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Original Research

Unraveling the Role of Midkine in Proliferative Diabetic Retinopathy: Implications from Hypoxia-Induced Angiogenesis

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Abstract

Objectives: This study aimed to compare the expression of midkine (MK) in the vitreous of patients with proliferative diabetic retinopathy (PDR) and non-diabetic individuals, elucidating its potential role in the pathogenesis of the disease.

Methods: This prospective cross-sectional study included three groups of patients who underwent pars plana vitrectomy (PPV) surgery. The first group (control) consisted of patients who underwent PPV for epiretinal membrane and macular hole and did not have diabetes mellitus (DM). The second group included patients who underwent PPV for vitreous hemorrhage (VH) and tractional retinal detachment (TRD) secondary to PDR without prior anti-VEGF treatment (No preoperative anti-VEGF application: NPa-VEGF). The third group comprised patients who underwent PPV for VH and TRD secondary to PDR and received a preoperative anti-VEGF injection one week before surgery (preoperative anti-VEGF application: Pa-VEGF). Vitreous samples were collected intraoperatively, and the concentrations of MK, interleukin (IL)-6, and IL-8 were measured using specific Enzyme-Linked Immunosorbent Assay (ELISA) kits.

Results: The study included a total of 49 eyes from 49 patients undergoing PPV. The concentrations of IL-6 and IL-8 in vitreous samples from the NPa-VEGF group (n=15) and the Pa-VEGF group (n=14) were not significantly different compared to the control group (n=20) ($p>0.05$). However, the vitreous fluid of patients in the NPa-VEGF group exhibited significantly higher MK concentrations compared to the control group ($p<0.007$). Similarly, MK concentrations were significantly elevated in the Pa-VEGF group compared to the control group ($p<0.046$). No significant difference in MK levels was detected between the NPa-VEGF and Pa-VEGF groups ($p>0.05$).

Conclusion: These findings suggest that increased MK expression in the vitreous may be associated with the pathogenesis of PDR. Further studies are warranted to elucidate the precise mechanisms underlying this association and to explore the potential of MK as a therapeutic target for PDR management.

Keywords: Interleukin 6, interleukin 8, midkine, pars plana vitrectomy, proliferative diabetic retinopathy

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Proliferative diabetic retinopathy (PDR) is a significant cause of blindness, driven by the formation of new blood vessels due to retinal ischemia.[1] The pathology of PDR involves microvascular occlusions, leading to retinal hypoxia and the development of ischemic spots.[2] In PDR patients, neuroprotective factors such as somatostatin and pigment epithelium-derived factor are reduced, while pro-inflammatory cytokines like interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) increase in the vitreous.[3] Vascular endothelial growth factor (VEGF) is a key

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player in PDR, with VEGF-A expression increasing under hypoxic conditions, promoting vascular permeability and new blood vessel formation.[4] Treatments targeting VEGF-A, such as aflibercept, play a crucial role in managing PDR.[5] However, the use of anti-VEGF therapy is limited by factors such as high cost, frequent injections, and complications like endophthalmitis.[6] Additionally, intravitreal aflibercept injections administered before pars plana vitrectomy (PPV) in PDR patients have been reported to reduce intraoperative complications, shorten surgical duration, and lower the incidence of postoperative vitreous hemorrhage (VH).[7]

Midkine (MK), a neurotrophic growth factor renowned for its vital roles in neuronal development and viability, is relatively understudied concerning its implications in the human retina.[8] Studies have shown that MK levels increase in rat retinas exposed to ischemia, providing neuroprotection.[9] Furthermore, in mice, MK has been demonstrated to reduce light-induced photoreceptor damage.[10] Moreover, MK has been found to promote angiogenesis, macrophage migration, and inflammation under hypoxic conditions.[11] Elevated MK levels have been associated with several diseases, including rheumatoid arthritis, cancer, and diabetic nephropathy.[12-14] MK's involvement in inflammation has been well-established, with its effects mediated through a signaling pathway involving various receptors. It has been shown to participate in the recruitment of inflammatory cells and support neutrophil recruitment during acute inflammation.[15] Additionally, MK expression is associated with hypoxic conditions and is linked to angiogenesis, the process of forming new blood vessels.[16] Several studies have demonstrated that MK promotes tumor growth, endothelial cell proliferation, and increased vascular density. Moreover, MK has been implicated in neointimal formation by recruiting inflammatory cells in ischemic vascular injury models.[11]

In this investigation, our hypothesis centered on the potential involvement of MK in the pathogenesis of PDR, considering its impact on angiogenesis and inflammatory responses. Consequently, we assessed MK levels alongside various pro-inflammatory cytokines in the vitreous of both PDR patients and non-diabetic individuals with diverse vitreoretinal conditions.

Methods

Subjects

This prospective, cross-sectional, controlled, and comparative study was conducted on patients who underwent PPV at our retina unit between January 2018 and January 2020. For the purpose of analysis, the cohort was divided into three distinct groups.

Control Group (Group 1): This group included patients who underwent PPV for idiopathic epiretinal membrane (ERM) or idiopathic macular hole (MH), with no history of diabetes mellitus (DM). Due to ethical constraints that prevented the use of vitreous samples from healthy individuals, patients with ERM and MH were included in the control group.

NPa-VEGF Group (Group 2): This group included patients with PDR who underwent PPV for long-standing VH (lasting more than 3 months) and/or tractional retinal detachment (TRD) that was either affecting or threatening the macula. These patients had not received any preoperative anti-VEGF treatment.

Pa-VEGF Group (Group 3): This group included patients with PDR-related VH and TRD who received an intravitreal aflibercept injection one week prior to PPV surgery.

Intravitreal aflibercept was exclusively administered to the Pa-VEGF group. Patients who had previously received preoperative bevacizumab or ranibizumab were excluded from participation. Additional exclusion criteria encompassed patients under the age of 18, those with a history of panretinal laser photocoagulation (PRP) or intravitreal injections within the preceding three months, any prior ocular surgeries, or the presence of ocular ischemic syndrome or other ocular pathologies. Furthermore, patients undergoing dialysis for renal insufficiency, those diagnosed with cancer or chronic inflammatory diseases, and individuals with uncontrolled hypertension despite antihypertensive therapy were also excluded. In the control group, patients with secondary ERMs or secondary MHs resulting from trauma or high myopia were similarly excluded from the study.

Each patient provided written informed consent after receiving a detailed explanation of the study. The study protocol was approved by the hospital's institutional review board and adhered to the principles of the Declaration of Helsinki. The local ethics committee also approved the study (approval code: TUTF-BAEK 2018/411). Hemoglobin A1c (HbA1c) levels, along with vitreous concentrations of midkine (MK), interleukin-6 (IL-6), and interleukin-8 (IL-8), were assessed.

Surgical Technique and Vitreous Samples Collection

The current methodology employed a 25-gauge transconjunctival vitrectomy technique. All pars plana vitrectomy (PPV) surgeries were conducted in an operating room setting by the same surgeon (SAO). During the initial stages of PPV, 0.5 mL of undiluted vitreous sample was aspirated into a 3-mL sterile syringe using a cutter and manual suction, prior to commencing intraocular infusion. Subsequently, the vitreous samples were transferred into sterile DNase and RNase-free tubes and immediately placed on ice. Cen-

trifugation was then performed at 3,000 rpm for 10 minutes at 4°C to eliminate any cellular debris. The resulting supernatant was collected in a new sterile microcentrifuge tube and subjected to a second centrifugation at 15,000 rpm for 10 minutes at 4°C. The supernatant obtained thereafter was divided into aliquots and stored at -80°C for subsequent measurement of various factors, including MK, IL-6, and IL-8. VEGF. EDTA venous blood samples were collected from all patients in the morning before PPV to assess HbA1c. The HbA1c determination in this study was conducted using the turbidimetric inhibition immunoassay (TINIA) method specifically designed for hemolyzed whole blood samples (COBAS C, Roche, CH). A threshold of 6.5% HbA1c is used to classify individuals as either non-diabetic or diabetic.

Determination of Midkine and Interleukin Concentrations

After the vitreous samples were removed from -80°C and thawed at room temperature, the concentration of MK, IL-6, and IL-8 in vitreous samples were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using specific kits (Bioassay Technology Laboratory, Shanghai, China). Samples and standard solutions were prepared following the manufacturer's instructions. The sensitivity of the kits was 2.49 pg/ml, 1.03 ng/L and 2.51 ng/L for MK, IL-6 and IL-8, respectively. For the analysis, 50 µl of each standard solution were added to standard wells, and 40 µl vitreous samples were pipetted to the sample wells. Subsequently, after adding 10 µl antibodies against MK, IL-6 and IL-8 to sample wells, 50 µl of streptavidin-HRP was pipetted into each well. Following incubation at 37°C for 1 h, the plates were washed with wash buffer, then 50 µl of substrate solutions A and B were added to each well. Finally, the plates were incubated at 37°C for 10 minutes, then the incubation was terminated by adding 50 µl of stop solution to each well. Then, the absorbance of the well was read at 450 nm within 5 minutes using a microplate reader (Multiskan GO, Thermo Scientific, Vantaa, Finland). The concentrations of MK, IL-6, and IL-8 were expressed in pg/mL.

Statistical Analysis

SPSS 22 (Statistical Package for Social Sciences version IBM, NY, US) was used for statistical analysis. The normality of the distribution of the data was evaluated by the Shapiro-Wilk test. The Kruskal-Wallis test was used to compare means in three independent groups with non-normal distribution, followed by the Mann-Whitney U test with the Bonferroni correction for post hoc pair-wise comparisons. The quantitative variables were described by the mean, and standard deviation (SD) values. The statistical significance was accepted as p value<0.05.

Results

The study comprised a total of 49 eyes from 49 patients who underwent PPV. Of the 20 patients in the control group, 12 patients (66.6%) had ERM and 8 patients (33.4%) had MH. Of the 15 patients in the NPa-VEGF group, 4 patients (26.7%) had VH, 4 patients (26.7%) had TRD and 7 patients (46.6%) had both VH and TRD. Of the 14 patients in the Pa-VEGF group, 4 patients (28.6%) had VH, 2 patients (14.3%) had TRD, and 8 (57.1%) had both VH and TRD. There were no other comorbidities in either the study or control groups. The main clinical characteristics of all patients included in the study are shown in Table 1. Mean HbA1c lev-

Table 1. Demographic characteristics of the patients included in the study

Parameter	NPa-VEGF	Pa-VEGF	Control	p
Age				
Mean±SD	62.4±7.6	59.1±9.5	63.2±10.7	0.453
Range	50–74	41-77	46-83	
Gender, n (%)				
Men	5 (66.7)	8 (57.1)	8 (40.0)	0.869
Women	10 (33.3)	6 (42.9)	12 (60.0)	
Duration of diabetes (years)				
Mean±SD	16.8±5.3	15.4±3.6	-	0.431
Range	10-28	11-23	-	
Type of diabetes, n (%)				
Type 1	2 (13.3)	2 (14.3)	-	0.943
Type 2	13 (86.7)	12 (85.7)	-	
HbA1c(%)	8.4	8.6	4.8	**<0.001**

Control: Macular hole or epiretinal membrane; NPa-VGEF: No preoperative anti-VEGF application; Pa-VEGF: Preoperative anti-VEGF application; SD: standard deviation.

els were significantly higher in both the NPa-VEGF (n=15) and Pa-VEGF (n=14) groups compared to the control samples (n=20) (p<0.001).

Vitreous cytokine and MK mean concentrations and median values are shown in Table 2. The level of IL-6 among the vitreous samples of NPa-VEGF and Pa-VEGF was not significantly changed compared to controls (p>0.05) (Table 2, Fig. 1A). Similar to IL-6, there was no difference in IL-8 concentrations between patient groups in vitreous samples (p>0.05) (Table 2, Fig. 1B). Unlike IL-6 and IL-8, NPa-VEGF and Pa-VEGF groups had significantly higher levels of MK in the vitreous fluid of the patients compared to the control group (Table 2, Fig. 1C).

Discussion

The development of PDR entails both structural and functional alterations in the retina and vitreous. Accumulation of pathological signaling molecules in the vitreous has been reported as a key factor in PDR development.[17] Therefore, studying the biological activity of vitreous material in PDR patients can help identify new molecules for diagnosis, treatment, and prognosis of the disease. As a result, this study sought to identify a novel molecule that could potentially play a role in PDR pathogenesis and serve as a treatment target. This study represents the initial demonstration of a statistically significant association between intravitreal levels of MK and PDR.

Midkine (MK), a heparin-binding growth factor, is involved in embryonic and neural development. Elevated MK levels have been reported in chronic inflammatory conditions such as tumor progression, atherosclerosis, diabetic nephropathy, rheumatoid arthritis, and inflammatory bowel diseases. Suppression of MK has shown healing effects in these conditions.[18] Elevated MK expression has been linked to diabetic nephropathy by activating monocyte chemoattractant protein (MCP)-1, promoting leukocyte recruitment and increasing capillary permeability.[19] Additionally, high levels of MCP-1 were found in the vitreous of PDR patients.[20] Similarly, elevated MK levels have been observed in rheumatoid arthritis[21] and atherosclerosis[11], where endothelial barrier damage triggers MK expression and leukocyte infiltration.

Epithelial-mesenchymal transition (EMT) and epidermal growth factor receptor (EGFR) play critical roles in preretinal membrane development in PDR and proliferative vitreoretinopathy. Although this study did not directly

Table 2. Vitreous IL-6, IL-8 and midkine concentrations of patients underwent vitrectomy

	Control n=20 (1)	NPa-VEGF n=15 (2)	Pa-VEGF n=14 (3)	p	p (1 vs. 2)	p (1 vs. 3)	p (2 vs. 3)
IL-6 (pg/mL)	225.75±31.35	245.54±31.52	223.61±44.49	0.182	0.086	0.699	0.084
IL-8 (pg/mL)	425.75±54.00	402.34±100.77	405.63±75.71	0.620	0.395	0.391	0.896
Midkine (pg/mL)	773.33±105.60	895.72±105.78	855.63±78.68	**0.003***	**0.007****	**0.046****	0.332

* Kruskal-Wallis test; ** Mann-Whitney U test with the Bonferroni correction; IL-6, IL-8 and midkine concentrations were determined by using specific ELISA kits. Control: Macular hole or epiretinal membrane; NPa-VGEF: No preoperative anti-VEGF application; Pa-VEGF: Preoperative anti-VEGF application.

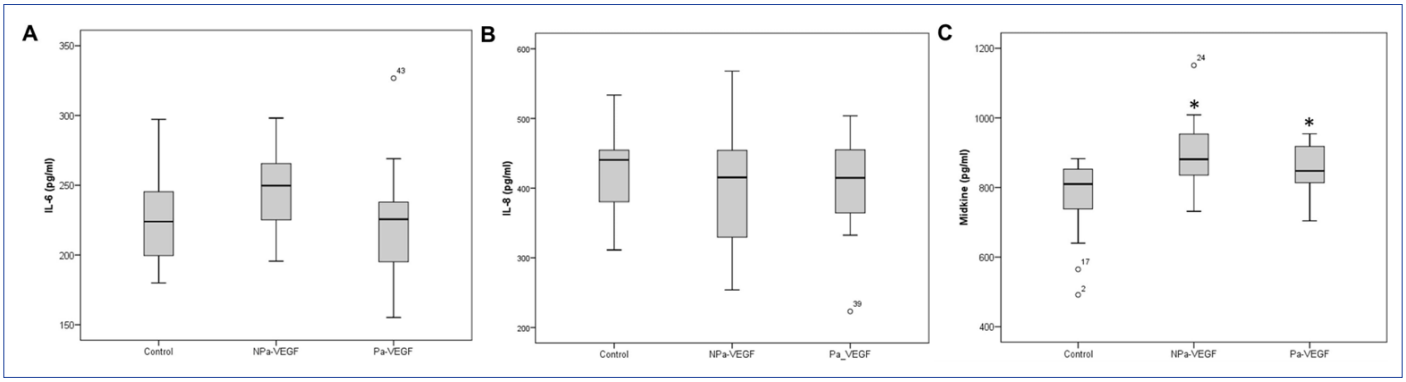

Figure 1. Levels of IL-6, IL-8, and midkine (MK) in vitreous samples from patients without preoperative anti-VEGF application (NPa-VEGF), patients with preoperative anti-VEGF application (Pa-VEGF), and the control group. **(a)** IL-6 levels in vitreous samples from NPa-VEGF, Pa-VEGF, and control groups. No statistically significant differences were observed between the groups (p>0.05). **(b)** IL-8 concentrations in vitreous samples from NPa-VEGF, Pa-VEGF, and control groups. Similar to IL-6, there were no significant differences in IL-8 levels among the groups (p>0.05). **(c)** MK levels in vitreous samples were significantly elevated in both NPa-VEGF and Pa-VEGF groups compared to the control group (*p<0.05).

demonstrate the effect of MK on EMT, previous research has shown its EMT-inducing capabilities in various cells, including pancreatic ductal adenocarcinoma.[22] Kinoshita et al.[23] found that MK promotes smooth muscle cell proliferation and migration through enhanced EGFR signaling. Reynolds et al.[24] reported that hypoxia-induced factor-1 (HIF-1) upregulates MK expression, contributing to pulmonary vascular remodeling under hypoxic conditions. HIF-1 levels are elevated in PDR patients due to reduced retinal perfusion[25], and this study suggests that increased vitreous MK levels in PDR patients may be linked to enhanced EGFR signaling and potential EMT induction. Weckbach et al.[16] further confirmed the angiogenic role of MK in a series of experiments, revealing robust angiogenesis in MK+/+ mice compared to MK-/- mice. Our findings of elevated vitreous MK in PDR patients suggest its association with PDR pathogenesis, potentially through increased VEGF levels. While anti-VEGF-treated patients showed lower vitreous MK levels compared to untreated patients, the difference was not statistically significant. Nonetheless, both treated and untreated PDR patients exhibited significantly higher MK levels compared to controls.

Inflammation and angiogenesis are closely intertwined processes in the pathogenesis of PDR. Studies have demonstrated leukocyte adhesion to capillary endothelial cells and microvascular occlusions in diabetic retinas, accompanied by increased expression of intercellular adhesion molecule-1.[26,27] Hypoxia in the retina initiates the release of several substances, such as VEGF, platelet-derived growth factor, insulin-like growth factor-1, fibroblast growth factor-2, stromal cell-derived factor-1, TNF-α, and interleukins.[28] This cascade contributes to pathological neovascularization within the retina. Inflammatory cytokines like IL-1, IL-6, IL-8, and TNF-α not only trigger leukostasis and angiogenesis but also play a role in neurodegeneration and the activation of microglial cells. Multiple studies have reported elevated levels of IL-6 and IL-8 in the vitreous of patients diagnosed with PDR.[29-32] Contrary to some reports, several studies, in line with our findings, have shown no significant elevation in IL-6 levels in the vitreous fluid of patients with PDR.[33] Additionally, it has been observed that IL-8 levels were notably reduced in PDR patients who received preoperative aflibercept treatment compared to those who did not undergo similar therapy.[34] Boss et al.[30] have provided evidence suggesting that levels of IL-6 and IL-8 in the vitreous were elevated in patients with non-proliferative diabetic retinopathy (NPDR) when compared to individuals diagnosed with PDR. The authors hypothesized that as NPDR advances to PDR, there is a subsequent increase in the levels of the mentioned cytokines in the vitreous. This elevation in IL-6 and IL-8 levels could be attributed to the proteins and cytokines that traverse the impaired blood-retinal barrier in PDR patients, potentially leading to a dilution effect.[33] In our investigation, although we detected elevated MK levels in individuals diagnosed with PDR, we did not detect a noteworthy variance in the levels of IL-6 and IL-8. We believe that the reason for this lack of significance could be related to the small sample size of patients with PDR. Additionally, the observed differences might be partially explained by the possibility that MK levels are more sensitive to changes in PDR than IL-6 and IL-8 levels.

This study demonstrates several notable strengths that significantly contribute to its importance in the realm of DR research. Firstly, it represents a pioneering endeavor in exploring the relationship between MK levels and PDR, shedding light on a relatively underexplored facet of the disease. The comprehensive analysis, encompassing not only MK but also IL-6 and IL-8 levels, offers a nuanced understanding of the inflammatory response underlying PDR. Moreover, the adoption of standardized procedures, including consistent surgical techniques and meticulous data collection methods, ensures the reliability and reproducibility of the findings. The study's robust insights have implications for clinical practice, potentially guiding the development of targeted therapeutic interventions for PDR. Overall, this study's contributions advance our comprehension of PDR pathogenesis and underscore avenues for further research and therapeutic innovation.

Although this study provides valuable insights into the relationship between MK levels and PDR, it is subject to several limitations. The small sample size may limit the generalizability of our findings, and as a single-center study, the lack of population diversity introduces potential bias. Additionally, the exclusion of certain subgroups and the presence of confounding factors could affect the validity of the results.

While we excluded PDR patients who had undergone intravitreal anti-VEGF or PRP within the three months prior to PPV, some participants had a prior history of these treatments, which may have influenced our outcomes. This represents a significant limitation of the study. Furthermore, the inability to measure serum MK levels and the constrained exploration of potential pathways due to budgetary restrictions further limit our findings. Despite these limitations, this study's rigorous methodology, including standardized procedures and ethical considerations, underscores the credibility of its findings.

Conclusion

In conclusion, our study sheds light on the previously unexplored relationship between MK levels in vitreous samples and PDR. The findings underscore the potential significance of MK in the pathogenesis of PDR and its implications for ocular health. Furthermore, being the pioneering study in this area, it fosters a deeper understanding of the role of MK in PDR and potentially guides novel therapeutic approaches. Moving forward, larger-scale studies are warranted to validate these findings and explore new approaches in the prevention or treatment of PDR.

Disclosures

Ethics Committee Approval: The study was approved by the Trakya University Faculty of Medicine Scientific Research Ethics Committee (date: 24.12.2018, no: TUTF-BAEK 2018/411).

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