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Original Article

High-mobility group box-1 regulates the expression of matrix metalloproteinase-9 in diabetic retina

Ghulam Mohammad[1], Mohammad Mairaj Siddiquei[1], Kaiser Alam[1], Mohammad Imtiaz Nawaz[1], Ahmed Mousa[1], Ghislain Opdenakker[2], Ahmed M Abu El-Asrar[1,3]

[1]Department of Ophthalmology, College of Medicine, King Saud University, Riyadh, Saudi Arabia; [2]Rega Institute for Medical Research, Laboratory of Molecular Immunology and Laboratory of Immunobiology, University of Leuven, KU Leuven, Belgium; [3]Dr. Nasser Al Rashid Research Chair in Ophthalmology

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Abstract: Purpose: To test the hypothesis that upregulated expression of the proinflammatory cytokine high-mobility group box-1 (HMGB1) in the eyes from patients with proliferative diabetic retinopathy (PDR) regulates the expression of matrix metalloproteinase-9 (MMP-9) in diabetic retina. Methods: Vitreous samples from 25 PDR and 17 nondiabetic patients, retinas from 1-month diabetic rats and normal rats intravitreally injected with HMGB1 and human retinal microvascular endothelial cells (HRMEC) were studied with the use of enzyme-linked immunosorbent assay, Western blot analysis and RT-PCR. We also studied the effects of HMGB1 inhibitor glycyrrhizin and targeted deletion of the MMP-9 gene on diabetes-induced biochemical changes in the retina. An assay for *in vitro* cell migration was performed on human retinal microvascular endothelial cells (HRMEC). Results: Levels of HMGB1 and MMP-9 were significantly higher in the vitreous fluid from PDR patients compared with nondiabetic patients ($P < 0.001$ for both comparisons) and these were significantly correlated ($r = 0.5$, $P = 0.003$). Diabetes induction and intravitreal injection of HMGB1 in normal rats induced significant upregulation of MMP-9 and downregulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA levels. Constant glycyrrhizin intake from onset of diabetes attenuated diabetes-induced upregulation of MMP-9, but did not affect TIMP-1 expression in the retina. Deletion of the MMP-9 gene in mice did not inhibit diabetes-induced upregulation of HMGB1 in the retina. However, the MMP-9 inhibitor inhibited HMGB1-induced MMP-9 upregulation and migration in HRMEC. Conclusions: Our findings suggest that MMP-9 acts downstream of HMGB1 and mediates the effect of HMGB1 in diabetic retinopathy.

Keywords: Proliferative diabetic retinopathy, high-mobility group box-1, matrix metalloproteinase-9, angiogenesis, TIMP-1

Introduction

Ischemia-induced angiogenesis is a hallmark feature of proliferative diabetic retinopathy (PDR) and is a critical step in the progression of the disease. Angiogenesis, the sprouting of new blood vessels from preexisting blood vessels, is a multistep process requiring the degradation of the basement membranes and extracellular matrix (ECM), endothelial cell migration, endothelial cell proliferation, and capillary tube formation [1]. Vascular endothelial growth factor (VEGF) is an important angiogenic factor in PDR that promotes neovascularization and vascular leakage [2]. The angiogenic switch involves in part the proteolytic degradation of basement membranes and ECM components by matrix metalloproteinases (MMPs) [1]. Several studies reported elevated levels of MMPs, in particular MMP-9, in the vitreous fluid from patients with PDR [3, 4]. This upregulation of MMP-9 is linked to angiogenesis and progression of PDR. MMP-9 expression is low or absent in most normal tissues, and is markedly elevated during inflammatory, autoimmune, degenerative and neoplastic diseases and in angiogenic lesions [5]. In addition to removing the physical barriers against new vessel growth, MMP-9 releases biologically active VEGF from ECM and increases its bioavailability resulting in VEGF-driven angiogenic switch [6-8]. MMP-9 may facilitate pathologic neovascularization through stimulation of the production of VEGF [9]. MMP-9 has also been shown to be important in

mobilizing endothelial and other progenitor cells from the bone marrow niche by releasing soluble kit-ligand [10]. Most of the MMPs are inhibited by specific endogenous tissue inhibitors which are known as tissue inhibitors of matrix metalloproteinases (TIMPs) [11].

Chronic, low-grade subclinical inflammation is responsible for many of the vascular lesions in diabetic retinopathy [12]. The causal relationship between inflammation and angiogenesis is now widely accepted [13]. Sustained proinflammatory responses are associated with increased angiogenesis that contributes to tissue disruption and disease progression [13]. An emerging issue in diabetic retinopathy research is the focus on the mechanistic link between chronic low-grade inflammation and angiogenesis. Recently, it was demonstrated that the pro-inflammatory cytokine high-mobility group box-1 protein (HMGB1) seems to be involved in a positive feedback mechanism that may help to sustain inflammation and angiogenesis in several pathological conditions, thereby contributing to disease progression [13, 14]. These findings suggest that HMGB1 might provide a mechanistic link between chronic low-grade inflammation and angiogenesis.

HMGB1 was originally identified as a nonhistone DNA binding nuclear protein produced by nearly all cell types. It plays an important role in chromatin organization and transcriptional regulation. HMGB1 is released passively from necrotic cells, and is actively secreted by different cell types, including activated immune effector cells and endothelial cells. Extracellular HMGB1 functions as a proinflammatory cytokine that triggers inflammation and recruits leukocytes to sites of tissue damage [13, 15-19] and exhibits angiogenic effects [19-23]. Treatment of endothelial cells with HMGB1 induced a proangiogenic gene expression program evidenced by the induction of VEGF and its receptors, platelet-derived growth factor receptors, integrins and MMPs. In addition, HMGB1 induced endothelial cell migration and sprouting. HMGB1 was also identified as a specific marker of tumor endothelium and as a tumor angiogenesis marker. Moreover, anti-HMGB1 antibodies inhibited tumor angiogenesis [19]. Another interesting role of HMGB1 in neovascularization is its ability to attract endothelial progenitor cells to sites of tissue injury and tumors to improve neovascularization in a RAGE-dependent manner [22]. Several receptors have been implicated in HMGB1-mediated functions, including receptor for advanced glycation end products (RAGE) and toll-like receptors TLR2, TLR4 and TLR9. Signaling through these receptors leads to activation of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathway and the transcription factor nuclear factor-kappa B (NF-kB) which may alter gene transcription and lead to the upregulation of proinflammatory cytokines, angiogenic factors, chemokines and adhesion molecules and intensify cellular oxidative stress [13, 15-18, 24], processes that play a role in the pathogenesis of diabetic retinopathy development and progression [12, 25-28]. In previous studies, we demonstrated increased levels of HMGB1 in the vitreous samples from patients with PDR [30, 31] and the *in situ* localization of the expression of HMGB1 and RAGE in vascular endothelial cells and stromal cells in fibrovascular epiretinal membranes from patients with PDR [31]. In addition, we demonstrated significant correlations between the vitreous levels of HMGB1 and the levels of the inflammatory biomarkers monocyte chemoattractant protein-1 (MCP-1) and soluble intercellular adhesion molecule-1 (sICAM-1) [29] and between the level of vascularization in PDR epiretinal membranes and the expression of HMGB1 and RAGE [31]. Moreover, we demonstrated that diabetes and intravitreal injection of HMGB1 in normal rats induced significant upregulation of the expression of HMGB1, RAGE, activated ERK1/2 and activated NF-kB in the retinas of rats and mice [29, 32-34]. Recently, a great deal of evidence has indicated the critical importance of HMGB1 in mediating inflammation, breakdown of the blood-retinal barrier, oxidative stress and neuropathy in the diabetic retina [32-35].

Several studies demonstrated that increased HMGB1 expression is linked to increased MMP-9 expression and activation [36-39]. In previous studies we showed HMGB1 (31) and MMP-9 [4] localization in vascular endothelial cells in PDR fibrovascular epiretinal membranes suggesting a cross-talk between HMGB1 and MMP-9 in the pathogenesis of PDR angiogenesis and progression. These findings suggest that HMGB1-induced angiogenesis in

PDR is, at least in part, through upregulation of MMP-9. To test this hypothesis, we measured and correlated the levels of HMGB1 and MMP-9 in the vitreous fluid from patients with PDR and investigated the expression of MMP-9 and TIMP-1 in the retinas of diabetic rats and in the retinas of normal rats after intravitreal administration of HMGB1. We also examined the effect of constant glycyrrhizin intake, a potent HMGB1 inhibitor [40], on diabetes-induced changes in the expression of MMP-9 and TIMP-1 in the retinas of rats. In addition, we examined the effect of targeted deletion of the MMP-9 gene in mice on diabetes-induced changes in the expression of HMGB1 in the retinas. Moreover, we investigated the expression of MMP-9 in human retinal microvascular endothelial cells (HRMEC) following exposure to HMGB1, and whether an MMP-9 inhibitor blocked HMGB1-induced upregulation of MMP-9, and migration in HRMEC.

Methods

Vitreous samples

Undiluted vitreous fluid samples (0.3-0.6 ml) were obtained from 25 patients with PDR during pars plana vitrectomy. The indications for vitrectomy were traction retinal detachment, and/or nonclearing vitreous hemorrhage. The control group consisted of 17 patients who had undergone vitrectomy for the treatment of rhegmatogenous retinal detachment (RD) with no proliferative vitreoretinopathy. Controls were free from systemic disease. Vitreous samples were collected undiluted by manual suction into a syringe through the aspiration line of vitrectomy, before opening the infusion line. The samples were centrifuged (5000 rpm for 10 min, 4°C) and the supernatants were aliquoted and frozen at -80°C until assay. The study was conducted according to the tenets of the declaration of Helsinki, and informed consent was obtained from all patients. The study was approved by the Research Centre, College of Medicine, King Saud University.

Enzyme-linked immunosorbent assay kits

Enzyme-linked immunosorbent assay (ELISA) kit for Human MMP-9 (Cat No: ab100610) was purchased from Abcam, UK. ELISA kit for human HMGB1 (Cat No: ST51011), was purchased from IBL international, GMBH, Hamburg, Germany.

The minimum detection limits for MMP-9 and HMGB1 ELISA kit were 10 pg/mL, and 200 pg/mL respectively. The ELISA plate readings were done using a Stat Fax-4200, Awareness Technology, Inc., Palm City, FL.

Measurement of MMP-9 and HMGB1

The quantification of human MMP-9 and HMGB1 in the vitreous fluid was determined using ELISA kits according to the manufacturer's instruction. For each ELISA, the undiluted standard served as the highest standard and calibrator diluents served as the zero standard. Depending upon the detection range for each ELISA the supernatant vitreous obtained were used either directly or diluted with calibrator diluents supplied with ELISA.

For the measurement of MMP-9, 100 µL of undiluted vitreous were used and added to the respective well of ELISA plate. As instructed in the kit manual, samples were incubated into the each well of ELISA plates. After incubation with biotinylated MMP-9 detection antibody, horseradish peroxidase (HRP)-Streptavidin solution was added to each well of the ELISA plate. After incubation and washing, TMB (tetramethylbenzidine) substrate solution was added for colour development. The reaction was stopped by the adding stop solution and OD (optical density) was read at 450 nm in a microplate reader. Each assay was performed in duplicate.

For the measurement of HMGB1, 50 µL of 2 fold diluted vitreous samples (sample diluents, supplied with the kit) were used in the ELISA assay for their analysis. As instructed in the kit manual, samples were incubated into the each well of ELISA plates. Antibody against HMGB1 conjugated to HRP was added to each well of the ELISA plate. After incubation and washing, substrate mix solution (1:1, hydrogen peroxide: tetramethylbenzidine) was added for colour development. The reaction was stopped by the addition of 2N sulfuric acid and the optical density (OD) was read at 450 nm in the microplate reader. Each assay was performed in duplicate.

Using the 4-parameter fit logistic (4-PL) curve equation, the actual concentration for each sample was calculated. For the vitreous fluids, that were diluted, the correction read from the

standard curve obtained using 4-PL were multiplied by the dilution factors to obtain the actual reading for each sample.

Animals

Rats: All procedures with animals were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for 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 of 8-9 weeks of age (200-220 g) were overnight fasted and streptozotocin (STZ) 55 mg/kg in 10 mM sodium citrate buffer, pH 4.5; (Sigma, St. Louis, MO) was injected intraperitoneally. Equal volumes of citrate buffer were injected in age-matched control rats. Rats were considered diabetic if their blood glucose was greater than 250 mg/dl.

Glycyrrhizin (GA) treatment

Diabetic rats were divided into 2 groups: the rats in group I received normal drinking water without any supplementation, and group II received drinking water supplemented with glycyrrhizic acid (150 mg/kg/day, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) immediately after establishment of diabetes. After 4 weeks of diabetes, the rats were euthanized by an overdose of chloral hydrate, the eyes were removed, and the retinas were isolated and frozen immediately in liquid nitrogen and stored at -80°C until analysis.

Intravitreal injection of HMGB1

Sprague Dawley rats (220-250 g) were kept under deep anesthesia, and a sterilized solution of recombinant HMGB1 (5 ng/5 µl; R&D Systems, Minneapolis, MN) was injected into the vitreous of the right eye as previously described [32]. For the control, the left eye received 5 µl of sterile phosphate buffer saline (PBS). Each group consisted of 7-10 rats. The animals were sacrificed 4 days after intravitreal administration, and the retinas were carefully dissected, snap frozen in liquid nitrogen, and stored at -80°C until analysis.

Mice: Wild-type (WT) and MMP-9 knock out (MMP-9 KO) mice [41] were backcrossed 13 times into C57BL6 background, were made diabetic by intraperitoneal injection of streptozotocin for five consecutive days. Mice with blood glucose above 200 mg/dL, 3 days after the last injection of streptozotocin were considered as diabetic. Age-matched normal WT mice served as controls. Mice were sacrificed by pentobarbital overdose and the retinas were removed from eye, snap frozen in liquid nitrogen, and stored at -80°C for RNA isolation. All experiments were performed in accordance to the Association of Research in Vision and Ophthalmology (ARVO) and King Saud University's Animal Care and Use Committee Guidelines on handling and treatment of animals for basic scientific research.

Cell culture

HRMEC were purchased from Cell Systems Corporation (Kirkland, WA) and maintained in complete serum free media (Cat. No. SF-4Z0-500, Cell System Corporation) supplemented with Rocket Fuel (Cat No. SF-4Z0-500, Cell System Corporation), Culture Boost (Cat. No. 4CB-500, Cell System Corporation) and antibiotics (Cat. No. 4Z0-643, Cell System Corporation) at 37°C in a humidified atmosphere with 5% CO_2. We used HRMEC cells up to passage 8 for all the experiments. When HRMEC cells became ~80% confluent, cells were starved in medium without growth factors (Rocket Fuel and Culture Boost) overnight to eliminate any residual effects of growth factors. Following starvation, HRMEC were either left untreated or treated with 10 µg/ml of cytokine-HMGB 1, lipopolysaccharide free (Cat. No. REHM120, IBL International Corp, Toronto, ON) or HMGB1 plus 50 ng/ml of MMP-9 inhibitor I (CAS 1177749-58-4; Cat. No. sc-311437, Santa Cruz Biotechnology, Inc). This dose of cytokine-HMGB 1 was based on our previous studies [23]. Cells were harvested after 24 hour in western 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) for Western blot analysis.

Real time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from retina using TRI reagent (Ambion, TX), according to the manufacturer's protocol. cDNA were synthesized from 1 µg RNA, using an high capacity cDNA reverse transcription kit (Applied Biosystem,

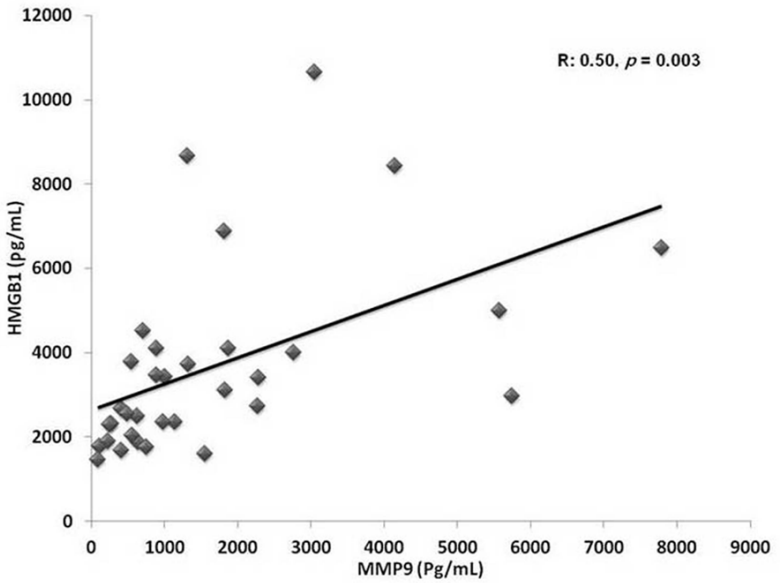

Figure 1. Correlations. Significant positive correlation between vitreous fluid levels of high-mobility group box-1 protein (HMGB1) and levels of MMP-9 in vitreous samples from 25 patients with proliferative diabetic retinopathy and 17 nondiabetic control patients.

CA) following manufacturer's instruction. Real time RT-PCR was performed using a SYBR green PCR master mix. The PCR primers for rat were: MMP-9 forward 5'-CCACCGAGCTATCCACTCAT-3' reverse 5'-GTCCGGTTTCAGCATGTTTT-3'; TIMP1 Forward 5'-CTGGCATCCTCTTGTTGCT-3' and reverse 5'-CACAGCCAGCACTATAGGTCTTT-3' and primers for mice were: MMP-9 forward 5'-GCAACGGAGACGGCAAACC-3' and reverse 5'-GACGAAGGGGAAGACGCA-3'; β-actin forward 5'-CCTCTATGCCAACACAGT GC-3' reverse 5'-CATCGTACTCCTGCTTGCTG-3'. 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 seconds and one min at 60°C. Threshold lines were automatically adjusted to intersect amplification lines in the linear portion of the amplification curves and cycle to threshold (Ct) values were recorded automatically. Data were normalized with β-actin-mRNA levels (housekeeping gene) and the fold change in gene expression relative to normal was calculated using the ddCt method.

Western blot analysis

Retinas and cell lysates were homogenized in a Western Blot lysis buffer. The lysate was centrifuged at 14,000 ×g for 15 min at 4°C and the supernatants was collected and equal amounts of protein (50 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Immunodetection was performed using antibodies against MMP-9 (1:500; R and D systems,) and HMGB-1 (Abcam, UK). Membranes were stripped and reprobed with β-actin to evaluate the lane-loading control. Bands were visualized with the use of 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.).

In vitro migration assay

HRMEC were plated at 1×10^5 cells/well on 6-well culture plates and allowed to grow as described above till 80-90% confluence. Quiescence was induced by incubating the cells in minimal media overnight. Scratches were made with a sterile pipette tip and then cells were rinsed with PBS. Cells were either left untreated or treated either with HMGB1 (10 μg/ml) or HMGB1 plus MMP-9 inhibitor I (50 ng/ml each) for 18 hrs at 37°C in a CO_2 incubator. Cell migration was monitored by visual examination using an inverted microscope (Olympus 1 X 81). Analysis of migration was done using Image J software.

Statistical analysis

The results are presented as mean ± s.d. and analyzed statistically using the nonparametric Kruskal-Wallis test, followed by the Mann-Whitney test for multiple group comparisons. Pearson correlation coefficients were computed to investigate correlation between variables. A *p*-value less than 0.05 indicated statistical significance. SPSS version 12.0 was used for the statistical analyses.

Results

Levels of HMGB1 and MMP-9 in vitreous samples

HMGB1 was detected in all vitreous samples from patients with PDR (n = 25) and nondiabetic control patients (n = 17). The mean level of

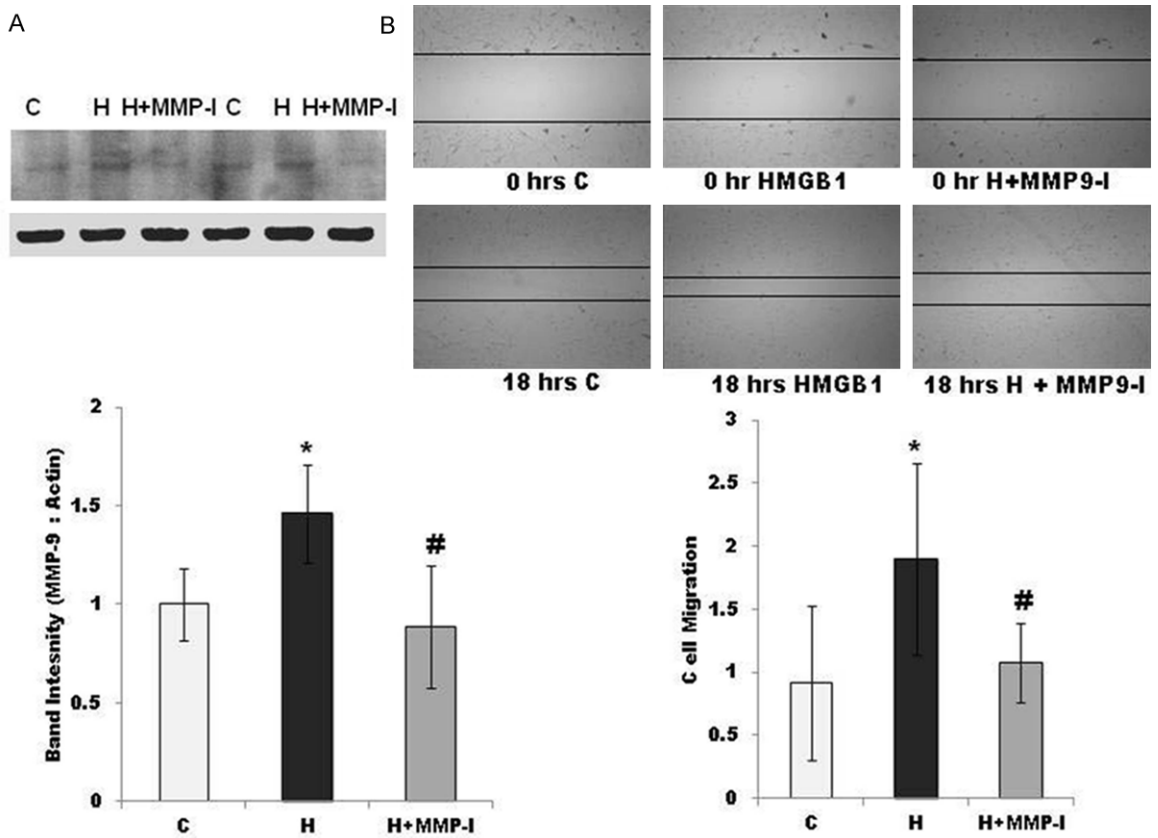

Figure 2. Effect of MMP-9 inhibitor on HMGB1-induced MMP-9 expressions and migration in HRMEC: A. Protein expressions of MMP-9 in cell lysates were determined by Western blotting. β-actin was used as a loading control. Control = untreated cells, H = cells treated with HMGB1 and H + MMP-I = cells treated with HMGB1 plus MMP-9 inhibitor. B. HMGB1-induced migration of the HRMEC was inhibited by the MMP-9 inhibitor. Cells were visualized using inverted microscope and six independent field images were taken from each treated group for migration analysis which was done by using Image J software. One image from each group is illustrated and the bar graphs show the analysis of all six images from each group. *The difference between the two means was statistically significant at the 5% level. Data are mean ± SD from three different experiments, each is performed in triplicate. *$P < 0.05$ compared to control and #$P < 0.05$ compared to diabetic.

HMGB1 in vitreous samples from PDR patients (4436.76 ± 2144.7 pg/mL) was significantly higher than the mean level in nondiabetic control patients (1990.99 ± 363.7 ng/ml) ($P < 0.0001$; Mann-Whitney test).

The levels of MMP-9 were measured in 21 vitreous samples from patients with PDR and in 17 vitreous samples from nondiabetic patients. The mean level of MMP-9 in vitreous samples from PDR patients (2312.14 ± 1955.7 pg/ml) was significantly higher than the mean level in nondiabetic control patients (720.29 ± 582 pg/ml) ($P < 0.0001$; Mann-Whitney test).

A significant positive correlation was observed between vitreous fluid levels of HMGB1 and MMP-9 ($r = 0.5$, $P = 0.003$) (**Figure 1**). These findings suggested the presence of a mechanistic link between HMGB1 and MMP-9 in the pathogenesis of diabetic retinopathy. To validate these clinical observations, we used *in vitro* and *in vivo* model experiments.

Effect of HMGB1 on MMP-9 expressions in HRMEC

To validate the significant positive correlation between the vitreous fluid levels of HMGB1 and MMP-9, we performed induction experiments on HRMEC with HMGB1. Stimulation of HRMEC with HMGB1 significantly increased MMP-9 expression by 42% as compared to untreated cells. Co-treatment of HMGB1 and MMP-9 inhibitor I attenuated significantly HMGB1-induced upregulation of MMP-9 expression (**Figure 2A**).

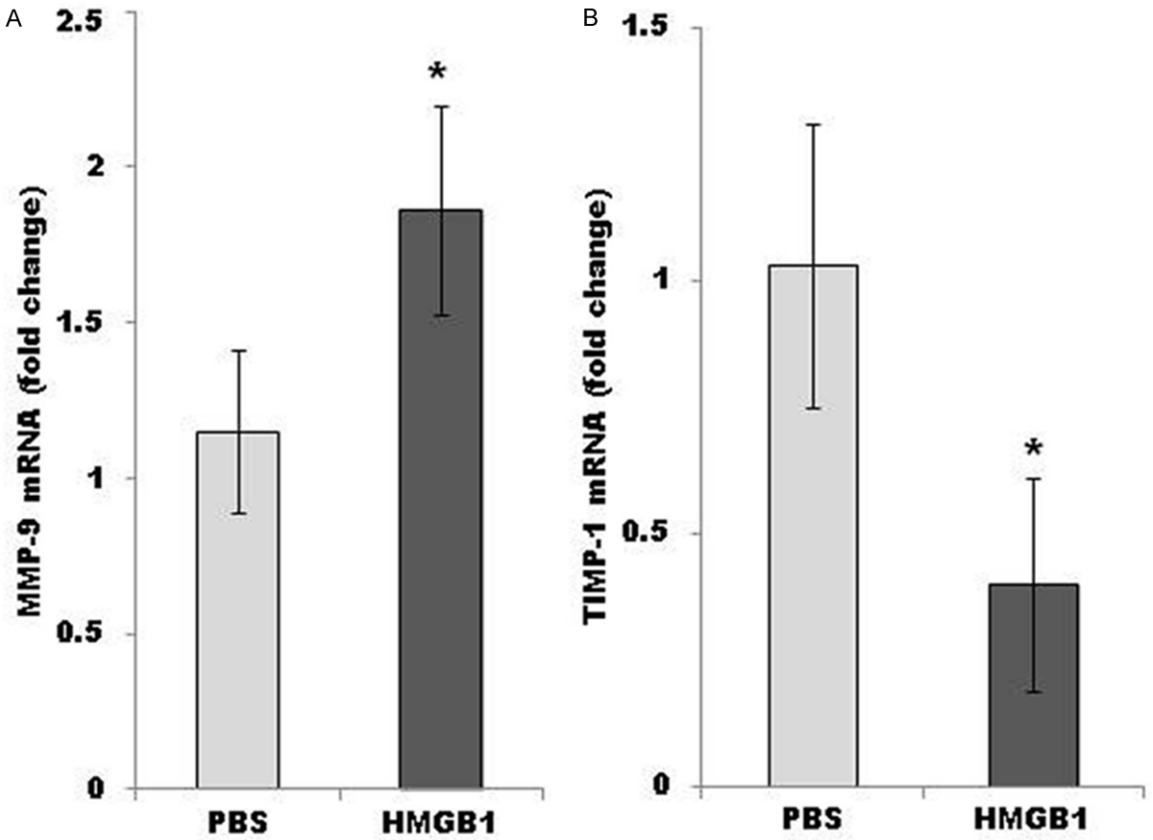

Figure 3. Effect of intravitreal administration of MMP-9 and TIMP-1 mRNA levels in retinas: Gene expression of MMP-9 (A) and TIMP-1 (B) was quantified by real-time PCR and was adjusted to the mRNA levels of β-actin in each sample. Each measurement was made in duplicate or triplicate. PBS = phosphate buffer saline (PBS) injected eye, HMGB1 = HMGB1 injected eye. Data are mean ± SD from retina from 6 rats in each group, *P < 0.05 compared to PBS injected eye.

Effect of HMGB1 and MMP-9 inhibitor on HRMEC migration

Stimulation of HRMEC with HMGB1 significantly enhances the cell migration. Co-treatment of HMGB1 and MMP-9 inhibitor I significantly inhibited the migration induced by HMGB1 in HRMEC suggesting that MMP-9 mediated the HMGB1-induced cell migration in HRMEC (**Figure 2B**).

Intravitreal administration of HMGB1 increased MMP-9 and decreased TIMP-1 mRNA levels in the retinas

RT-PCR analysis demonstrated significant upregulation of MMP-9 and downregulation of TIMP-1 levels in the retinas of HMGB-injected eyes compared to PBS-injected eyes. HMGB1 injection resulted in a 1.5-fold increase in MMP-9 mRNA levels when compared with the values obtained from contralateral eye that received PBS alone (**Figure 3A**). In the same HMGB1 injected eyes samples, TIMP-1 retinal mRNA level were decreased compared to PBS-injected eyes (**Figure 3B**).

Severity of hyperglycemia in rats

The body weights of the diabetic rats were lower and their blood glucose levels were more than four-fold higher compared with age-matched normal control rats (164 ± 25 vs 297 ± 18 g and 449 ± 27 vs 109 ± 12 mg/dl, respectively). Treatment of the diabetic rats with glycyrrhizin for one month did not change these metabolic variables in the diabetic rats (173 ± 27 vs 164 ± 25 g and 463 ± 32 vs 449 ± 27 mg/dl, respectively).

Glycyrrhizin attenuates diabetes-induced upregulation of MMP-9 but not downregulation of TIMP-1 in the retinas

RT-PCR analysis demonstrated significant upregulation of MMP-9 mRNA and down regula-

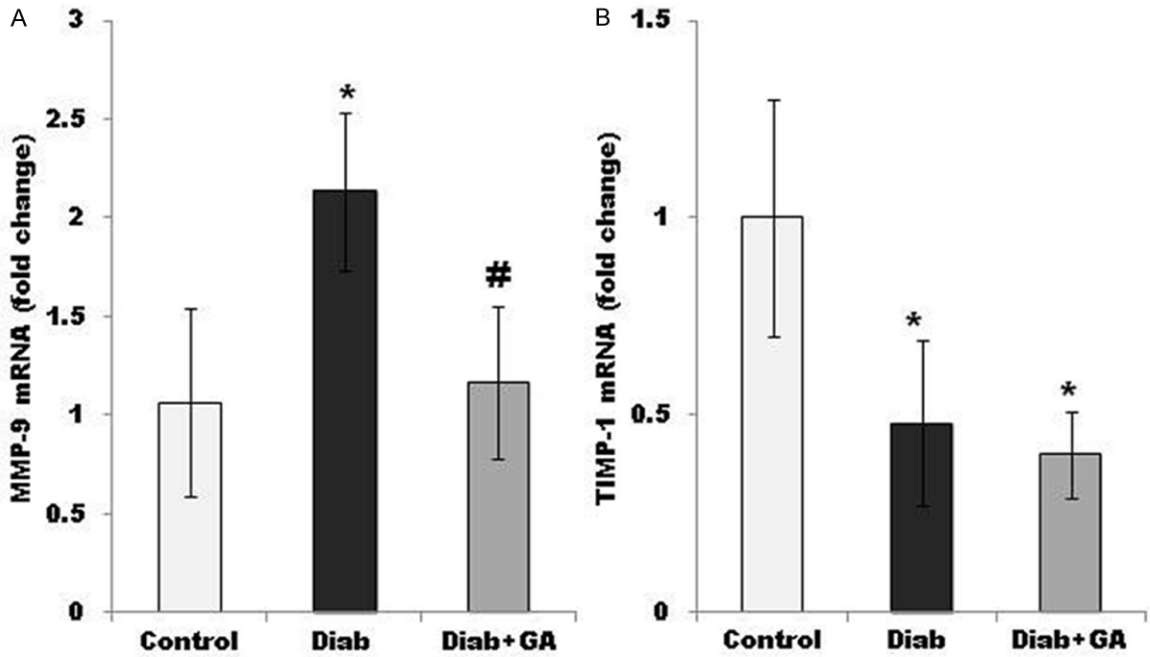

Figure 4. Effect of glycyrrhizin on diabetes-induced alteration MMP-9 and TIMP-1 in the retinas: Gene expression of MMP-9 (A) and TIMP-1 (B) in the retinas was quantified by RT-PCR using the specific primers given in the materials and methods section and were adjusted to the mRNA level of β-actin in each sample. Each measurement was performed at least three times. Results are presented as mean ± SD of 6 rats in diabetes (Diab) and 7 rats each in normal and diabetic + Glycyrrhizin (Diab + GA) groups. $^{*}P < 0.05$ compared to normal, and $^{\#}P < 0.05$ compared to diabetes.

tion of TIMP-1 in diabetic retinas compared to nondiabetic retinas. The mRNA levels of MMP-9 in the retinas of diabetic rats were significantly increased by about twofold as compared to nondiabetic rats. In the same diabetic retina samples, TIMP-1 mRNA level was decreased by 0.5 fold when compared with the nondiabetic retinas (**Figure 4A**, **4B**). The mRNA levels of MMP-9 in the glycyrrhizin-fed diabetic rats were significantly lower than those in the untreated diabetic rats ($P = 0.03$) (**Figure 4A**). Interestingly, the level of TIMP-1 mRNA in the retinas from glycyrrhizin treated diabetic rats did not change as compared to the diabetic rats ($P = 0.38$) but were significantly lower than nondiabetic rats ($P = 0.02$) (**Figure 4B**).

Effect of targeted deletion of the MMP-9 gene on the HMGB1 expression

MMP-9 mRNA level in retina of WT diabetic mice was about 1.8 fold higher compared with the WT nondiabetic mice. As a control, in MMP9-KO mice diabetes had no effect on retinal MMP-9 expression (**Figure 5A**).

There was a significant increase in the protein expression of HMGB1 in retina of WT diabetic mice compared to WT nondiabetic and the expression level of HMGB1 were did not changed in MMP9-KO diabetic mice compared with WT diabetic mice (**Figure 5B**).

Discussion

In the present study, we corroborated that HMGB1 and MMP-9 were significantly upregulated in the vitreous fluid from patients with PDR. Our results are consistent with previous reports in different patient cohorts that demonstrated increased levels of HMGB1 [29, 30] and MMP-9 [3, 4] in the vitreous fluid of PDR patients. In addition, we showed a significant positive correlation between vitreous fluid levels of HMGB1 and the levels of MMP-9. These findings suggest the presence of a mechanistic link between HMGB1 and MMP-9 in the pathogenesis of PDR. Similarly, previous reports demonstrated that increased HMGB1 expression is linked to MMP-9 expression and activation in several diseases. Significant correlation was reported between serum levels of HMGB1 and MMP-9 in patients with diabetes [36]. In patients with ischemic stroke, increased plasma levels of HMGB1 and MMP-9 are associat-

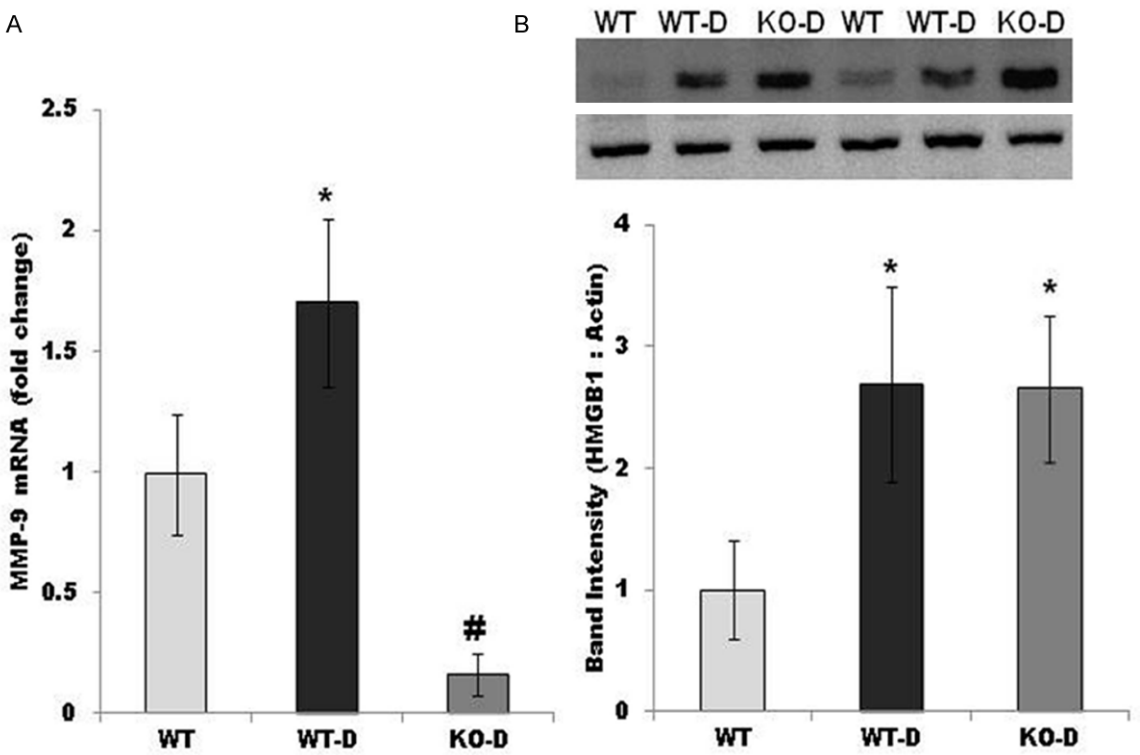

Figure 5. Effect of MMP-9 knocked out on retinal expression of MMP-9 and HMGB1. A. The mRNA level of MMP-9 was quantified in the retinal homogenate by RT-PCR. B. The expression of HMGB1 was quantified by western blotting using specific antibody and was adjusted to the protein levels of β-actin in each sample. Each measurement was performed at least three times. Results are expressed as mean ± s.d. of at least 6 mice in each group. *P < 0.05 compared with WT nondiabetic mice and #P < 0.05 compared to WT diabetic mouse. WT and WT-D = wild-type nondiabetic and diabetic, respectively, and KO-D = MMP-9 knockout diabetic mice.

ed with a poor functional outcome and are significantly correlated with each other [37]. In non-small cell lung cancer specimens, high HMGB1 expression was significantly associated with clinically advanced stages and was correlated to the expression of MMP-9 [38]. In a mouse model of abdominal aortic aneurysm, the expression of HMGB1 was increased, and was positively correlated with MMP-9 activity [39].

To corroborate the findings at the cellular level, stimulation with HMGB1 caused upregulation of MMP-9 in HRMEC. In addition, diabetes and intravitreal injection of HMGB1 in normal rats induced significant upregulation of the mRNA levels of MMP-9 and downregulation of the mRNA levels of TIMP-1 in the retinas. Consistent with our data, increased expression of MMP-9 and decreased expression of TIMP-1 have been reported in the retinas of diabetic rats [42, 43]. *In vitro* studies showed that HMGB1 upregulated MMP-9 in neuronal and glial cell cultures [44], astrocytes [45] and hepatocarcinoma cells [46]. In addition, stereotactic injection of HMGB1 directly into normal striatum caused a significant upregulation of MMP-9 in striatal homogenates [44].

In previous studies, we demonstrated that diabetes and intravitreal administration of HMGB1 in normal rats induced significant upregulation of the expression of HMGB1, RAGE, activated ERK1/2 and activated NF-kB in the retinas [29, 32-34]. In addition, diabetes increased the interaction between HMGB1 and RAGE in the retina of rats [32, 35]. Furthermore, in our laboratory, we recently demonstrated that exposure of HRMEC to HMGB1 and intravitreal administration of HMGB1 in normal rats induced significant upregulation of reactive oxygen species [47]. In the context of PDR, these pathways may be especially relevant because NF-kB and ERK1/2 signaling pathways [43, 48] and reactive oxygen species [48, 49] play pivotal role in the production and release of MMP-9. We also

demonstrated that HMGB1 induced significant upregulation of the angiogenic biomarker vascular endothelial growth factor (VEGF) in HRMEC and in the retinas of rats [35]. VEGF has been shown to induce MMP-9 expression in different cells [9, 50].

Constant glycyrrhizin intake significantly reduced diabetes-induced upregulation of MMP-9, but did not affect the expression of TIMP-1 in the retinas of rats. Glycyrrhizin has been shown to bind to, and inhibit mitogenic, chemo-attractant and cytokine-like activities of HMGB1 [40]. Similarly, previous studies demonstrated that HMGB1 blockade markedly reduced MMP-9 expression and activity. Treatment with neutralizing anti-HMGB1 monoclonal antibody suppressed the expression and activity of MMP-9, and ameliorated brain infarction induced by middle cerebral artery occlusion in rats [51], and suppressed aneurysm formation in a mouse model of abdominal aortic aneurysm [39]. Treatment with HMGB1 small interfering RNA reduced MMP-9 expression and cellular metastatic ability in gastric cancer cells [52] and in non-small cell lung cancer cells [38]. Blockade of HMGB1-RAGE axis by ethyl pyruvate decreased the expression of MMP-9 and inhibited gall bladder cancer cell proliferation and invasion [53]. In addition, knockdown of RAGE in cancer cells diminished HMGB1-induced increased production of MMP-9 suggesting that RAGE is required for HMGB1 promotion of tumor invasion by increased production of active MMP-9 [44].

The results of the present study also show that deletion of the MMP-9 gene in MMP-9 gene knockout mice did not inhibit diabetes-induced HMGB1 upregulation in the retina. In addition, our *in vitro* studies showed that exposure of HRMEC to HMGB1 induced MMP-9 expression and stimulated HRMEC migration, a key early step in angiogenesis. A MMP-9 inhibitor decreased MMP-9 expression and inhibited HRMEC migration induced by HMGB1. Our results indicate that MMP-9 acts downstream of HMGB1 and that MMP-9 plays a significant role in mediating HMGB1-induced angiogenesis in PDR.

In conclusion, on the basis of our clinical and *in vitro* and *in vivo* studies, we hypothesize that MMP-9 is a downstream effector that plays a critical role in HMGB1-dependent angiogenic activity in PDR. The ability of HMGB1 to upregulate MMP-9 provides a novel mechanism by which retinal injury is amplified in patients with PDR. Targeting the HMGB1-MMP-9 pathway may be a novel therapeutic approach for PDR.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ghulam Mohammad, Department of Ophthalmology, College of Medicine, King Saud University, P.O. Box 245, Riyadh 11411, Saudi Arabia. Tel: 966-1-4775723; Fax: 966-1-4775724; E-mail: mghulam@KSU.edu.sa; gmkbiochembhu@gmail.com

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