Assessment of Neurotrophins and Inflammatory Mediators in Vitreous of Patients With Diabetic Retinopathy

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JDB and PKS contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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PURPOSE. To assess vitreous levels of inflammatory cytokines and neurotrophins (NTs) in diabetic retinopathy (DR) and elucidate their potential roles.

METHODS. A prospective study was performed on 50 vitreous samples obtained from patients with DR (n = 22) and the nondiabetic controls (n = 28). All patients were candidates for vitrectomy. Inflammatory cytokine and NT levels were determined with ELISA. Potential source and role of NTs was determined by using human retinal Müller glia and mouse photoreceptor cells and challenging them with TNF-α or IL-1β, followed by detection of NTs and cell death.

RESULTS. Vitreous NT levels of all DR patients were significantly higher than those of nondiabetic controls (nerve growth factor [NGF; P = 0.0001], brain-derived neurotrophic factor [BDNF; P = 0.009], neurotrophin-3 [NT-3; P < 0.0001], neurotrophin-4 [NT-4; P = 0.0001], ciliary neurotrophic factor [CNTF; P = 0.0001], and glial cell–derived neurotrophic factor [GDNF; P = 0.008]). Similarly, the levels of inflammatory mediators IL-1β (P < 0.0001), IL-6 (P = 0.0005), IL-8 (P < 0.0001), and TNF-α (P < 0.0001) were also higher in eyes with DR. Interestingly, inflammatory cytokine and NT levels, particularly NT-3 (P < 0.05), IL-8 (P < 0.004), NT-3 (P = 0.012), NGF (P = 0.04), GDNF (P = 0.005), and CNTF (P = 0.002), were higher in eyes with nonproliferative diabetic retinopathy (NPDR) than in eyes with active proliferative diabetic retinopathy (PDR). Cytokine stimulation of Müller glia resulted in production of NTs, and GDNF treatment reduced photoreceptor cell death in response to inflammation and oxidative stress.

CONCLUSIONS. Together, our study demonstrated that patients with DR have higher levels of both inflammatory cytokines and NTs in their vitreous. Müller glia could be the potential source of NTs under inflammatory conditions to exert neuroprotection.

Keywords: diabetic retinopathy, inflammation, neurotrophins, vitreous humor, human
ciliary neurotrophic factor (CNTF), have not been evaluated in DR. Therefore, the objectives of our study were (1) to analyze and compare NT levels in the vitreous of patients with or without DR, (2) to find correlation between the levels of vitreous NTs and inflammatory mediators in patients with DR, (3) to analyze the effect of panretinal photocoagulation (PRP) ablation of the ischemic peripheral retina in DR on NT levels, and (4) to determine the potential mechanism of NT production and its functional role under inflammatory/oxidative stress conditions.

MATERIALS AND METHODS

Patient Population and Vitreous Sample Collection

This was a prospective study conducted according to the tenets of the Declaration of Helsinki. All patients were candidates for vitrectomy and had signed a preoperative informed consent with their approval to use the excised vitreous fluid for analysis and clinical research. The study design and protocol were approved by the Wayne State University School of Medicine Institutional Review Board. Exclusion criteria included patients younger than 18 years, a history of previous intravitreal anti-vascular endothelial growth factor (VEGF) injections, a history of current or current uveitis, current or recent topical or systemic steroid use, a history of penetrating eye injury, a history of blunt trauma to the eye, or previous intraocular surgery within the past 6 months. Patients were excluded from analysis of intravitreal inflammatory cytokines if they underwent a pars plana lensectomy for retained lens fragments due to complicated cataract surgery.

Undiluted vitreous fluid samples were obtained from 50 eyes from 50 patients (DR, N = 22; Nondiabetic, N = 28) from 2013 to 2015 during pars plana vitrectomy. The indications for vitrectomy were rhegmatogenous and tractional retinal detachment, nonclearing vitreous hemorrhage, epiretinal membrane peel, macular hole repair, or retained lens fragments. (Table 1) The preoperative degree of DR was graded from the current preferred practice patterns outlined by the American Academy of Ophthalmology. Undiluted vitreous samples were collected by manual suction into a syringe through the aspiration line of vitrectomy before opening the infusion line. Samples were transferred to sterile polypropylene screw cap conical bottom vials and were frozen at −80°C freezer for storage and assays.

Vitreous Neurotrophins and Cytokine Analysis

Vitreous levels of NGF (Cat. No. DY 256), BDNF (Cat. No. DY248), NT-3 (Cat. No. DY267), NT-4 (Cat. No. DY268), CNTF (Cat. No. DY257), GDNF (Cat. No. DY212), interleukin 1β (IL-1β, Cat. No. DY201), IL-6 (Cat. No. DY206), IL-8 (Cat. No. DY208), and TNF-α (Cat. No. DY210) were determined by ELISA as per manufacturer’s instruction (R&D Systems, Minneapolis, MN, USA). For ELISA assay, total protein concentration in the vitreous was determined by the Micro BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) and equal amount (20 μg) of protein was used for ELISA assay.

Cell Culture and Treatment

The immortalized human Müller glial cells (MIO-M1 cell line) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin, and L-glutamine (10 μg/mL). Cells were grown in serum-free media before TNF-α challenge. For NT expression, MIO-M1 cells were challenged with recombinant TNF-α (100 ng/mL), IL-1β (100 ng/mL), IL-4 (100 ng/mL), or dexamethasone (100 μM) for indicated time points. The conditioned media was centrifuged at 10,000g for 10 minutes to remove cell debris. The clear supernatant was used for dot blot to measure the expression of various NTs.

For the neuroprotection study, mouse cone photoreceptor cell line 661W, as described in our previous study, was used. The 661W cell line was maintained in DMEM supplemented with 10% FBS, 10 μg/mL L-glutamine, 1% penicillin-streptomycin, 40 μg/L hydrocortisone, 40 μg/L progesterone, 32 mg/L putrescine, and 40 μg/L β-mercaptoethanol. Photoreceptor cell death was induced by challenging 661W cells with H2O2 (100 μM) or TNF-α (100 ng/mL) for 24 hours in the presence and absence of GDNF (100 ng/mL). The cell death and protection by GDNF treatment was assessed by TUNEL staining using an ApopTag fluorescein in situ apoptosis detection kit as described previously.

Dot Blot Analysis

To measure the NT level in MIO-M1, dot blot was performed as described previously. Briefly, 50 μL conditioned media was loaded onto a 0.2-μm nitrocellulose membrane by using a BIODOT apparatus (Bio-Rad, Hercules, CA, USA) and vacuum suction. The membrane was fixed in 10% formaldehyde in Tris buffer saline (TBS) for 1 hour at room temperature (RT). The membrane was blocked in 5% skim milk made in TBST (TBS containing 0.05% Tween 20) for 1 hour at RT and incubated with primary antibody for various NTs (GDNE NGE NT-3, and NT-4) overnight at 4°C. On the following day, the blot was washed three times in TBST and incubated with respective anti-mouse or anti-rabbit horseradish peroxidase (HRP) conjugates (Bio-Rad) for 1 hour at RT. The blot was developed using SuperSignal West Femto maximum Sensitivity Substrate (Thermo Scientific) via chemiluminescence using a Kodak image station, 4000R Pro molecular imaging system (Carestream Health, Inc., Rochester, NY, USA). Dot intensity was quantified using ImageJ analysis software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Values of vitreous concentrations of cytokines and NTs were reported as mean ± standard deviation (SD). All of the analyses were performed by using the Statistical Package for the Social

Table 1. Surgical Indications for Vitrectomy

<table>
<thead>
<tr>
<th>Surgical Indication</th>
<th>No. of Eyes</th>
<th>No. of Eyes</th>
<th>No. of Eyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRD</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>RRD</td>
<td>4</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>ERM</td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>NCVH</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>MH</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>RLF</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

Five eyes with PDR met surgical criteria for having both an NCVH and TRD. ERM, epiretinal membrane; MH, macular hole; NCVH, nonclearing vitreous hemorrhage; RLF, retained lens fragment; RRD, rhegmatogenous retinal detachment; TRD, tractional retinal detachment.
Science software version 16.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad prism V7.02 (GraphPad Software, La Jolla, CA, USA). The unpaired t-test was used for the normally distributed continuous variables, comparing the means from two independent groups, and a P value < 0.05 was considered statistically significant. One-way ANOVA was performed for the dot blot densitometry analysis. The relationship between NTs and cytokines was determined by Spearman rank correlation.

RESULTS

Demographics
A total of 50 vitreous samples from 50 patients (50 eyes) were collected. Among these 22 patients had DR and 28 patients were nondiabetic controls. The mean age of all subjects was 64.7 years (range, 42–90) and 39 patients were male and 11 were female. Among diabetics, the mean age was 67.8 years (range, 46–90), whereas the mean age of nondiabetics was 61.5 years (range, 42–78). Among patients with DR, at the time of vitrectomy 3 had mild NPDR, 4 had moderate NPDR, 3 had severe NPDR, and 12 had PDR (Table 1). All patients were candidates for vitrectomy performed at the Kresge Eye Institute of Wayne State University, Detroit, Michigan, United States.

Vitreous Levels of Neurotrophins and Inflammatory Cytokines
Our analysis showed that NTs, including NGF, BDNF, NT-3, NT-4, CNTF, and GDNF, were present in all vitreous samples of diabetic and nondiabetic eyes, but levels were on average higher in DR eyes than in nondiabetic eyes. There was a significant increase in levels of NGF (P = 0.0001), BDNF (P = 0.0091), NT-3 (P < 0.0001), NT-4 (P = 0.0001), CNTF (P = 0.0001), and GDNF (P = 0.0079) in eyes with DR compared to nondiabetic eyes (Fig. 1A; Table 2). Among eyes with DR, eyes with NPDR compared to eyes with active PDR had higher vitreous concentrations of NGF (P = 0.04), BDNF (P = 0.15), NT-3 (P = 0.01), NT-4 (P = 0.48), CNTF (P = 0.001), and GDNF (P = 0.004) (Fig. 1B). NTs in eyes with NPDR compared to nondiabetic eyes had higher average vitreous concentrations of NGF (P < 0.0001), BDNF (P < 0.0001), NT-3 (P < 0.0001), NT-4 (P < 0.0001), CNTF (P < 0.0001), and GDNF (P < 0.0001). The Spearman correlation analysis revealed a strong correlation (0.513; P < 0.0001) between NT and cytokine levels in patients with DR. The subgroup analysis also revealed a strong correlation between NTs and cytokines in NPDR (0.366; P = 0.02) and PDR (0.535; P = 0.001).

The assessment of inflammatory cytokines revealed overall higher levels in eyes with DR than in nondiabetic controls (Fig. 2). There was a significant increase in levels of IL-1β (P < 0.0001), IL-6 (P = 0.0005), IL-8 (P < 0.0001), and TNF-α (P < 0.0001) in eyes with DR compared to nondiabetic eyes (Fig. 2A). The comparative analysis of cytokine levels in eyes with
NPDR (N = 12, excluding eyes with PDR) and in nondiabetic control eyes (N = 21, excluding retained lens fragment eyes) also revealed a significant increase in levels of IL-1β (P < 0.0001), IL-6 (P = 0.0011), IL-8 (P < 0.0001), and TNF-α (P < 0.001). Interestingly, levels of some cytokines were higher in diabetic eyes with NPDR than with active PDR, with an increase in levels of IL-8 (P = 0.004) and TNF-α (P < 0.05), while IL-1β (P = 0.08) and IL-6 (P = 0.41) concentrations were comparable (Fig. 2B).

In a subgroup analysis of eyes with severe NPDR and PDR, nine (60%) vitreous samples were collected from eyes with a history of PRP, and six (40%) control samples were collected from eyes without a history of PRP (No PRP). The average patient age was 57.9 years (range, 44–76) in the PRP group and 65.3 years (range, 42–71) in No PRP. The comparative analysis of PRP versus No PRP revealed no significant difference in the vitreous levels of either NTs (Fig. 3A; Table 3) or inflammatory cytokines (Fig. 3B).

**Table 2. Quantitative Analysis and Comparison of Intravitreal Levels of Various Neurotrophins in Eyes With Diabetic Retinopathy (N = 22) and Nondiabetic Eyes (N = 28)**

<table>
<thead>
<tr>
<th>Neurotrophin</th>
<th>Diabetic, Mean ± SD, pg/mL</th>
<th>Nondiabetic, Mean ± SD, pg/mL</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>90.6 ± 35.1</td>
<td>51.0 ± 31.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>BDNF</td>
<td>80.2 ± 48.3</td>
<td>45.5 ± 41.8</td>
<td>0.009</td>
</tr>
<tr>
<td>NT-3</td>
<td>122.9 ± 57.0</td>
<td>53.9 ± 49.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NT-4</td>
<td>71.4 ± 12.3</td>
<td>52.7 ± 13.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>CNTF</td>
<td>270.4 ± 110.0</td>
<td>161.8 ± 71.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>GDNF</td>
<td>129.0 ± 50.8</td>
<td>89.5 ± 49.4</td>
<td>0.008</td>
</tr>
<tr>
<td>IL-1β</td>
<td>12.9 ± 5.8</td>
<td>4.0 ± 1.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>212.5 ± 179.6</td>
<td>43.3 ± 84.7</td>
<td>0.0005</td>
</tr>
<tr>
<td>IL-8</td>
<td>53.6 ± 28.1</td>
<td>18.2 ± 9.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>155.8 ± 82.0</td>
<td>63.9 ± 23.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

SD, standard deviation; pg/mL, picograms/milliliter; NGF, nerve growth factor; BDNF, brain derived neurotrophic factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; CNTF, ciliary neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; IL-8, Interleukin 8; TNF-α, tumor necrosis factor alpha.

**Müller Gla Secrete Neurotrophins in Response to Inflammatory Stimuli**

We previously reported that in response to infectious or inflammatory stimuli, retinal glial cells (Müller glia and microglia) get activated and produce more inflammatory mediators along with protective molecules.15,18,19 We hypothesized that diabetes-induced increase in inflammatory mediators triggers the production of NTs by Müller glia, the major glial cell types in the retina.20 To test this, we challenged human retinal Müller glia (MIO-M1 cell line) with recombinant TNF-α or IL-1β for 8 and 24 hours. Our data showed that both TNF-α and IL-1β–challenged Müller glia secrete NTs in conditioned media (Figs. 4A, 4B). To determine whether TNF-α or IL-1β-stimulated NT production is specific to inflammatory stimuli, MIO-M1 cells were challenged with an

**Figure 2. Box-and-whisker plot analysis showing comparison of vitreous IL-6, IL-1β, IL-8, and TNF-α in diabetics (n = 22) compared to nondiabetics (n = 28) (A) and diabetic patients with NPDR compared to PDR (B).**
anti-inflammatory cytokine, IL4 (Fig. 4C), and an anti-inflammatory drug, dexamethasone (Fig. 4D). To this end our results indicate that Müller glia were unable to secrete NTs in the conditioned media in response to these anti-inflammatory agents.

These findings indicate that Müller glia could be one of the potential sources of increased NT levels in the vitreous of patients with DR under chronic inflammatory conditions. Since NTs are known to exert neuroprotection, we then investigated whether NTs can diminish inflammation or oxidative stress–induced neuronal cell death, using mouse cone photoreceptor cell line 661W. Our data showed that GDNF treatment attenuated cell death (TUNEL stain) of 661W cells in response to inflammatory (TNF-α) and oxidative stress (H₂O₂) conditions, as in the case of DR (Fig. 5).

**DISCUSSION**

DR is a multifactorial disease affecting the retina with an extremely complex pathobiology involving a variety of cell types (both resident and infiltrated), molecules, and factors. From the clinical presentation, DR is broadly classified as NPDR or PDR.²¹ NPDR represents an early stage of the disease in which symptoms are either mild or nonexistent. This progresses to PDR, a more advanced form of the disease where neovascularization is evident in the retina. Because of the severity of PDR, significant research effort has been devoted in determining the pathogenesis of transition from NPDR to PDR and to identify potential biomarkers to aid in early diagnosis, including ETDRS.²²,²³ Clearly, a plethora of studies have implicated inflammation in the pathogenesis of DR, with progression to PDR being strongly correlated with increased inflammatory mediators.²⁴ While inflammation is a protective host response to infection or tissue injury, it must be resolved quickly to prevent collateral tissue damage, especially in the retina.²⁵ How inflammation is initiated and persists in DR or whether it is a cause or effect still remains the key questions in the field. Similarly, how increased inflammatory milieu in DR influences other growth factors, including NTs, is not clearly understood. We postulate that increased inflammatory mediators in DR could trigger the production of protective agents to counterregulate the harmful effects of chronic inflammation and induce neuroprotection. In the current study, we showed that patients...
with DR have high levels of both inflammatory mediators and NTs in their vitreous. Moreover, a comparative analysis revealed higher vitreous inflammatory mediators in NPDR than in PDR. Furthermore, in a proof-of-principle in vitro study, we demonstrated the mechanistic basis of NT production and its potential role in protecting retinal neurons under inflammatory and oxidative stress conditions.

Previous studies analyzing intravitreal inflammatory cytokines in DR have shown a significant increase in levels of IL-6, IL-8, IL-1β, and TNF-α.26–28 These studies, however, have mainly focused on PDR. In the present study, the inflammatory cytokines IL-6, IL-8, IL-1β, and TNF-α were shown to be significantly elevated in vitreous samples not only of PDR, but also of patients with mild to severe NPDR. Interestingly, levels of cytokines were higher in diabetic eyes with NPDR than with active PDR. Because most (8 of 15) of our PDR patients had vitreous hemorrhage and because of the known compromised blood–retinal barrier in PDR, there is a possibility that serum/plasma proteins might have leaked into the vitreous and diluted the relative amount of NTs and cytokines. However, we did not find a significant difference in average concentration of total vitreous protein in PDR (4.72 ± 3.1 μg/μL) versus NPDR (4.86 ± 2.4 μg/μL) patients. Our findings also corroborated with those of Loukovaara et al.,29 showing no major differences in vitreous protein levels in a large cohort of PDR and NPDR patients. Hence, we concluded that differential levels of inflammatory mediators or NTs, in our study, are unlikely due to altered concentration of total vitreous proteins in PDR. Overall, these results highlight the increased activity of these inflammatory cytokines in the early stages of DR. One of the potential implications of these findings is to therapeutically target inflammatory milieu in NPDR patients to prevent progression to PDR. Indeed, a recent study by Wykoff and colleagues30 supports this notion whereby intravitreal corticosteroid injections in NPDR eyes have been shown to decrease the rate of progression from NPDR to PDR in patients in FAME A and B trials receiving intravitreal fluorozone. Additionally, a DRCR.net study called Protocol W is being started in order to compare the reduction in progression from NPDR to PDR when anti-VEGF and PRP laser are applied to severe NPDR patients. Moreover, new Food and Drug Administration (FDA) indications for intravitreal ranibizumab have changed to now incorporate any degree of DR. Hence, an early indication of increased inflammatory mediators in eyes of diabetic patients, as reported in our study, may allow initiating therapeutic strategies to prevent or halt the progression to PDR.

Another unique aspect of our study is the simultaneous assessment of inflammatory mediators and NTs in vitreous of patients with DR. Neurotrophins, released from glial cells such as Müller cells, are a group of functionally and structurally related growth factors that play a critical role in the development, survival, maintenance, and repair of the nervous system, as well as play essential roles in angiogenesis and fibrosis.31 Several studies have shown that inflammation alters the expression of neurotrophins. In the brain, administration of proinflammatory cytokines or lipopolysaccharides (LPS) causes a significant reduction in BDNF expression.32 Similarly, BDNF levels are reported to be reduced in DR,33 whereas GDNF levels are increased.14,33 This study provided evidence that NTs are present in the vitreous in both diabetics and nondiabetic patients. Moreover, this human study showed vitreous levels of NTs, including NGF, BDNF, NT-3, NT-4, CNTF, and GDNF, to be statistically significantly elevated in patients with DR when compared to patients without a history of diabetes mellitus. In previous animal studies of NT levels, both NGF and BDNF are undetected in both diabetic and control samples15; however,
FIGURE 5. GDNF protects oxidative stress and inflammation-induced photoreceptor cell death. Mouse cone photoreceptor cells (661W cell line) were challenged with H$_2$O$_2$ (100 µM) and TNF-α (100 ng/mL) in the presence and absence of GDNF (100 ng/mL) for 24 hours. The neuroprotective effect of GDNF was measured by measuring the cell death by TUNEL staining (blue, 4',6-diamidino-2-phenylindole [DAPI] nuclear stain; green, TUNEL+ve cells).
Neurotrophin Levels in DR

Figure 6. Schematic of the relationship between inflammatory mediators and NTs in diabetic retinopathy. Patients with diabetic retinopathy have increased levels of inflammatory mediators in vitreous. This increased inflammatory milieu triggers the production of NTs in retinal cells, such as Müller glia, which in turn exert neuroprotective and anti-inflammatory effects to prevent neuronal cell death in the retina.

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