$), MMP-9 ($P = 0.04$), and MMP-10 ($P = 0.003$). At 1 week after surgery, the value of MMP-10, but not MMP-2 and MMP-9, was significantly higher than that of controls. Each of the MMP levels returned to insignificant levels, to their status before surgery at 1 week and 8 weeks in control and diabetic groups, respectively.

In 27% of patients with diabetes, corneal disorders including corneal epithelial erosion (Fig. 2A) or severe superficial punctate keratopathy (Fig. 2B) after vitrectomy, occurred 2 weeks after vitrectomy; no instances of the corneal disorder occurred in the control group. In the diabetic group, the MMP-10 levels in the tears of patients who developed corneal epithelial disorder after vitrectomy were significantly higher than those in patients without a corneal epithelial disorder at 1 day after surgery ($P = 0.04$, Mann-Whitney nonparametric test; Fig. 3C). However, no significant differences were found in the MMP-2 or -9 levels between the patients with corneal epithelial disorder after vitrectomy and those without corneal epithelial disorder at 1 day after surgery (Figs. 3A, 3B).

DISCUSSION

Increased MMP levels in tears are involved in the pathogenesis of various ocular surface diseases.[27–29] In this study, we found that the MMP concentration in the tears of patients with diabetes was significantly higher than that in patients without diabetes after vitrectomy. Among the MMPs in the tears, MMP-10 was associated with a corneal epithelial disorder after vitrectomy for patients with diabetes. These data suggested that vitrectomy could cause abnormally higher concentrations of MMP-10 in the tears of patients with diabetes, which resulted in the association with corneal disorders after surgery.

Disorders of corneal epithelium have been frequently observed in patients with diabetes after vitrectomy.[7–10] Actually, our data showed that postoperative corneal erosion occurred in 27% of the patients with diabetes. In addition, these patients had higher levels of MMP-10 in their tears, but not higher levels of MMP-2 or MMP-9. Previous evidence has supported the view that MMP-10 is important in the pathogenesis of diabetic keratopathy including corneal epithelial disorder. The MMP-10 weakens the attachment of the human corneal epithelium.[24] Moreover, MMP-10 protein delays wound healing of the corneal epithelium in a diabetic rat model; however, this does not occur with MMP-2 and -9.[25] These experiments demonstrate that high glucose levels enhance MMP-10 expression in the corneal epithelium, which can be followed by the degradation of integrin subunits and fragile attachment of the corneal epithelium. In the present study, the patients who had developed corneal erosions after vitrectomy had higher levels of MMP-10 in their tears. These findings suggested that aberrant levels of MMP-10 in the tears can cause a corneal epithelial disorder after vitrectomy in patients with diabetes.

Our data showed that the levels of tear MMP-10 obtained at 1 day after surgery were significantly higher in the diabetic patients with corneal disorders, while corneal disorders were observed within 2 weeks after surgery. Based on these results, it seems that there is a time lag between the peak in MMP-10 levels and the onset of corneal disorders. This is perhaps because several days may be required for the degradation of the basement membrane secondary to the enhanced levels of tear MMP-10.[18–21] Since the amount of tear MMP-10 at 1 week after surgery remained higher than that of control, prolonged high levels of MMP-10 in tears probably contributed to the occurrence of the corneal disorders.

On the other hand, MMP-2, -9, and -10 in tears were shown to have a transient elevation after vitrectomy. These changes during the early postoperative period may reflect the inflammatory response to the surgical invasion at the ocular surface induced by vitrectomy. The values of MMPs in tears have been used as a biomarker of the inflammatory reaction at ocular surface microenvironment. Actually, the

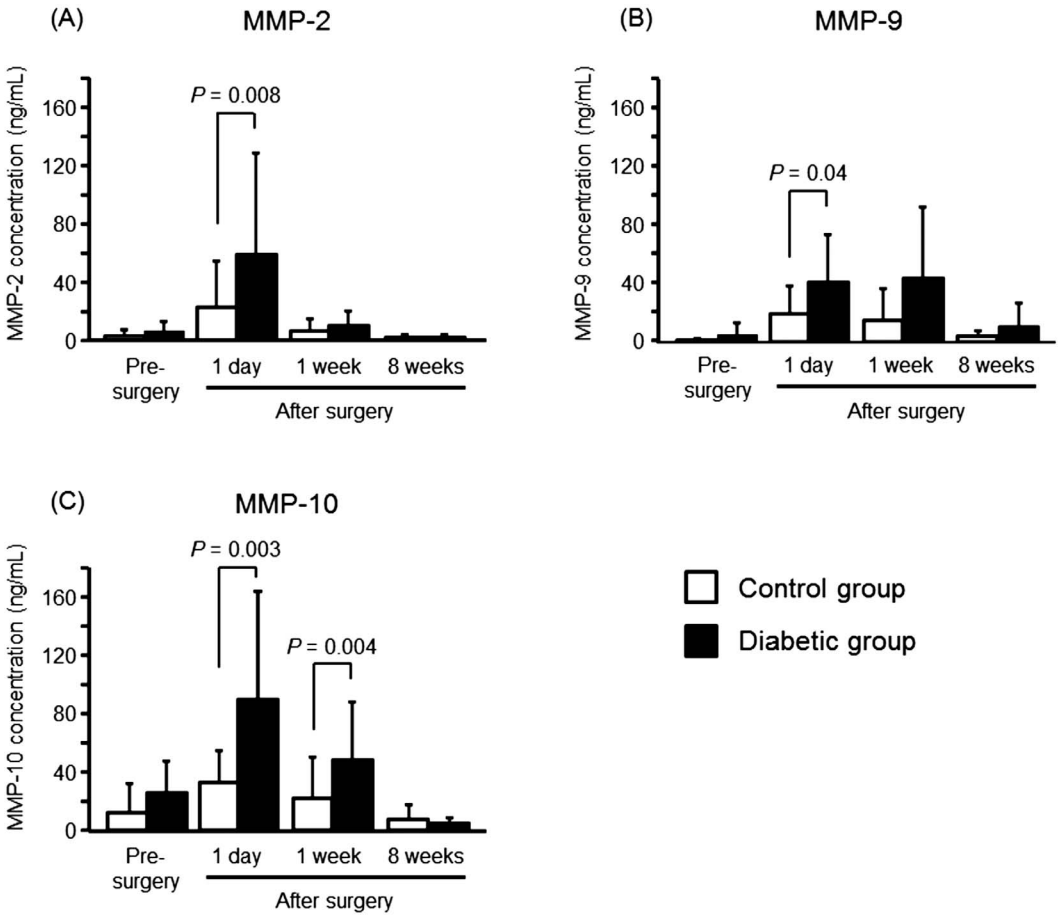

Figure 1. The levels of MMPs in the tears of patients with or without diabetes after vitrectomy. (**A**) The MMP-2 level in the tears was significantly increased in the group of patients with diabetes at 1 day after vitrectomy ($P = 0.008$). (**B**) The MMP-9 level in the tears was significantly increased in the group of patients with diabetes at 1 day after vitrectomy ($P = 0.04$). (**C**) The MMP-10 levels in the tears were significantly increased in the group of patients with diabetes, at 1 day, and at 1 week after vitrectomy ($P = 0.003$, $P = 0.004$, respectively). All data (means ± SD) were compared with the control patients without diabetes by using the Mann-Whitney nonparametric test.

evaluation of the level of MMP-9 in tears can contribute to the diagnosis of inflammatory dry eye disease[36] and conjunctivochalasis.[29] In the present study, all MMPs (-2, -9, and -10) were amplified transiently after vitrectomy; however, only the levels of MMP-10 in the diabetic group remained significantly higher than those in the control group at 1 week after surgery. Although vitrectomy can induce the elevation of MMPs levels in tears, the change in MMP-10 was a more specific finding for the patients with diabetes. Only MMP-10 was overexpressed in the diabetic corneal epithelium[22]; therefore, these changes were likely to occur in the tears of patients with diabetes.

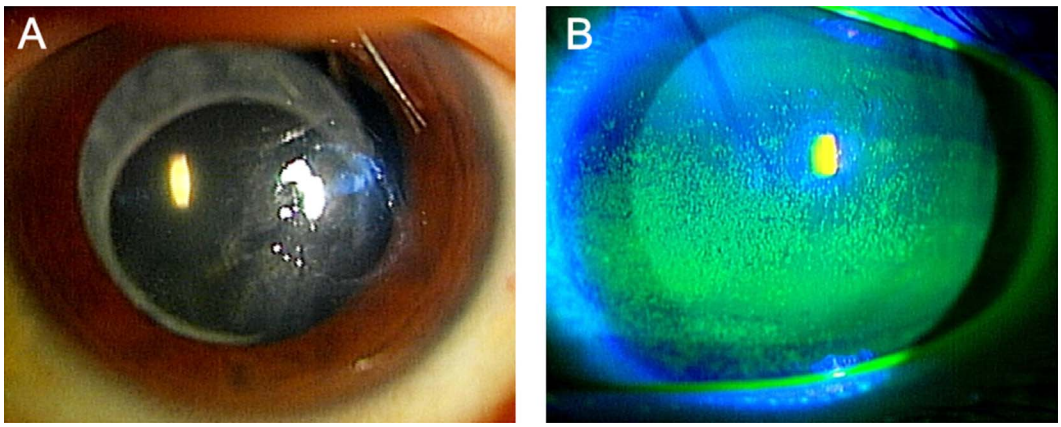

Figure 2. Representative cases of corneal disorders of patients with diabetes. (**A**) Slit-lamp appearance of a case of corneal epithelial erosion. (**B**) A case of severe superficial punctate keratopathy seen with fluorescein stain.

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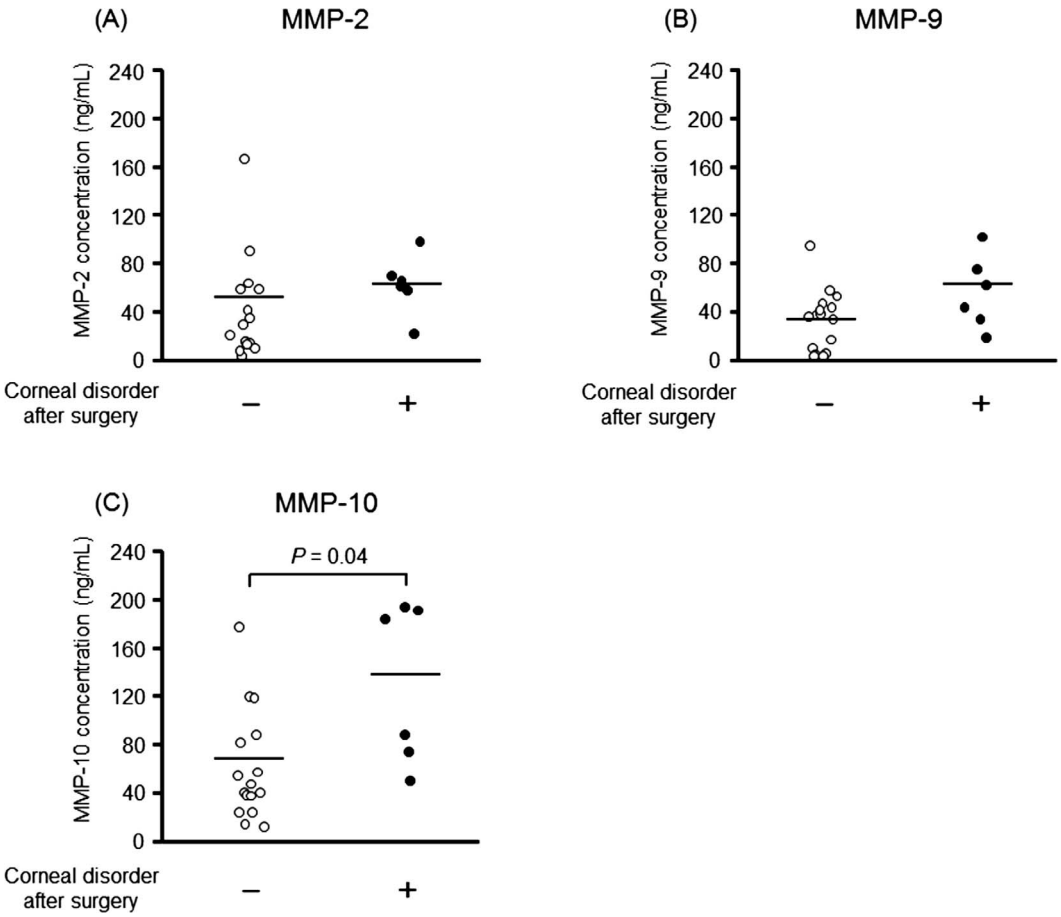

FIGURE 3. The levels of MMPs in the tears of the diabetic group with or without corneal epithelial disorder after vitrectomy. Significantly higher levels of MMP-10 (**C**) were observed in the tears of patients who developed a corneal epithelial disorder after vitrectomy (*filled circles*) in comparison to those in the patients without a corneal disorder (*open circles*). There were no significant differences in the levels of MMP-2 (**A**) and MMP-9 (**B**) in the tears between the patients with and without corneal disorders. Statistical comparisons were performed by using the Mann-Whitney nonparametric test. *Solid bar* indicates the mean value.

Although we observed changes in MMPs and investigated the involvement with corneal epithelial disorder after vitrectomy, corneal disorder can also be accelerated by dysfunctional tear syndrome, which has been frequently noticed in patients with severe diabetic retinopathy.[37] Since we excluded patients with lower tear menisci, short tear break-up time, and corneal epithelial disorder before surgery, the epithelial damage may have been induced as a result of intraocular surgical invasion and was independent of dry eye disease. Matrix metalloproteinase-9 could be a marker of dry eye,[36] because abnormally elevated MMP-9 levels ($\geq$40 ng/mL) in tears have been shown to correspond with moderate to severe dry eye disease. In fact, all samples that we collected before surgery had values below 40 ng/mL; therefore, these data supported the criteria we used to exclude the patients with dry eye.

There were several limitations in this study. We did not measure other inflammatory cytokines, such as interleukin-1β or tumor necrosis factor-α, which also can injure corneal epithelium. Interleukin-1β and tumor necrosis factor-α can disrupt the barrier function of cultured human corneal epithelial cells by impairing the tight junction proteins,[38,39] and the level of interleukin-1β in tears increases after 20- and 23-gauge pars plana vitrectomy.[34] Therefore, these factors may also influence the development of corneal epithelial disorder after vitrectomy. In addition, it remains unclear whether other intraocular surgeries, such as glaucoma surgery or lens extraction, can upregulate the expression of MMPs in patients with diabetes because we recruited patients who were scheduled for vitrectomy. Furthermore, the increased levels of MMPs in tears may be secreted from corneal tissue; however, we cannot rule out other possibilities, such as secretion from the lacrimal gland, because we did not obtain tissue samples from patients owing to ethical reasons.

In conclusion, MMP concentrations in the tears of patients with diabetes were higher than those of nondiabetic patients after vitrectomy. High MMP-10 levels in tears were observed in patients with diabetes who had corneal epithelial disorders after vitrectomy. These observations suggested that aberrant levels of MMP-10 in tears may cause these corneal epithelial disorders after vitrectomy. Tear samples could be helpful for evaluating the status of diabetic keratopathy.

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