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Matrix metalloproteinase-14 is a biomarker of angiogenic activity in proliferative diabetic retinopathy

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Purpose: Matrix metalloproteinase-14 (MMP-14) is a transmembrane MMP that plays a critical role in promoting angiogenesis. We investigated the expression levels of MMP-14 and correlated the levels with clinical disease activity and with the levels of the angiogenic factors vascular endothelial growth factor (VEGF) and MMP-9 in proliferative diabetic retinopathy (PDR). To reinforce the findings at the functional level, we examined the expression of MMP-14 in the retinas of diabetic rats.

Methods: Vitreous samples from 34 patients with PDR and 18 nondiabetic patients and epiretinal membranes from 13 patients with PDR and the retinas of rats were studied with enzyme-linked immunosorbent assay, immunohistochemistry, western blotting, and real-time reverse transcription PCR (RT–PCR).

Results: The MMP-14, VEGF, and MMP-9 levels were statistically significantly higher in the vitreous samples from patients with PDR than in the samples from the nondiabetic controls ($p<0.001$ for all comparisons). The MMP-14 levels in patients with PDR with active neovascularization were statistically significantly higher than those in patients with inactive PDR ($p<0.001$). There were statistically significant positive correlations between levels of MMP-14 and levels of VEGF ($r = 0.3$; $p = 0.032$) and MMP-9 ($r = 0.54$; $p<0.001$). In the epiretinal membranes, MMP-14 was expressed in vascular endothelial cells, leukocytes, and myofibroblasts. Statistically significant positive correlations were detected between the numbers of blood vessels expressing CD31 and the numbers of blood vessels ($r = 0.74$; $p = 0.004$) and stromal cells ($r = 0.72$; $p = 0.005$) expressing MMP-14. Statistically significant increases of MMP-14 mRNA and protein were detected in rat retinas after induction of diabetes.

Conclusions: These results suggest that MMP-14 is involved in PDR angiogenesis.

Ischemia-induced angiogenesis and expansion of extracellular matrix (ECM) in association with the outgrowth of fibrovascular epiretinal membranes at the vitreoretinal interface are the pathological hallmark features in the ocular microenvironment of patients with proliferative diabetic retinopathy (PDR). Pathologic growth of new blood vessels, proliferation of α-smooth muscle actin (α-SMA)-expressing myofibroblasts, infiltration of inflammatory leukocytes, and local production of proinflammatory cytokines, chemokines, and matrix metalloproteinases play considerable roles in disease progression [1-7]. These findings reinforced the paradigm that neovascularization, inflammation, and fibrosis are critical mechanisms for the development and progression of PDR.

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Angiogenesis, the formation of new blood vessels from preexisting ones, is controlled by a dynamic balance between proangiogenic and antiangiogenic factors. An upset in the balance in favor of angiogenic factors leads to the formation of new vessels, whereas the prevalence of antiangiogenic factors shifts the equilibrium to vessel quiescence [8,9]. Of the many angiogenic factors produced in the ocular microenvironment of patients with PDR, vascular endothelial growth factor (VEGF) has been the subject of extensive research because of the factor's selective mitogenic effect on endothelial cells, promoting retinal neovascularization and vascular leakage [10,11]. However, angiogenesis depends on multiple factors, and when the activity of one angiogenic factor such as VEGF is suppressed, the expression of other angiogenic factors may appear [12].

Angiogenesis is a multistep process requiring the degradation of basement membranes and the ECM, the proliferation, migration, and differentiation of endothelial cells, and the formation of capillary tubes [13]. Activated endothelial cells progress through the ECM by local proteolysis involving matrix metalloproteinases (MMPs) [13].

In the ocular microenvironment of patients with PDR, the levels of the MMPs MMP-1, MMP-7, and MMP-9 [6] and extracellular matrix metalloproteinase inducer (EMMPRIN) [14] are increased. This upregulation is linked to angiogenesis of PDR. In addition, MMPs may facilitate pathologic neovascularization through stimulation of the production of VEGF [15] and the proteolytic release of VEGF from the ECM-associated reservoirs [16,17], resulting in increased VEGF bioavailability.

MMP-14, also called membrane type 1 MMP, is a transmembrane collagenase that plays a critical role in conferring cells with the ability to remodel and penetrate the ECM. MMP-14 is shown to be expressed in highly migratory cells and drives invasion by functioning as a pericellular collagenase. MMP-14 has a direct activity against different ECM proteins, including gelatin, fibronectin, vitronectin, fibrillar collagens, and aggrecan [18-24]. Several authors demonstrated that MMP-14 is a critical regulator of angiogenesis [19,20,25]. MMP-14 is upregulated in many tumor types and has been implicated in tumor progression, angiogenesis, and metastasis [26-30].

Regulation of angiogenesis has emerged as a potential therapeutic strategy for the treatment of PDR [31]. To aid the progress of these strategies, a more comprehensive understanding of the molecules that regulate angiogenesis in PDR is of value to identify additional therapeutic targets. Given the critical roles of MMP-14 in regulating angiogenesis, we hypothesized that MMP-14 may be involved in the pathogenesis of PDR. Therefore, we investigated the expression of MMP-14 in the vitreous fluid and epiretinal fibrovascular membranes from patients with PDR and nondiabetic controls and correlated MMP-14 levels with the levels of the known angiogenic factors VEGF and MMP-9. In addition, to corroborate a functional link between MMP-14 and diabetes, we investigated the expression of MMP-14 in the retinas of diabetic rats.

METHODS

Vitreous samples and epiretinal membrane specimens: Undiluted vitreous fluid samples (0.3–0.6 ml) were obtained from 34 patients with PDR during pars plana vitrectomy, for the treatment of tractional retinal detachment, and/or nonclearing vitreous hemorrhage. The diabetic patients were 21 (61.8%) men, and 13 (38.2%) women, whose ages ranged from 25 to 75 years with a mean of 51.8±12.8 years. The control group consisted of 18 patients who underwent vitrectomy for the treatment of rhegmatogenous retinal detachment with no proliferative vitreoretinopathy (PVR). Control subjects were free from diabetes or other systemic disease and were 11 (61%) men and seven (39%) women whose ages ranged from 23 to 70 years with a mean of 48.5±14.5 years. The ages (p= 0.418; Mann–Whitney test) and male/female ratios (p = 0.859; chi-square test) did not differ statistically significantly between nondiabetic control patients and patients with PDR. Vitreous samples were collected undiluted by manual suction into a syringe through the aspiration line of vitrectomy, before the infusion line was opened. The samples were centrifuged (700 ×*g* for 10 min, 4 °C), and the supernatants were aliquoted and frozen at −80 °C until assay. Epiretinal fibrovascular membranes were obtained from 13 patients with PDR during pars plana vitrectomy for the repair of tractional retinal detachment. The severity of retinal neovascular activity was graded clinically at the time of vitrectomy using previously published criteria [32]. Neovascularization was considered active if there were visible perfused new vessels on the retina or optic disc and present within tractional epiretinal membranes. Neovascularization was considered inactive (involuted) if only nonvascularized white fibrotic epiretinal membranes were present. For comparison, fibrocellular epiretinal membranes were obtained from ten patients without diabetes undergoing vitreoretinal surgery for the treatment of retinal detachment complicated by PVR. PDR epiretinal membranes were obtained from eight (61.5%) men and five (38.5%) women, whose ages ranged from 25 to 74 years with a mean of 48.9±19.4 years. PVR epiretinal membranes were obtained from seven (70%) men and three (30%) women, whose ages ranged from 12 to 71 years with a mean of 44.6±19.6 years. The ages (p = 0.535; Mann–Whitney test) and male/female ratios (p = 0.673; chi-square test) did not differ statistically significantly between patients with PDR and patients with PVR. Membranes were fixed for 2 h in 10% formalin solution and embedded in paraffin.

The study was conducted according to the tenets of the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology (ARVO) statement on human subjects. All the patients were candidates for vitrectomy as a surgical procedure. All patients signed a preoperative informed written consent and approved the use of the excised epiretinal membranes and vitreous fluid for further analysis and clinical research. The study design and the protocol were approved by the Research Centre and Institutional Review Board of the College of Medicine, King Saud University.

Enzyme-linked immunosorbent assays: Enzyme-linked immunosorbent assay (ELISA) kits for human MMP-14 (Cat No: DY918–05) and human VEGF (Cat No: SVE00) were purchased from R&D Systems (Minneapolis, MN). ELISA kits for human MMP-9 (Cat. No: ab100610) was purchased from Abcam (Cambridge, UK). The quantification of human

MMP-14, VEGF, and MMP-9 in vitreous fluid was determined using ELISA kits according to the manufacturer's instructions. The minimum detection limits for the VEGF and MMP-9 ELISA kits were 9 pg/ml and less than 10 pg/ml, respectively. The ELISA plate readings were performed using a Stat Fa×-4200 microplate reader from Awareness Technology, Inc. (Palm City, FL).

Western blot analysis: Retinas from diabetic and control rats were homogenized in western blot lysis buffer (30 mM Tris-HCl; pH 7.5, 5 mM EDTA, 1% Triton X-100, 250 mM sucrose, 1 mM Sodium vanadate, and protease inhibitor cocktail). The protease inhibitor used was "Complete without EDTA" (Roche, Mannheim, Germany). The homogenates were centrifuged at 14,000 ×*g* for 15 min at 4 °C, the supernatants were collected, and the protein concentrations were measured with the use of the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of extracted protein (50 µg) were subjected to sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE) electrophoresis in 10% gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). To determine the presence of MMP-14 in the vitreous samples, equal volumes (15 µl) of vitreous samples were boiled in Laemmli's sample buffer (1:1, v/v) under reducing conditions for 10 min and analyzed. Nonspecific binding sites were blocked (1.5 h, room temperature) with 5% non-fat milk made in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Blots were then incubated at 4 °C overnight with anti-MMP-14 antibody (1:1,000; ab53712, Abcam). Three TBS-T washings (5 min each) were performed before the secondary antibody treatment at room temperature for 1 h. Finally, the immunodetection was performed with the use of the chemiluminescence western blotting luminol reagent (sc-2048; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Membranes were stripped and reprobed with β-actin-specific antibody (1:2,000; sc-47778; Santa Cruz Biotechnology Inc.) used as the lane-loading control. Bands were visualized with the use of a high-performance chemiluminescence machine (G: Box Chemi-XX8 from Syngene, Synoptic Ltd., Cambridge, UK), and the intensities were quantified by using GeneTools software (Syngene by Synoptic Ltd.).

Immunohistochemical staining for epiretinal membranes: For CD31 and α-smooth muscle actin (α-SMA), antigen retrieval was performed by boiling the sections in citrate-based buffer (pH 5.9–6.1; BOND Epitope Retrieval Solution 1; Leica Biosystems, Buffalo Grove, IL) for 10 min. For CD45 and MMP-14 detection, antigen retrieval was performed by boiling the sections in Tris/EDTA buffer (pH 9; BOND Epitope Retrieval Solution 2; Leica) for 20 min. Subsequently, the sections were incubated for 60 min with mouse monoclonal anti-CD31 (ready-to-use; clone JC70A; Dako, Glostrup, Denmark), mouse monoclonal anti-CD45 (ready-to-use; clones 2B11+PD7/26; Dako), mouse monoclonal antibody against α-SMA (ready-to-use: clone 1A4; Dako), and rabbit monoclonal anti-MMP-14 antibody (1:100; ab51074, Abcam). Optimal working conditions for the antibodies were determined in pilot experiments on kidney sections. The sections were then incubated for 20 min with a post-primary immunoglobulin G (IgG) linker followed by an alkaline phosphatase conjugated polymer. The reaction product was visualized by incubation for 15 min with the Fast Red chromogen, resulting in bright-red immunoreactive sites. The slides were then faintly counterstained with Mayer's hematoxylin (BOND Polymer Refine Red Detection Kit; Leica).

To identify the phenotype of the cells expressing MMP-14, sequential double immunohistochemistry was performed. The sections were incubated with the first primary antibody (anti-CD45) and subsequently treated with peroxidase conjugated secondary antibody. The immunoreactivities were visualized with 3,3′-diaminobenzidine tetrahydrochloride. Incubation of the second primary antibody (anti-MMP-14) was followed by treatment with alkaline phosphatase conjugated secondary antibody. The sections were visualized with Fast Red. No counterstain to visualize the cell nuclei was applied.

Negative controls were by omission of the primary antibody from the staining protocol. Instead, the ready-to-use DAKO Real Antibody Diluent (Agilent Technologies, Dako, Glostrup, Denmark, product code S2022) was applied.

Quantitation: Immunoreactive blood vessels and cells were counted in five representative fields, using an eyepiece calibrated grid in combination with the 40X objective. These representative fields were selected based on the presence of immunoreactive blood vessels and cells. With this magnification and calibration, immunoreactive blood vessels and cells present in an area of 0.33 mm × 0.11 mm were counted.

Rat streptozotocin-induced diabetes model: All procedures with animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the College of Pharmacy, King Saud University. Adult male Sprague-Dawley rats, 8–9 weeks of age weighing in the range of 220–250 g were overnight fasted, and streptozotocin (STZ; 65 mg/kg in 50 mM sodium citrate buffer, pH 4.5; Sigma, St. Louis, MO) was injected intraperitoneally as a single bolus. Equal volumes of citrate buffer were injected in non-diabetic control animals. Measurement of blood glucose concentrations and body-weights were started 3 days after injection of STZ. Diabetes

was confirmed by assaying the glucose concentration in blood taken from the tail vein. Rats with glucose levels >250 mg/dl were categorized as diabetic. After 4 weeks of diabetes, animals were anesthetized by intraperitoneal injection of an overdose of chloral hydrate and euthanized by decapitation. Retinas were dissected, flash frozen, and stored at −80 °C until use. Similarly, retinas were obtained from age-matched nondiabetic control rats.

Real-time reverse transcription polymerase chain reaction: Total RNA was extracted from the retinas using TRI reagent (Ambion, Austin, TX), according to the manufacturer's protocol. cDNA were synthesized from 1 µg RNA, using an high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer's instruction. Real-time reverse transcription PCR (RT–PCR) was performed using a TaqMan PCR Master Mix. TaqMan primers were used to quantify MMP-14 gene expression (assay ID: Rn00675906_g1) in the rat retina. The standard PCR conditions included 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of extension at 95 °C for 15 s and 1 min at 60 °C. Threshold lines were automatically adjusted to intersect the amplification lines in the linear portion of the amplification curves, and the cycle to threshold (Ct) values were recorded automatically. Data were normalized with *β-actin* (housekeeping gene; assay ID: Rn00667869_m1), and the fold-change in gene expression relative to normal was calculated using the ddCt method.

Statistical analysis: Data are presented as the mean ± standard deviation. Values below the detection limit were treated as "zero" in terms of the analysis due to their trivial decimal values which were quite close to zero. The non-parametric Kruskal–Wallis test was used to compare means among patients with active PDR, patients with inactive PDR, and nondiabetic control patients. The non-parametric Mann–Whitney test and the chi-square test were used to compare means from two independent groups. Spearman's correlation coefficients were computed to investigate correlation between variables. A p value of less than 0.05 indicated statistical significance. SPSS version 20.0 for Windows (IBM Inc., Chicago, IL) was used for statistical analysis.

RESULTS

ELISA levels of MMP-14, VEGF, and MMP-9 in vitreous samples: MMP-14 was detected in ten of 18 (55%) vitreous samples from nondiabetic control patients and in all vitreous samples from patients with PDR. The mean MMP-14 levels in the vitreous samples from patients with PDR were statistically significantly higher than the mean levels in the vitreous samples from nondiabetic control patients ($p<0.001$; Mann–Whitney test; Table 1). VEGF was detected in nine of 18 (50%) vitreous samples from nondiabetic control patients, and in 31 of 34 (92.2%) vitreous samples from patients with PDR. The mean VEGF levels in patients with PDR were statistically significantly higher than the mean levels in nondiabetic control patients ($p<0.001$; Mann–Whitney test; Table 1). MMP-9 was detected in all vitreous samples from nondiabetic control patients and patients with PDR. The mean MMP-9 levels in vitreous samples from patients with PDR were statistically significantly higher than the mean levels in nondiabetic control patients ($p<0.001$; Mann–Whitney test; Table 1). With the use of western blot analysis of equal volumes of vitreous fluid, we confirmed the presence of MMP-14 in vitreous samples (Figure 1).

Relationship between ELISA levels of MMP-14 in vitreous samples and angiogenic activity of PDR: Comparisons of the mean levels of MMP-14 among patients with active PDR (n = 20), patients with inactive PDR (n=14), and nondiabetic control patients (n = 18) was conducted with the Kruskal–Wallis test. The mean levels differed statistically significantly between the three groups ($p<0.001$). Pairwise comparisons (Mann–Whitney test) indicated that the mean MMP-14 levels were statistically significantly higher in the patients with active PDR (6.2±1.2 ng/ml) than in the patients with inactive PDR (4.5±0.8 ng/ml; $p<0.001$) and the control patients (2.5±2.5 ng/ml; $p<0.001$). In addition, the mean MMP-14 levels in the patients with inactive PDR were statistically

Table 1. Comparisons of mean matrix metalloproteinase-14 (MMP-14), vascular endothelial growth factor (VEGF) and MMP-9 levels in proliferative diabetic retinopathy (PDR) and nondiabetic patients with rhegmatogenous retinal detachment (RD).

Disease Group	MMP-14 (ng/ml)	VEGF (pg/ml)	MMP-9 (pg/ml)
PDR (n=34)	5.5±1.4	675.1±521.7	654.8±376
RD (n=18)	2.5±2.5	26.9±44.8	98.2±112.4
P value (Mann–Whitney test)	<0.001*	<0.001*	<0.001*

*Statistically significant at 5% level of significance.

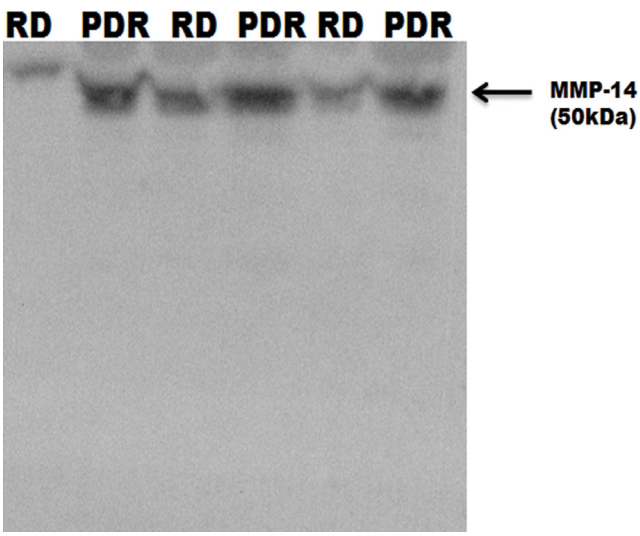

Figure 1. The expression of matrix metalloproteinase-14 (MMP-14) in equal volumes (15 µl) of vitreous fluid samples obtained from patients with proliferative diabetic retinopathy (PDR) and from control patients with rhegmatogenous retinal detachment (RD) was determined with western blot analysis.

significantly higher than the mean levels in the control patients (p = 0.022).

Correlations: Statistically significant positive correlations were found between vitreous fluid levels of MMP-14 and levels of VEGF (r = 0.3; p = 0.032) and MMP-9 (r = 0.54; p<0.001). In addition, there was a statistically significant positive correlation between vitreous fluid levels of VEGF and levels of MMP-9 (r = 0.51; p<0.001).

Immunohistochemical analysis of epiretinal fibrovascular membranes from patients with PDR: To identify the cellular source of vitreous fluid MMP-14, epiretinal fibrovascular membranes from patients with PDR were studied with immunohistochemistry. No staining was observed in the negative control slides (the same procedure without the primary antibody; Figure 2A). The level of vascularization and proliferative activity in epiretinal membranes was determined with immunodetection of the endothelial cell marker CD31. All membranes showed pathologic new blood vessels, positive for the endothelial cells marker CD31 (Figure 2B,C), with a mean number of 77.7±53.7 per section (range, 14–190). Leukocytes expressing the leukocyte common antigen CD45 (Figure 2D) and spindle-shaped cells expressing the myofibroblast marker α-SMA (Figure 2E) were detected in the stroma of all membranes. Strong immunoreactivity for MMP-14 was present in all membranes and was noted in vascular endothelial cells, stromal cells, and intravascular leukocytes (Figure 3A–D). MMP-14-positive stromal cells were CD45-expressing leukocytes and spindle-shaped cells. In serial sections, the distribution and morphologies of spindle-shaped cells expressing MMP-14 (Figure 3B) were similar to those of spindle-shaped stromal cells expressing the myofibroblast marker α-SMA (Figure 2E). Double immunohistochemistry analysis confirmed that stromal cells and intravascular leukocytes expressing MMP-14 coexpressed CD45 (Figure 3E).

The number of blood vessels that were immunoreactive to MMP-14 ranged from eight to 110, with a mean of 52.7±26.0 per section. The number of MMP-14-positive stromal cells ranged from ten to 155 with a mean of 78.4±44.4 per section.

Correlations between MMP-14 expression and angiogenic activity in PDR fibrovascular epiretinal membranes: Statistically significant positive correlations were detected between the numbers of blood vessels expressing CD31 and the numbers of blood vessels (r = 0.74; p = 0.004) and stromal cells (r = 0.72; p = 0.005) expressing MMP-14. The mean numbers of blood vessels expressing CD31 were statistically significantly higher in membranes from patients with active PDR (n = 6; 122.5±44.60) than in membranes from patients with inactive PDR (n = 7; 39.3±19.5; p = 0.003; Mann–Whitney test; Figure 2B,C). The mean numbers of blood vessels expressing MMP-14 were statistically significantly higher in membranes from patients with active PDR (73.0±22.0) than in membranes from patients with inactive PDR (35.5±16.2; p = 0.004; Mann–Whitney test; Figure 3A–D). Similarly, the mean numbers of stromal cells immunoreactive for MMP-14 were statistically significantly higher in membranes from patients with active PDR (108.3±36.40) than in membranes from patients with inactive PDR (52.7±34.2; p = 0.015; Mann–Whitney test).

Immunohistochemical analysis of epiretinal fibrocellular membranes from patients with PVR: No immunostaining was noted in the negative control slides (Figure 4A). All membranes showed spindle-shaped cells expressing the myofibroblast marker α-SMA (Figure 4B) and cells expressing the leukocyte common antigen CD45 (Figure 4C). Immunoreactivity for MMP-14 was noted in spindle-shaped cells (Figure 4D). In serial sections, the distribution and morphology of spindle-shaped cells expressing MMP-14 were similar to those of spindle-shaped cells expressing the myofibroblast marker α-SMA (Figure 4B). In addition, double immunohistochemistry demonstrated that cells expressing MMP-4 coexpressed CD45 (Figure 4E).

Severity of hyperglycemia and effect of diabetes on retinal expression of MMP-14 in experimental rats: After induction of diabetes with a single dose of streptozotocin, the bodyweights of the diabetic rats were statistically significantly lower, and their blood glucose levels were more than fourfold higher compared with age-matched normal control rats (182±19.0 g versus 256±17.0 g and 419±29.0 mg/

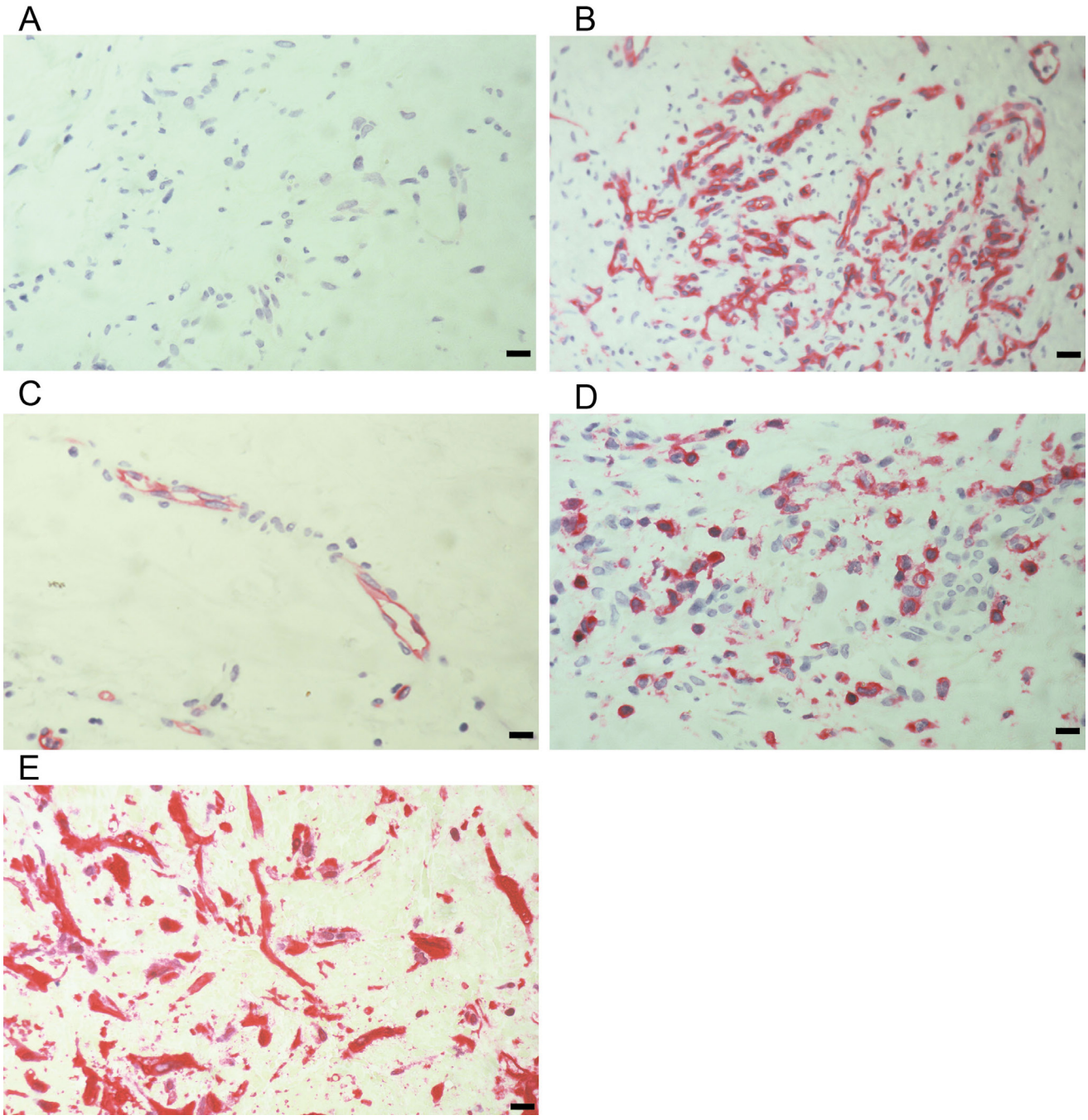

Figure 2. Proliferative diabetic retinopathy (PDR) fibrovascular epiretinal membranes. **A**: Negative control slide showing no labeling. Immunohistochemical staining for CD31 showing pathologic new blood vessels expressing this endothelial cell marker in (**B**) a membrane from a patient with active PDR and in (**C**) a membrane from a patient with inactive PDR. **D**: Immunohistochemical staining for CD45 showing numerous leukocytes in the stroma. **E**: Immunohistochemical staining for α-smooth muscle actin showing immunoreactivity in myofibroblasts (scale bar, 10 µm).

dl versus 105±11.0 mg/dl, respectively; n = 7–10 per group). We quantified the expression of MMP-14 in the rat retinas with western blot analysis. Densitometric analysis of the bands revealed a statistically significant increase in MMP-14 in diabetic retinas compared to nondiabetic control retinas ($p = 0.02$; Mann–Whitney test; Figure 5A). In addition, the retinal mRNA levels of MMP-14 in the diabetic rats increased

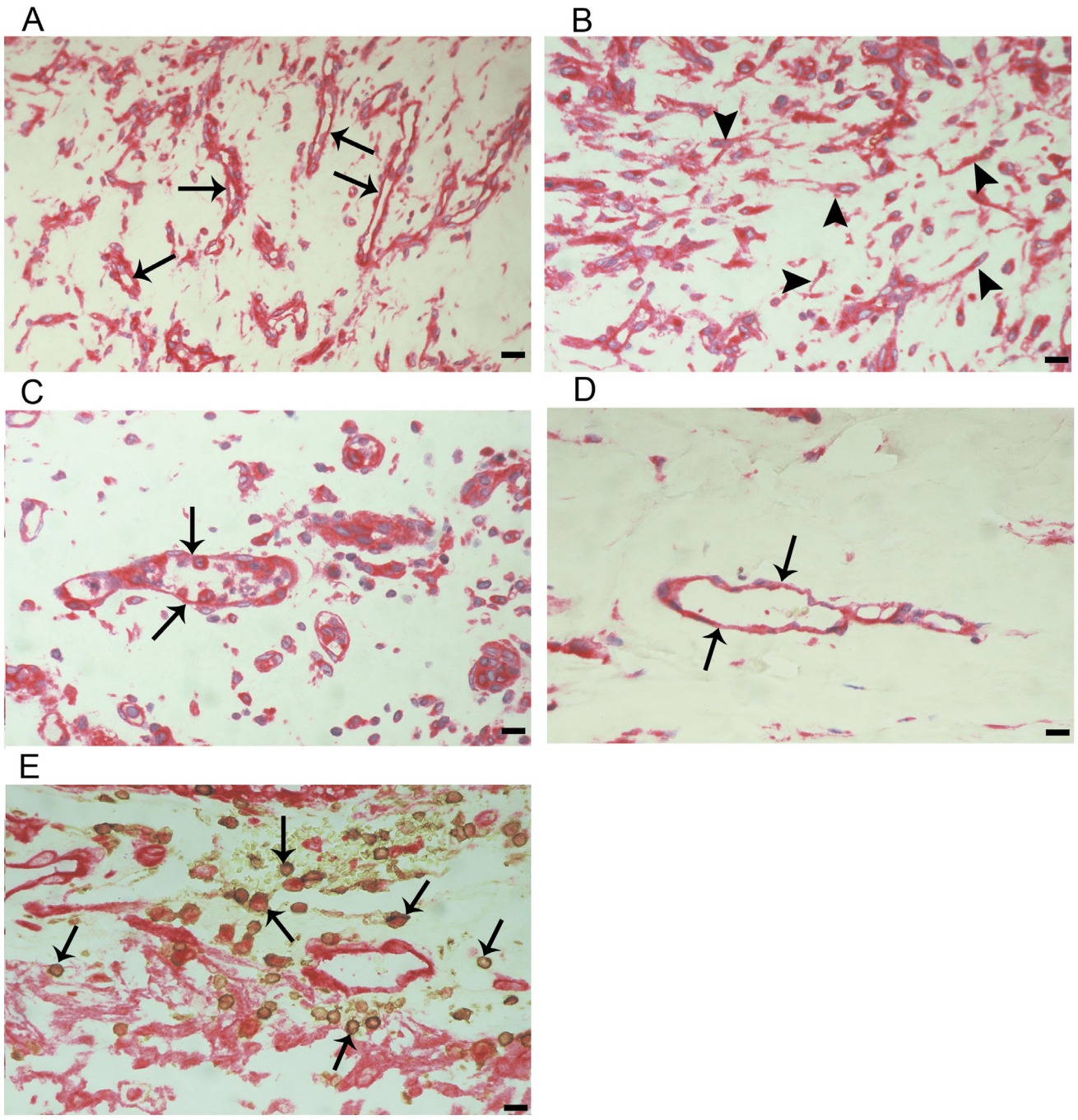

Figure 3. Proliferative diabetic retinopathy (PDR) fibrovascular epiretinal membranes. Immunohistochemical staining for matrix metalloproteinase-14 (MMP-14) showing immunoreactivity in the vascular endothelial cells (arrows), in intravascular leukocytes, in stromal cells, and in stromal spindle-shaped cells (arrowheads) in a membrane from a patient with active PDR (**A**, **B**, **C**) and in a membrane from a patient with inactive PDR (**D**). Notice that the membrane from the patient with inactive PDR is composed mostly of fibrous tissue. Double immunohistochemistry for CD45 (brown) and MMP-14 (red) in a membrane from a patient with active PDR demonstrated stromal cells coexpressing CD45 and MMP-14 (arrows; **E**). No counterstain to visualize the cell nuclei was applied (scale bar, 10 µm).

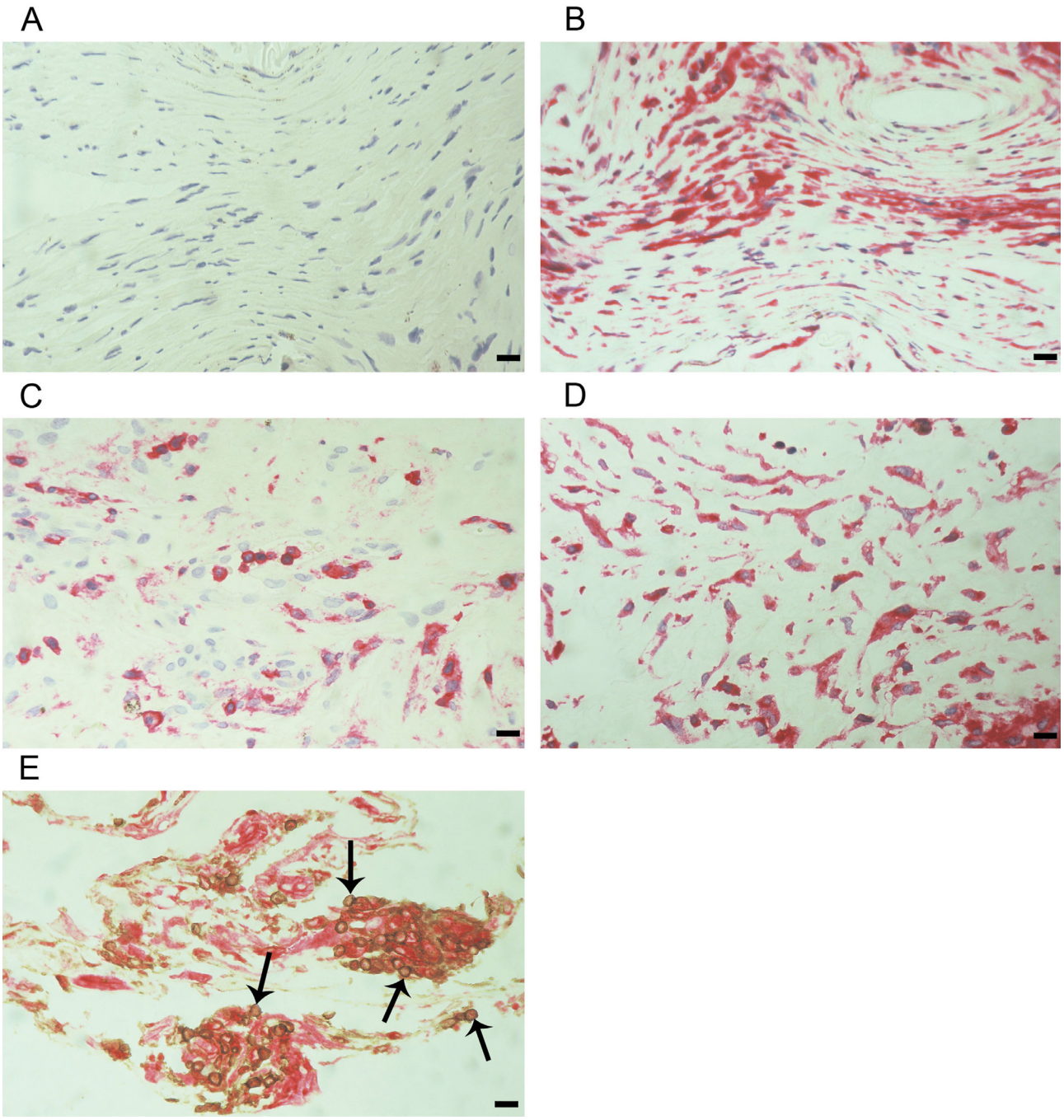

Figure 4. Proliferative vitreoretinopathy fibrocellular epiretinal membranes. **A**: Negative control slide showing no labeling. **B**: Immunohistochemical staining for α-smooth muscle actin showing immunoreactivity in spindle-shaped myofibroblasts. **C**: Immunoreactivity staining for CD45 showing immunoreactivity in leukocytes. **D**: Immunohistochemical staining for matrix metalloproteinase-14 (MMP-14) showing immunohistochemistry in spindle-shaped myofibroblasts. **E**: Double immunohistochemistry for CD45 (brown) and MMP-14 (red) demonstrates cells coexpressing CD45 and MMP-14 (arrows; scale bar, 10 µm).

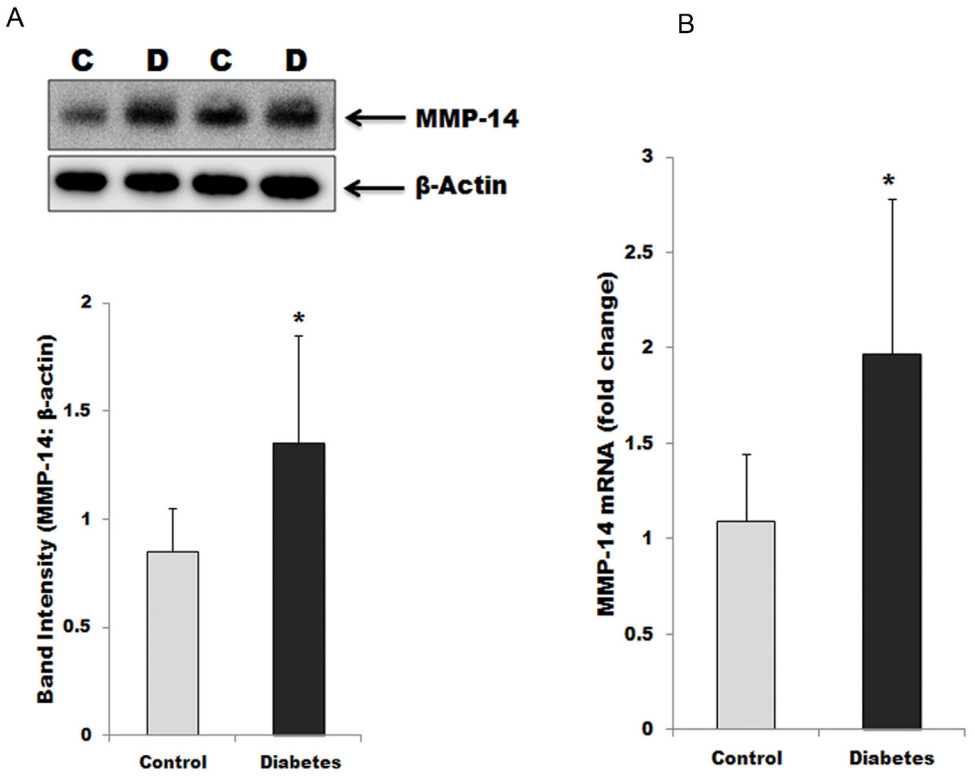

Figure 5. Matrix metalloproteinase-14 (MMP-14) expression in diabetic rat retinas. **A**: MMP-14 expression was determined by western blot analysis on lysates of diabetic (**D**) and nondiabetic control (**C**) rats. After determination of the intensity of the MMP-14 protein band, intensities were adjusted to those of β-actin in each sample. Each measurement was performed at least three times. **B**: In addition, cell extracts were used to quantify the mRNA of MMP-14 by quantitative-RT-PCR. Results are expressed as mean ± standard deviation of 7-10 rats in each group. *The difference between the two means was statistically significant at 5% level of significance.

by about twofold compared to the levels in the nondiabetic control rats (p = 0.01; Mann–Whitney test; Figure 5B).

DISCUSSION

In the current study, we demonstrated for the first time that the transmembrane MMP-14 was statistically significantly upregulated in the vitreous fluid from patients with PDR and in the retinas of diabetic rats. Similarly, in a previous study, Giebel et al. [33] demonstrated increased expression of MMP-14 in the retinas of diabetic rats. The present subgroup analysis demonstrated that MMP-14 levels in vitreous samples from PDR eyes with active neovascularization were higher than those in quiescent cases. MMP-14 is a secreted membrane-tethered MMP. For this reason, it is surprising to observe MMP-14 in vitreous solution. However, we postulate that proteolytic activities, for example, from other MMPs, present in the PDR microenvironment might cleave the extracellular domain of MMP-14 and allow for its detection in vitreous fluid. We confirmed the increased presence of MMP-14 in vitreous fluid from patients with PDR using ELISA and western blot analysis. Similarly, in previous studies, it was demonstrated that MMP-14 levels are statistically significantly increased in the serum and ascites of patients with malignant ovarian tumors suggesting that MMP-14 could be a useful tumor biomarker for monitoring response to therapy [30]. Using immunohistochemistry, we demonstrated that the MMP-14 protein was specifically localized in endothelial cells of pathologic new blood vessels, leukocytes expressing the leukocyte common antigen CD45, and myofibroblasts in fibrovascular epiretinal membranes from patients with PDR. These findings suggest that endothelial cells, leukocytes, and myofibroblasts are the source of MMP-14 detected in the vitreous fluid. In addition, we found a statistically significant positive correlation between the level of vascularization in PDR fibrovascular epiretinal membranes and the numbers of blood vessels and stromal cells expressing MMP-14. Furthermore, the expression of MMP-14 in membranes from patients with active neovascularization was statistically significantly higher than that in membranes from patients with quiescent PDR. Collectively, these results suggest that MMP-14 might be a useful biomarker of angiogenesis in patients with PDR.

Angiogenesis involves the activation and invasion of endothelial cells through their basement membrane, migration to distal sites, and tube formation [13]. It has been demonstrated that endothelial MMP-14 is a critical regulator of angiogenesis. The membrane- anchored collagenase MMP-14 confers endothelial cells with the ability to proteolytically remodel type I collagen and to express invasive

and tubulogenic activity in a type I collagen-rich milieu in vitro or in vivo [20]. It was also demonstrated that MMP-14 expression is markedly increased in endothelial cells under conditions that induce capillary-like tubular structures in a collagen-containing fibrin matrix in vitro [19]. Selective inhibition of MMP-14 impairs migration and invasion of endothelial cells in vitro [27]. In the aortic ring model of angiogenesis, neovessels expressed MMP-14 and MMP-14 neutralizing antibodies block angiogenesis [25]. The presence of MMP-14 was also demonstrated in vivo in the newly formed vessels of a recanalized arterial mural thrombus [19]. Consistent with these previous reports, we demonstrated MMP-14 expression in vascular endothelial cells of pathologic new blood vessels in epiretinal membranes from patients with PDR. Downregulation of MMP-14 expression in cancer cell lines blocks activation of pro-MMP-2, inhibits angiogenesis, and slows tumor invasion and formation of metastatic lesions [27-30].

In the present study, we detected overlapping expression of MMP-14, VEGF, and MMP-9 in vitreous samples from patients with PDR. Moreover, there were statistically significant positive correlations between the levels of MMP-14 and the levels of MMP-9 and the angiogenic biomarker VEGF, a key angiogenic factor in PDR [10,11]. These findings are in accordance with previous reports that demonstrated active involvement of MMP-14 in the regulation of MMP-9 activation and expression [24,30,34-36] and that treatment of cancer cells with monoclonal MMP-14 antibody decreases MMP-9 levels [30]. The present findings are also in agreement with a previous report that demonstrated a strong correlation between MMP-14 and VEGF expression levels in human glioblastomas. In addition, MMP-14 and VEGF proteins colocalize in tumor and endothelial cells [37]. The MMP-14 proangiogenic effect was mediated at least in part by the upregulation of VEGF. MMP-14 overexpression in human cancer cells is associated with enhanced VEGF expression. This effect is associated with increased endothelial cell response and subsequent accelerated tumor angiogenesis and growth [37-40]. However, downregulation of MMP-14 expression in cancer cell lines downregulates the expression levels of VEGF and attenuates tumor angiogenesis, growth, and metastasis [29,30].

In the present study, MMP-14 was localized in vascular endothelial cells and stromal cells in PDR fibrovascular epiretinal membranes. In a previous report, we demonstrated VEGF [41] and MMP-9 [6] localization in vascular endothelial cells and stromal cells in PDR epiretinal membranes. The coexpression of MMP-14, VEGF, and MMP-9 in the ocular microenvironment of patients with PDR suggests an interplay between these regulatory factors in the pathogenesis of PDR angiogenesis and progression.

Myofibroblasts, the key cellular mediators of fibrosis, are contractile cells, characterized by the expression of α-SMA, and the presence of myofibroblasts is a marker of progressive fibrosis [42]. Using immunohistochemistry, we demonstrated that MMP-14 and α-SMA are colocalized to the same cells in epiretinal membranes from patients with PDR and PVR. These immunohistochemical data are in agreement with previous studies demonstrating that activated fibroblasts express MMP-14 [21,22]. Current data show that in addition to MMP-14's well-characterized role in angiogenesis, MMP-14 activity may represent an important determinant in the pathogenesis of fibrotic disorders. MMP-14, but not other collagenases, confers the focal collagenolytic activity necessary for supporting fibroblast invasive activity [21]. In addition, MMP-14 was identified as the dominant protease responsible for type I collagenolytic activity mediated by fibroblasts. Furthermore, MMP-14 has been shown to be essential for fibroblast migration within three-dimensional hydrogels of cross-linked type I collagen that recapitulate ECM barriers encountered in the in vivo environment [22]. Recently, MMP-14 has been implicated in the progression of fibrosis in various organs [43-46]. MMP-14 plays a critical role in facilitating activation of profibrotic synthetic pathways that result in fibrosis. MMP-14 can hydrolyze latency-associated transforming growth factor binding protein and release transforming growth beta [43,45,46]. With the availability of neutralizing antibodies against MMP-14, it will be possible to evaluate their effect in well-planned and controlled clinical trials of diabetic retinopathy.

In conclusion, we demonstrated that MMP-14 is upregulated in the intraocular microenvironment of patients with PDR, particularly in patients with active neovascularization. Given that MMP-14 promotes angiogenesis along with increased fibrosis, selective targeting of MMP-14 may not only limit PDR angiogenesis but also attenuate fibrosis.

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