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A cross-sectional study of vitreous and serum high mobility group box-1 levels in proliferative diabetic retinopathy

Yinchen Shen,* Hui Cao,* Feng'e Chen, Yan Suo, Ning Wang and Xun Xu

Department of Ophthalmology, Shanghai General Hospital, School of medicine, Shanghai Jiaotong University, Shanghai, China

ABSTRACT.

Purpose: **We determined vitreous and serum levels of high mobility group box-1 (HMGB-1) in patients with proliferative diabetic retinopathy (PDR) and elucidate their relationship with receptor for advanced glycation end products (RAGE), vascular endothelial growth factor (VEGF) and interleukin-1β (IL-1β).**

Methods: **In this cross-sectional study, patients with PDR who underwent vitrectomy were enrolled, and the control group included non-diabetic eyes. Vitreous and serum samples were analysed for HMGB-1, RAGE, VEGF and IL-1β by ELISA. We investigated the correlation between serum and vitreous levels of each cytokine, and we analysed the influence of intravitreal anti-VEGF treatment prior to vitrectomy on the cytokine levels in PDR.**

Results: **Of 78 eyes of 78 patients enrolled consecutively, there were 32 PDR eyes and 46 control eyes. The serum levels were higher in diabetic than in non-diabetic subjects for HMGB-1, RAGE, VEGF and IL-1β (all $p < 0.001$), respectively. Similarly, the vitreous levels were higher in diabetic than in non-diabetic subjects for HMGB-1 ($p < 0.001$), RAGE ($p = 0.001$), VEGF ($p < 0.001$) and IL-1β ($p < 0.001$), respectively. We found a positive correlation between serum and vitreous levels of HMGB-1 in patient with PDR ($p = 0.047$, $R = 0.353$). There was a negative correlation between serum and vitreous levels of VEGF in patient with PDR ($p = 0.001$, $R = -0.546$). For the subgroup analysis, we detected that the vitreous levels of RAGE were significantly lower in patients who underwent anti-VEGF injection prior to vitrectomy than those who did not ($p < 0.001$).**

Conclusions: **Our findings suggest that HMGB-1 is involved in PDR disorders, and it may be a novel therapeutic target to inhibit progression of PDR.**

Key words: diabetic retinopathy – HMGB-1 – human – inflammation – vitreous humour

*These authors contributed equally to this work and are listed as cofirst authors.

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Introduction

Diabetic retinopathy (DR) is the most frequent cause of legal blindness among working-age individuals in developed countries (Antonetti et al. 2012). The incidence of DR in adult diabetic patients is higher than 40%, and approximately 5%–10% of DR cases progress to severe visual impairment (Kempen et al. 2004). Proliferative diabetic retinopathy (PDR) is a severe stage of DR, characterized by neovascularization followed by fibrovascular changes resulting in vitreous haemorrhage (VH) or tractional retinal detachment (TRD) (Gologorsky et al. 2012). A growing body of scientific evidence supports a pathogenic role of chronic low-grade inflammation in the development of DR (Adamis & Berman 2008). Several clinical studies have demonstrated that diabetics have increased serum levels of inflammatory markers, including C-reactive protein, interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-α), suggesting that inflammatory processes play a considerable role in the pathogenesis of DR (Tang & Kern 2011). Moreover, in the vitreous of patients with advanced stages of DR, a number of angiogenic cytokines, for example endothelin 1 (EDN1), vascular endothelial growth factor (VEGF), are elevated consistently (Zhou et al. 2012). The causal relationship between inflammation and angiogenesis is now widely accepted (van Beijnum et al. 2008).

High mobility group box-1 (HMGB-1) protein is a nuclear DNA-binding protein released passively from necrotic cells and actively from monocytes/macrophages and endothelial cells (Dvoriantchikova et al. 2011). Release of chromatin protein HMGB-1 by necrotic cells triggers inflammation and recruits leucocytes to the site of tissue damage (van Beijnum et al. 2008). Other studies demonstrated that HMGB-1 exhibits angiogenic effects (Mitola et al. 2006; Schlueter et al.

2005). Previous studies indicated that the receptor for advanced glycation end products (RAGE) mediates the inflammatory (Luan et al. 2010) and angiogenic (Mitola et al. 2006; Sasahira et al. 2007) activities of HMGB-1. During inflammation, HMGB-1 signals through RAGE, resulting in activation of the transcription factor nuclear factor κB (NF-κB), and induces the expression of various pro-inflammatory cytokines and chemokines (van Beijnum et al. 2008; Luan et al. 2010). As for angiogenesis, RAGE blockade inhibits HMGB1-induced neovascularization in vivo and endothelial cell proliferation in vitro, identifying HMGB1/RAGE interaction as a potent proangiogenic stimulus (Sasahira et al. 2007).

To our knowledge, there are no published studies demonstrating elevated vitreous and serum HMGB-1 levels in Chinese patients with PDR. Therefore, the primary aim of our study was to analyse and compare HMGB-1 levels in the vitreous as well as in the serum of patients with and without diabetic retinopathy. Our secondary aim was to determine the relationship of HMGB-1 with its receptor RAGE and other cytokines (VEGF and interleukin 1 beta (IL-1β)) that have been implicated in the pathogenesis of DR.

Patients and Methods

This cross-sectional study was approved by the ethics committee of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China (permit No. 2017KY169) and conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all the patients.

Subjects

All participants were recruited from Department of Ophthalmology, Shanghai General Hospital, School of Medicine, Shanghai Jiaotong University from 31 August 2017 to 17 April 2018. Inclusion criteria were as follows: (1) age ≥18 years; (2) Han Chinese ethnicity with PDR undergoing vitrectomy for VH and/or TRD. Exclusion criteria were as follows: (1) those who had prior ophthalmic surgeries (e.g. pars plana vitrectomy), intravitreal triamcinolone acetonide (IVTA) treatment, trauma or endophthalmitis; (2) with clinical histories that may cause VH or TRD (e.g. uveitis and retinal venous occlusion); (3) with diabetic complications other than DR; and (4) pregnancy or breast-feeding. The control subjects without DM were randomly chosen using random number table from patients who underwent vitrectomy. The inclusion criteria of the control group were as follows: (1) age ≥18 years; (2) Han Chinese ethnicity without DM or family history of DM undergoing vitrectomy for rhegmatogenous retinal detachment, macular hole, etc. The exclusion criteria were the same as the PDR group.

Examination procedures

The diagnosis of diabetes was based on criteria outlined by the American Diabetes Association in 1997. The evaluation of DR was according to the diagnostic criteria of the American Academy of Ophthalmology (AAO) 2001 Annual Meeting by retinal examination using Ophthalmoscope by a senior ocular fundus specialist (Xu X). Age, gender, type of diabetes, duration of diabetes, history of hypertension, regular medication, history of prior photocoagulation and/or anti-VEGF therapy were recorded.

Collection of vitreous and serum samples

Blood was collected on an empty stomach in the morning one day before vitrectomy for measurements of fasting blood glucose, haemoglobin (HbA1c), total cholesterol, triglyceride, creatinine and serum levels of HMGB-1, RAGE, VEGF and IL-1β. Whole blood was collected, and after centrifugation (1789 ***g*** for 20 min, 4°C), serum samples were stored at −80°C until assay.

Vitreous was collected for the analysis of HMGB-1, RAGE, VEGF and IL-1β. Vitrectomy was conducted under nerve block anaesthesia by two ocular fundus specialists with over twenty years' surgery experience (Feng'e Chen and Hui Cao). In order to prevent severe complications such as explosive choroidal haemorrhage, 0.3–0.6 ml undiluted vitreous samples was obtained at the onset of vitrectomy by manual suction to a syringe through the aspiration line of vitrectomy, before opening the infusion line. The core vitreous was obtained to eliminate the influence of blood cells deposited in the inferior vitreous cavity, and care was taken not to aspire any blood if avoidable. Vitreous samples were passed into sterile tubes, immediately placed on ice and centrifuged (2795 ***g*** for 10 min, 4°C), and the supernatants were aliquoted and frozen at −80°C until assay.

Analytical techniques

Enzyme-linked immunosorbent assay (ELISA) kits for human HMGB-1, RAGE, VEGF and IL-1β (Shanghai X-Y biotechnology Company, Ltd., China) were used. The detection range for each ELISA kit for HMGB-1, RAGE, VEGF and IL-1β is 5–100 μg/L, 130–4000 pg/ml, 30–1200 pg/ml and 1–40 pg/ml, respectively. The ELISA plate readings were done using DNM-9602 microplate reader (Pu Lang technology Company, Ltd., Beijing, China). We followed the serial dilution methods to prepare the series of standards. For each ELISA kit, the undiluted standard served as the highest standard and calibrator diluents served as the zero standard. Depending on the detection range for each ELISA kit, the supernatant vitreous obtained was used either directly or diluted with calibrator diluents supplied with the ELISA kit. In order to normalize the ELISA data with equal protein, we use the BCA protein quantitative kit to measure the protein level of every vitreous sample. Total concentration in the vitreous was determined by the BCA protein assay kit (Jiancheng technology Company, Ltd., Nanjing, China), and equal amount (40 μg) of protein was used for ELISA assay.

Vitreous and corresponding serum cytokines were analysed in a 100 count microplate ELISA kit. Quantification of human HMGB-1, RAGE, VEGF and IL-1β in the vitreous fluid and serum was determined by standard clinical laboratory methods in accordance with the manufacturer's instructions, and all standards were within limits of detection.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation or median (first-third quartile). The comparisons of cytokine levels were performed using independent-samples *t*-test or Mann-Whitney *U*-test. Categorical variables were expressed as number (percentage). Comparison was made by using chi-

square or Fisher's extract test. Spearman's correlation coefficients were computed to investigate correlations between serum and vitreous levels of each cytokine, and we did subgroup analysis for the influence of intravitreal anti-VEGF treatment prior to vitrectomy on the cytokine levels in PDR. All statistical analysis was performed with SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

Results

Study population

A total of 78 vitreous samples and 78 serum sample from 78 patients (78 eyes) were collected. Among these, 32 eyes of 32 patients (41.0%) underwent vitrectomy for complications due to PDR, including VH and/or TRD. Among the 32 PDR eyes, 27 had VH, and 5 had a TRD; 9 eyes (28.1%) had a history of intravitreal anti-VEGF (Lucentis; Genentech, South San Francisco, CA, USA) injection (3–5 days prior to the vitrectomy). The purpose of anti-VEGF treatment was to reduce the postoperative VH. Eight eyes (25.0%) had prior panretinal photocoagulation (PRP) in order to suppress retinal neovascularization. The remaining 46 non-diabetic controls (46 eyes) underwent vitrectomy for rhegmatogenous retinal detachment (RRD, 39 eyes), macular hole (MH, 2 eyes) and VH with retinal hole or age-related macular degeneration (5 eyes).

Demographic characteristics of all patients included in the study were shown in Table 1. The mean age of all subjects was 56.83 ± 12.71 years (range, 24–84 years), and 40 patients were male, and 38 were female. Diabetic subjects were younger than non-diabetic subjects ($p = 0.044$). Among patients with PDR, the mean age was 52.88 ± 14.48 years (range, 27–79 years), whereas the mean age of non-diabetics was 59.59 ± 10.63 years (range, 24–84 years). There were no significant differences between the PDR and control groups in regard to gender ($p = 0.233$), history of hypertension ($p = 0.078$), total cholesterol ($p = 0.205$) and triglyceride ($p = 0.151$). The fasting blood glucose ($p < 0.001$) and creatinine ($p = 0.030$) were significantly higher in patients with PDR than in the control group.

Table 1. Demographic characteristics of the patients with proliferative diabetic retinopathy (PDR) and control subjects.

Variables	PDR ($n = 32$)	Control ($n = 46$)	p value
Gender, male (%)	19 (59.4%)	21 (45.7%)	0.233
Age (years)	52.88 ± 14.48	59.59 ± 10.63	0.044
Duration of diabetes (years)	11.28 ± 6.04	–	–
Types of diabetes			
Type 1 (%)	0	–	–
Type 2 (%)	32 (100%)		
Hypertension (%)	19 (59.4%)	18 (39.1%)	0.078
Fasting blood glucose (mmol/l)	9.90 ± 4.50	5.86 ± 1.25	<0.001
HbA1c (%)	7.89 ± 1.30	–	–
Total cholesterol	4.37 ± 1.13	4.68 ± 0.94	0.205
Triglyceride	2.07 ± 1.16	1.63 ± 0.98	0.151
Creatinine	87.77 ± 42.03	66.48 ± 13.54	0.030

Cytokine levels in serum samples

HMGB-1, RAGE, VEGF and IL-1β were detected in all serum samples. The serum levels were higher in diabetic than in non-diabetic subjects for HMGB-1 (185.69 ± 59.20 μg/l versus 133.42 ± 49.04 μg/L, $p < 0.001$), RAGE (5189.07 ± 1560.61 pg/ml versus 3000.52 ± 1119.56 pg/ml, $p < 0.001$), VEGF (1485.21 ± 497.97 pg/ml versus 884.74 ± 365.22 pg/ml, $p < 0.001$) and IL-1β (65.94 ± 20.43 pg/ml versus 31.52 ± 13.64 pg/ml, $p < 0.001$), respectively. Subject characteristics are described in Table 2.

Cytokine levels in vitreous samples

HMGB-1, RAGE, VEGF and IL-1β were detected in all vitreous samples. The vitreous levels were higher in diabetic than in non-diabetic subjects for HMGB-1 (197.34 ± 67.60 μg/l versus 126.74 ± 52.84 μg/l, $p < 0.001$), RAGE (4447.36 ± 1909.27 pg/ml versus 2973.61 ± 1262.68 pg/ml, $p = 0.001$), VEGF (1149.44 ± 501.40 pg/ml versus 677.10 ± 236.29 pg/ml, $p < 0.001$) and IL-1β (59.01 ± 19.79 pg/ml versus 36.24 ± 15.76 pg/ml, $p < 0.001$), respectively. Subject characteristics are described in Table 2.

Correlation between cytokine levels in the vitreous and in the serum

We found a positive correlation between serum and vitreous levels of HMGB-1 in patient with PDR ($p = 0.047$, $R = 0.353$). On the contrary, there was a negative correlation between serum and vitreous levels of VEGF in patient with PDR ($p = 0.001$, $R = -0.546$). In the control group, we found a positive correlation between serum and vitreous levels of VEGF ($p = 0.025$, $R = 0.329$). None of the other cytokine levels in vitreous samples were associated with those in

Table 2. Cytokine levels in serum and vitreous of the patients with proliferative diabetic retinopathy (PDR) and control subjects.

Cytokines	PDR ($n = 32$)	Control ($n = 46$)	p value
Serum			
HMGB-1 (μg/l)	185.69 ± 59.20	133.42 ± 49.04	<0.001
RAGE (pg/ml)	5189.07 ± 1560.61	3000.52 ± 1119.56	<0.001
VEGF (pg/ml)	1485.21 ± 497.97	884.74 ± 365.22	<0.001
IL-1β (pg/ml)	65.94 ± 20.43	31.52 ± 13.64	<0.001
Vitreous			
HMGB-1 (μg/l)	197.34 ± 67.60	126.74 ± 52.84	<0.001
RAGE (pg/ml)	4447.36 ± 1909.27	2973.61 ± 1262.68	0.001
VEGF (pg/ml)	1149.44 ± 501.40	677.10 ± 236.29	<0.001
IL-1β (pg/ml)	59.01 ± 19.79	36.24 ± 15.76	<0.001

HMGB-1 = high mobility group box-1, IL-1β = interleukin-1β, PDR = proliferative diabetic retinopathy, RAGE = receptor for advanced glycation end products, VEGF = vascular endothelial growth factor.

Table 3. Correlation between cytokine levels in the vitreous and in the serum of the patients with proliferative diabetic retinopathy (PDR) and control subjects.

	PDR (n = 32)		Control (n = 46)	
Cytokines	Correlation coefficient	p value	Correlation coefficient	p value
HMGB-1	0.353	0.047	−0.030	0.841
RAGE	0.087	0.635	0.165	0.274
VEGF	−0.546	0.001	0.329	0.025
IL-1β	−0.155	0.396	−0.095	0.536

HMGB-1 = high mobility group box-1, IL-1β = interleukin-1β, PDR = proliferative diabetic retinopathy, RAGE = receptor for advanced glycation end products, VEGF = vascular endothelial growth factor.

serum samples. The results were described in Table 3.

Influence of intravitreal anti-VEGF injection prior to vitrectomy on the cytokine levels in patients with PDR.

The vitreous levels of RAGE were significantly lower in patients who underwent intravitreal anti-VEGF treatment prior to vitrectomy than those who did not (2863.89 ± 1176.25 pg/ml versus 5066.98 ± 1790.63 pg/ml, p = 0.002). However, there was no significant difference in serum levels of RAGE between patients with and without anti-VEGF treatment prior to vitrectomy (5056.91 ± 1964.72 pg/ml versus 5240.78 ± 1420.64 pg/ml, p = 0.770). For other cytokines, we did not find influence of intravitreal anti-VEGF treatment on the cytokine levels either in the vitreous or in the serum. The results were described in Table 4.

Discussion

An emerging issue in DR research is the mechanistic link between angiogenesis and inflammation. Previous studies indicated that a number of angiogenic cytokines are elevated in the vitreous of patients with advanced stages of DR, that is PDR. On the other hand, inflammatory pathway also plays pivotal role in the progression of DR. DR is recognized as a chronic, low-grade subclinical inflammatory disorder. Inflammatory cytokines mediate the synthesis of acute phase proteins which are able to initiate and support inflammatory process in the vascular wall (Ozturk et al. 2009), which aggravate the progression of PDR.

The purpose of this study was to gain a better understanding of the cellular and molecular processes underlying the pathogenesis of PDR disorders, especially the 'link-bridge' between angiogenesis and inflammation. We compared HMGB-1, inflammatory parameters (RAGE, IL-1β) and angiogenesis parameter (VEGF) in patients with PDR and non-diabetic control patients. As a result, HMGB-1 levels were significantly elevated in the vitreous fluid of patients with PDR. Increased levels of inflammatory parameters and VEGF were also observed in PDR. In serum samples, the levels of the above parameters were also high in PDR group compared with control group. We found a positive correlation between serum and vitreous levels of HMGB-1 in patient with PDR. Furthermore, the results showed that there was a significant decrease in RAGE levels in the vitreous in PDR after intravitreal anti-VEGF injection. However, we did not find effects of anti-VEGF treatment on other cytokines.

Previous study found that HMGB1 induced HRMEC migration which was a key early step in angiogenesis (Mohammad et al. 2017). Stimulation with HMGB1 caused the upregulation of VEGF, a key angiogenic factor in PDR (Spranger & Pfeiffer 2001). In our study, HMGB1 and VEGF levels were significantly elevated in the vitreous fluid of patients with PDR, suggesting that upregulation of HMGB1 might contribute to the initiation and progression of angiogenesis in PDR. This result consisted with that of Ahmed et al. who reported HMGB-1 was significantly higher in vitreous samples from patients with PDR than those from non-diabetics (Abu El-Asrar et al. 2017). In addition, prior researches indicated that HMGB-1 mediates inflammatory responses by increasing the cell surface expressions of ICAM-1, VCAM-1 on the surface of endothelial cells, thereby promoting barrier disruption and the adhesion of leucocytes (Abu El-Asrar et al. 2017; Fiuza et al. 2003; Liu et al. 2016). Moreover, this pro-inflammatory phenotype was mediated by the activation of NF-κB and was RAGE-dependent (Treutiger et al. 2003; Luo et al. 2013). In the present study, we demonstrated increased levels of RAGE and IL-1β in the vitreous and serum of patients with PDR. Our result complied with prior studies conducted by Doganay et al. (2002) who found the mean serum IL-8 and TNF-α levels increased with the stage of DR and, the highest levels were found in patients with PDR. Ahmed M. et al. detected in PDR membranes that stromal cells expressing HMGB1 and RAGE were significantly higher in active membranes than in inactive membranes (El-Asrar et al. 2011). As RAGE is one of the ligand of HMGB-1, the above findings suggested the role for the HMGB-1/RAGE signalling axis in the progression of PDR.

Furthermore, we have investigated the impact of intravitreal anti-VEGF injection on the vitreous cytokine levels in patients with PDR. Postoperative VH is a significant complication

Table 4. Influence of intravitreal antivascular endothelial growth factor (VEGF) injection prior to vitrectomy on the cytokine levels in patients with proliferative diabetic retinopathy (PDR)

	Anti-VEGF (n = 9)	No anti-VEGF (n = 23)	p value
Serum			
HMGB-1 (μg/l)	197.90 ± 70.19	197.12 ± 68.16	0.977
RAGE (pg/ml)	2863.88 ± 1176.25	5066.97 ± 1790.63	0.002
VEGF (pg/ml)	1171.30 ± 597.14	1140.88 ± 473.61	0.880
IL-1β (pg/ml)	66.53 ± 24.40	56.06 ± 17.41	0.183
Vitreous			
HMGB-1 (μg/l)	205.83 ± 51.61	177.81 ± 61.14	0.235
RAGE (pg/ml)	5056.91 ± 1964.72	5240.78 ± 1420.64	0.770
VEGF (pg/ml)	1477.93 ± 410.55	1488.05 ± 536.75	0.960
IL-1β (pg/ml)	72.08 ± 17.90	63.54 ± 21.21	0.295

HMGB-1 = high mobility group box-1, IL-1β = interleukin-1β, PDR = proliferative diabetic retinopathy, RAGE = receptor for advanced glycation end products, VEGF = vascular endothelial growth factor.

following vitrectomy for PDR. It delays visual recovery and can make further treatment difficult if the view of the fundus is significantly obscured (Smith & Steel 2015). A number of interventions to reduce the incidence of VH have been proposed, including the perioperative use of anti-VEGF. Anti-VEGFs reduce vascular proliferation and the vascularity of neovascular tissue, which is often the source of bleeding following vitrectomy. In our study, there was a significant decrease in RAGE levels in the vitreous in PDR after intravitreal anti-VEGF injection. However, we did not find effects of anti-VEGF treatment on other cytokines. We guess there might be two reasons. Firstly, the patients who underwent anti-VEGF treatment suffered from more severe PDR than those who did not, and thus, their vitreous cytokines levels prior to anti-VEGF treatment might be even higher. Secondly, the relative small sample size of patients who underwent anti-VEGF injections may influence the results.

The limits of this study were its cross-sectional nature and relative small sample size. Our results are preliminary and need further corroboration by larger sample studies. A longitudinal study would clarify the relationship of HMGB-1 and other cytokines with the development of PDR. Thus, further prospective clinical trials for evaluation and treatment of PDR are needed.

In conclusion, our finding suggests the presence of a pro-inflammatory and angiogenesis state in PDR as evidenced by increased levels of HMGB-1, RAGE, VEGF and IL-1β in the vitreous as well as in the serum. HMGB-1 might contribute to the cross-talk links between angiogenesis and inflammation and could be a novel therapeutic target to inhibit progression of PDR.

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Correspondence:
Ning Wang, MD
Department of Ophthalmology
Shanghai General Hospital
School of Medicine
Shanghai Jiaotong University
No.100 Haining Road
Hongkou District
Shanghai 200080
P.R. China
Tel: 86-021-63240090
Fax: 86-021-63240825
Email: drwangning@126.com

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