Increased Levels of Dickkopf 3 in the Aqueous Humor of Patients With Diabetic Macular Edema

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Purpose. To investigate associations between diabetic macular edema (DME) and levels of the Wnt modulator Dickkopf 3 (DKK-3) in the aqueous humor (AH) of patients with DME and to analyze the clinical implications of this association.

Methods. Forty-four eyes of 39 patients with DME and 27 eyes of 27 controls were studied. Aqueous humor DKK-3 levels were measured by ELISA before the first intravitreal injection of bevacizumab (IVB). Visual acuity assessments and spectral-domain optical coherence tomography (SD-OCT) were performed before and 3 months after the first IVB. DKK-3 expression in high glucose–treated human Müller cells was examined by Western blot and immunofluorescence. Concentration of secreted DKK-3 in conditioned medium from human Müller cell cultures were analyzed by ELISA.

Results. ELISA showed increased DKK-3 levels in the eyes of DME patients compared with control subjects (median 207.86 ng/mL, range, 66.75–499.64 vs. 94.94 ng/mL, 33.34–164.45 ng/mL; $P < 0.001$). Based on multivariate analyses, elevated DKK-3 levels were associated with increased inner nuclear layer (INL) volume on SD-OCT before IVB. Western blot and immunofluorescence analyses showed higher DKK-3 expression in high glucose–treated Müller cells than in control cells, with DKK-3 secretion also being increased.

Conclusions. DKK-3 expression was elevated in the AH of DME patients and in high glucose–treated human Müller cells. The observation of increased DKK-3 expression levels in the AH of DME patients with prominent edema in the INL suggests that the area of INL thickening might correlate with the area of reactive responses by Müller cells in these patients.

Keywords: diabetic macular edema, Müller cell, aqueous humor

Diabetic macular edema (DME), which is caused by the breakdown of the inner blood–retinal barrier, is a leading cause of severe visual impairment in patients with diabetic retinopathy (DR).[1,2] However, the causative pathogenic mechanisms of DR are multifactorial and incompletely determined. The underlying pathologies of DR reported in previous studies include aldose reductase gene polymorphisms, protein kinase C activation, VEGF upregulation, pigment epithelium-derived factor (PEDF) downregulation, and enhanced insulin-like growth factor 1 and para-inflammatory responses.[3–8] Although anti-VEGF agents and steroids have become the predominant therapies for DME in recent years, due to the decreased leakage of fluid from blood vessels in the macula,[9,10] the reported responses to these treatments in terms of both anatomic and functional improvements are not satisfactory.[11,12] Moreover, because anti-VEGF agents appear to be less effective in treating DME than in treating neovascular age-related macular degeneration (AMD) due to the increased complexity and multifactorial nature of DME, new treatment strategies for DME targeting multiple or novel signaling pathways are being investigated.[13–15]

Among these pathways, the Wnt signaling pathway is associated with multiple physiological and pathological processes, including cell survival, development, differentiation, inflammation, and angiogenesis.[16–19] In the retina, Wnt signaling activation has been reported to protect photoreceptors in vitro and in vivo, including in cultures of primary retinal cells subjected to oxidative stress and in degenerating retinas from rd1 animals.[20]

Using donor eyes from diabetic patients and three diabetic animal models, Chen and associates[21] demonstrated that overactivation of Wnt/β-catenin signaling plays a pathogenic role in DR, and their finding suggests that targeting Wnt signaling could be a promising therapeutic strategy for DR. Qiu and associates[22] reported that the levels of the Wnt antagonist Dickkopf (DKK)-1 were decreased in the vitreous of patients with DR; their result indicates that this decrease in DKK-1 levels might be associated with the development and progression of DR. Consistent with this finding, intravitreal injection of DKK-1 effectively ameliorated retinal inflammation and vascular leakage in DR animal models.[21]

Müller glia, the predominant glial cell type in the retina, structurally and functionally support photoreceptors and neuronal cells.[23,24] These cells perform a secretory function, releasing glutamate and various other factors to maintain homeostasis in the retina.[24] Under pathological conditions, Müller cells protect photoreceptors and retinal neurons from cell death by releasing antioxidants, neurotrophic factors, and growth factors. Müller cells are also a major source of VEGF; therefore, the pathological changes observed in DR could be mediated by Müller cells.[25] In contrast, in diabetic retinas, Müller cells become reactive in response to disruptions of the blood–retinal barrier and to neuronal dysfunction, even at an early stage, and these cells secrete various factors, such as glial

cell–derived neurotrophic factor, thrombospondin-1, and PEDF, which enhance the barrier function of endothelial cells and provide protection to the neuronal cells in the retina.[26–29] However, whether the reactivity of Müller cells is a consequence of or a contributor to these pathological changes in the diabetic retina remains uncertain.

Wnt signaling in Müller cells is activated in response to photoreceptor injury.[20] DKK-3, a Wnt modulator in the DKK family, is expressed by and secreted from Müller cells, and it potentiates Wnt signaling in cultured Müller glia and protects retinal cells from apoptotic stimuli by inhibiting caspase activation.[30,31] DKK-3 expression was shown to be increased in rd1 animals compared with control animals and was found to be expressed in the inner nuclear layer (INL) and the ganglion cell layer (GCL) of the retina.[32] Although DKK-3 has been described as a positive or negative regulator of Wnt in a cell- and tissue-specific manner,[31] its biological function in relation to DR or DME has not been studied to date.

Considering the known role of Wnt signaling in DR and the lack of research regarding its association with DME in DR patients, we examined the changes in the abundance of secreted DKK-3. DKK-3 has been hypothesized to be secreted primarily by Müller cells in diabetic retinas during the course of DME. Previously, we demonstrated that DKK-3 is present in the aqueous humor (AH) of AMD patients.[33] In the present study, we examined DKK-3 levels in the AH from DME patients who had received no previous treatment compared with control subjects, and we determined the clinical implications of these findings to DME. In addition, DKK-3 expression was assessed in Müller cell cultures.

Methods

Subjects and AH Sample Collection

Aqueous humor samples were collected at the Department of Ophthalmology, Konkuk University Medical Center, Seoul, Korea. From July 1, 2013, to January 31, 2015, 44 eyes of 39 patients with DME and 27 eyes of 27 controls were enrolled in this study. All 39 patients had been untreated (i.e., they had not received any treatment for DR except retinal laser photocoagulation at least 3 months before their inclusion in the study) and had experienced recent onset of visual symptoms (<3 months). None of these patients had uncontrolled systemic diseases, such as ischemic heart diseases, chronic kidney diseases, or systemic inflammatory diseases. Eyes with the following characteristics were excluded: DME with epiretinal membrane or foveal traction, severe foveal hard exudate infiltration, vitreous hemorrhage or pre- and sub-retinal hemorrhage, AMD, or a history of uveitis. Aqueous humor samples from patients undergoing cataract surgery were used as control samples. Except for cataracts, the control subjects did not have diabetes or uncontrolled systemic diseases or any ophthalmic diseases. Aqueous humor samples from 44 eyes with DME were obtained immediately before performing the first intravitreal injection of bevacizumab (IVB, 1.25 mg/0.05 mL), and AH samples from 27 control subjects were obtained immediately before cataract surgery. These samples were used for ELISA of the DKK-3 concentration.

All sample collections and intravitreal injections were performed using standard sterile methods. Aqueous humor samples were obtained via anterior chamber paracentesis through a 30-G needle, and no complications occurred. Aqueous humor samples (~50–100 μL) were extracted into Safe-Lock microcentrifuge tubes (1.5 mL), immediately frozen at −80°C and then stored until ELISA. This study followed the guidelines of the Declaration of Helsinki, and informed written consent was obtained from all patients and control subjects. The procedure for AH collection was approved by the institutional review boards of Konkuk University Medical Center, Seoul, Korea.

Patient Data

The baseline (pre-IVB) data from the 39 patients with DME whose AH samples were used for ELISA included demographic and systemic characteristics (age, sex, diabetes duration, presence of controlled systemic hypertension, body-mass index, and smoking status) and ophthalmologic examination findings, which included best corrected visual acuity (VA, logMAR), lens status (phakia or pseudophakia), fluorescein angiography (FA) results, and spectral-domain optical coherence tomography (SD-OCT) results. A follow-up examination (VA and SD-OCT) of 28 patients was performed 3 months after one to three consecutive IVBs (post-IVB). The average number of IVBs was 2.0 (±0.92). The follow-up period ranged from 87 to 250 days. Based on the international clinical DR severity scale,[34] DR eyes were subdivided into three categories: no DR, nonproliferative DR (NPDR), or proliferative DR (PDR). Diabetic macular edema patients without new vessels in the retina were classified into the NPDR group. Diabetic macular edema patients with previous panretinal photocoagulation (at least 3 months before the inclusion date) but no active new vessels in the retina, vitreous hemorrhage or tractional retinal detachment were classified into the PDR group. Eyes with DME were subdivided into two categories according to previous descriptions.[35] Briefly, in focal edema, the retinal fluid is located in the outer retina (outer plexiform layer [OPL] to external limiting membrane [ELM]), as determined by SD-OCT. In diffuse (or combined) edema, the retinal fluid is located in the inner retina (internal limiting membrane [ILM] to inner nuclear layer [INL]), with or without retinal fluid in the outer retina, as determined by SD-OCT.

A 9 × 6 mm area of the macular region centered on the fovea was examined by SD-OCT (Spectralis HRA+OCT; Heidelberg Engineering, Heidelberg, Germany). In total, 25 volume scans (each with a length of 240 μm, centered on the fovea) were performed, with an average of nine replicates of the B-scan image of each section. A built-in real-time eye-tracking system was used to ensure the correct scanning position. For high-quality images, only images with a quality score greater than 16 dB were analyzed. The SD-OCT image from the horizontal foveal scans was reviewed in a masked fashion (blinded to the VA and ELISA results of the patients) by a retina specialist (HCK) for measurements of foveal microstructures. The integrities of the foveal photoreceptor layer, including the ELM, and the junction between the photoreceptor inner segment and outer segment (IS/OS) were measured using SD-OCT images within a 6000-μm diameter centered on the fovea. The disrupted lengths of the ELM and the IS/OS pre- and post-IVB were measured manually using a caliper built into the SD-OCT software. Disruption of the ELM or the IS/OS was defined as the loss of a continuous hyperreflective line. If this dim line was detectable, then the microstructure was considered intact. If the ELM was absent or disrupted, then the next visible reflective line was considered the outer border of the outer nuclear layer (ONL). The average retinal thickness within a circle with a 1-mm diameter centered on the fovea (the center circle of the Early Treatment Diabetic Retinopathy Study grid) was calculated automatically as the central macular thickness (CMT) (software version 6.0.9.0; Heidelberg Engineering).

The volumes of the total retinal layer (from the ILM to the basement membrane), the INL, and the OPL plus ONL (OPL/

ONL) were measured semiautomatically using SD-OCT segmentation software with manual correction. First, we used built-in automatic layer segmentation software (version 6.0.9.0) to obtain the retinal boundaries on each B-scan. Next, all 25 segmented B-scans were adjusted manually by a trained ophthalmologist (BJ). A retina specialist (HC) reviewed the retinal segmented boundaries for possible errors in marking. Finally, the volumes (mm^3) of the total retinal layer, the INL and the OPL/ONL in macular cube scans were calculated automatically within a 6-mm diameter circle centered on the fovea. Ten of 44 eyes were excluded from the volume analysis due to failure to record consistent and reproducible retinal boundaries as a result of segmentation produced by severe macular edema.

MIO-M1 Cell Culture and Treatment

Human Müller cells (MIO-M1 cells) were purchased from E-lucid (University College London, London, UK), cultured in Dulbecco's modified Eagle's medium (DMEM) containing Glutamax (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco), and maintained in an incubator at 37°C and 5% CO_2 to facilitate cell proliferation.

Cells were plated in 6-well culture plates (5×10^5 cells/well) for Western blot analysis and ELISA or in 24-well culture plates (8×10^4 cells/well) containing coverslips for immunofluorescence. Once the cells reached approximately 80% confluence, the cells were treated with 5.5 mM glucose as a control or with high glucose (25 mM or 50 mM) at 37°C for 24 hours. Mannitol (Sigma-Aldrich Corp., St. Louis, MO, USA)-containing culture medium was used as an osmotic control. Cell viability was measured using the trypan blue (Gibco) exclusion assay according to the manufacturer's protocol.

DKK-3 Measurement via ELISA

The levels of DKK-3 in the AH and in conditioned media from MIO-M1 cells were quantitatively assessed using a sandwich ELISA kit (Aviscera Bioscience, Inc., Santa Clara, CA, USA). All procedures were performed according to the manufacturer's protocol. The dilution factors of AH and conditioned media from MIO-M1 cells were 500- and 1000-fold, respectively. Color intensities were determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Duplicate samples were used in all assays. The standard concentration range was 31.3 to 1000 pg/mL and the detection limit was 15.6 pg/mL. Inter- and intra-assay variations were 4.1% and 6%, respectively.

Western Blot Analysis

MIO-M1 cells were lysed in RIPA buffer (Thermo Scientific, Waltham, MA, USA) containing protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitors (Thermo Scientific). Supernatants were obtained via centrifugation at 13,400*g* for 15 minutes and used for Western blot analysis.

Protein concentrations were quantified using the BCA protein assay. Samples containing equivalent quantities of proteins (25 μg) were separated via 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). These membranes were blocked at room temperature with 3% bovine serum albumin (BSA) in TBST (1× TBS containing 0.1% Tween 20) for 1 hour and then incubated in primary antibodies (anti–DKK-3, 1:1000; Thermo Scientific and anti-active β-catenin, 1:1000; Millipore) overnight at 4°C. Subsequently, the membranes were washed three times in TBST and incubated in corresponding horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technologies, Beverly, MA, USA) for 1 hour. After the membranes were washed another three times in TBST, immunoreactive protein bands were visualized using a chemiluminescent substrate (ECL Prime; Amersham Biosciences, Piscataway, NJ, USA). An LAS-4000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan) equipped with LAS-4000 Image Reader software was used as a digital imaging system. Levels of protein expression were quantified via densitometry using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

Immunofluorescence was performed on MIO-M1 cells. Cultured MIO-M1 cells were fixed in 4% paraformaldehyde/PBS for 10 minutes. The cells were incubated in blocking solution (1% BSA in PBS containing 0.1% Triton X-100) for 15 minutes before incubation in the primary antibody (anti-DKK-3, 1:50; Thermo Scientific) at 4°C for overnight. The samples were then incubated in the Alexa Fluor 488 goat anti-mouse secondary antibody (1:1000; Life Technologies, Carlsbad, CA, USA) for 1 hour. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:2000; Thermo Scientific) for 10 minutes. Samples treated only with secondary antibody were used as negative controls. Fluorescence images were obtained using a confocal microscope (LSM 710; Carl Zeiss, Jena, Germany).

Statistical Analysis

Statistical analysis was performed using SPSS software version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). All values are presented as the means ± SD, means ± SE, medians (range), or numbers (%). Depending on the distribution of the data, parametric or nonparametric statistical methods were used. Wilcoxon rank sum tests were used to compare distinct variables between groups. The χ^2 test or Fisher's exact test was applied to compare variables. The Wilcoxon signed rank test was performed to examine differences between pre- and post-IVB values. Multivariate analysis was performed using backward elimination of specific variables. Pre-IVB VA (logMAR) and age were adopted for adjustment in multivariate analysis. Spearman correlation coefficients were calculated to examine bivariate relationships. Student's *t*-tests were performed on the results of Western blot and trypan blue exclusion assays. Paired *t*-tests were performed on the ELISA results. *P* values less than 0.05 were considered statistically significant.

Results

Study Group and DKK-3 Measurements via ELISA

Aqueous humor samples were collected from 44 eyes of 39 patients with DME for ELISA. The age of the patients ranged from 30 to 76 years (median: 51 years), and the age of the control subjects ranged from 46 to 69 years (median: 60 years). The duration of DM ranged from 1 to 30 years (8.1 ± 7.2 years). ELISA demonstrated that the level of DKK-3 was higher in the 44 eyes with DME than in the control eyes (median 207.86 ng/mL, range, 66.75–499.64 ng/mL versus median 94.94 ng/mL, range, 33.34–164.45 ng/mL; $P < 0.001$, Wilcoxon rank sum test). The baseline characteristics of the 39 patients with DME and the 27 control subjects are listed in Table 1.

The patients were divided into subgroups according to edema type, clinical DR severity, and the presence or absence of hypertension. The type of DME was classified based on SD-

Table 1. Baseline Characteristics of DME Patients and Control Subjects

	DME Patients* $n = 44$	Control Subjects* $n = 27$	*P* Value
Age, y	51 (30–76)	60 (46–69)	0.003†
Male, *n* (%)	28 (63.6)	19 (70.4)	0.560‡
Hypertension, *n* (%)	11 (25.0)	12 (44.4)	0.089‡
Current smoker, *n* (%)	8 (18.2)	3 (11.1)	0.515‡
Lens status, phakia, *n* (%)	40 (90.9)	27 (100)	0.290‡
BMI, kg/m^2	23.75 (19.84–27.04)	24.26 (19.63–35.14)	0.375*
HbA1c (%)	8.785 ± 2.365		–
DM duration, y	8.05 ± 7.211		–
DKK-3, ng/mL	207.86 (66.75–499.64)	94.94 (33.34–164.45)	<0.001†

BMI, body-mass index.

* Data are shown as numbers (%) (male, hypertension, smoking status, and lens status), means ± SD (HbA1c, DM duration), or medians (ranges) (age, BMI, DKK-3).

† Wilcoxon rank sum test.

‡ χ^2 test.

OCT findings in the baseline examination as described in the Methods section. Twenty-five eyes exhibited diffuse (or combined) edema, and 19 eyes exhibited focal edema. As shown in Table 2, the level of DKK-3 in the diffuse (or combined) edema group was higher than that in the focal edema group (205.16 ng/mL, 129.82–499.64 vs. 121.56 ng/mL, 66.75–313.04 ng/mL; $P < 0.001$, Wilcoxon rank sum test). There was a statistically significant difference of the level of DKK-3 according to DR severity (254.24 ng/mL, 97.36–499.64 ng/mL for PDR versus 141.65 ng/mL, 66.75–480.92 ng/mL for NPDR). Among the 18 eyes with PDR, 16 exhibited diffuse edema (DKK-3 level: 259.10 ng/mL, 155.61-499.64 ng/mL) and 2 exhibited focal edema (97.36 and 217.20 ng/mL). Among the 26 eyes with NPDR, the levels of DKK-3 in the 9 eyes with diffuse edema were higher than those in the 17 eyes with focal edema (218.33 ng/mL, 129.82–480.92 ng/mL vs. 121.56 ng/mL, 66.75–313.04 ng/mL; $P = 0.003$, Wilcoxon rank sum test). The levels of HbA1c in the DME patients were not significantly different between those with diffuse edema and those with focal edema (Wilcoxon rank sum test, $P = 0.734$). The presence of hypertension was also not associated with the level of DKK-3.

Clinical data pre- and post-IVB were obtained for 28 eyes (Table 3). The mean CMT of these 28 eyes was decreased after IVB (pre-IVB 412.5 μm, 263–861 μm versus post-IVB 333.5 μm, 244–906 μm; $P = 0.009$, Wilcoxon signed rank test), and the lengths of disrupted IS/OS and ELM was not significantly altered post-IVB.

Association of DKK-3 Levels With SD-OCT Findings and Clinical Parameters

The associations of clinical variables, including age, DM duration, VA, and SD-OCT parameters, with increased DKK-3 levels in the AH of the eyes with DME were analyzed. Univariate analysis demonstrated that elevated DKK-3 levels were associated with CMT (pre-IVB), changes in CMT (between pre- and post-IVB), and the volumes of the total retina layer and the INL in a 6-mm diameter circle centered on the fovea (designated as retina volume and INL volume, respectively). No associations were found between DKK-3 levels and other variables, such as age, DM duration, VA, or disruption of the ELM or the IS/OS (pre- or post-IVB) based on univariate analysis (Table 4). The explanatory variables selected for inclusion in the multivariate analysis were age, CMT (pre-IVB), changes in CMT, retina volume, and INL volume ($P < 0.1$). Multivariate analysis I showed that elevated DKK-3 levels were associated with INL vol. Because retina volume, INL volume, and OPL/ONL volume were considered to show multicolinearity, in multivariate analysis II, INL volume, and OPL/ONL volume were selected as explanatory variables. This analysis also demonstrated that elevated DKK-3 levels were associated with increased INL volume. This association was not compromised after adjusting for VA pre-IVB and age in multivariate analyses III and IV. These results indicated that elevated DKK-3 expression was strongly associated with increased INL volume (pre-IVB; Table 5, Figs. 1, 2).

Table 2. Subgroup Analysis of DKK-3 Levels According to the Type of DME, the Degree of DR, and the Presence of Hypertension

	Eyes, *n*	DKK-3, ng/mL*	*P* Value†
Diffuse edema	25	250.16 (129.82–499.64)	<0.001
Focal edema	19	121.56 (66.75–313.04)	
NPDR	26	141.65 (66.75–480.92)	0.002
PDR	18	254.24 (97.36–499.64)	
With hypertension	11	151.05 (66.75–499.64)	0.408
Without hypertension	33	205.18 (76.55–480.92)	

* Data are shown as medians (ranges).

† Wilcoxon rank sum test.

Western Blot and Immunofluorescence Analyses of Human Müller Cells

Because the volume of the INL, which mainly consists of the nuclei of Müller and bipolar cells, was increased in DME patients' eyes, which displayed high levels of DKK-3 in the AH, we examined the expression of DKK-3 in cultured human Müller cells (MIO-M1 cells) that were exposed to high-glucose media. The expression of DKK-3 was significantly increased in

Table 3. Changes in Clinical and SD-OCT Parameters in Patients With DME

	Pre-IVB* $n = 28$	Post-IVB* $n = 28$	*P* Value†
logMar	0.22 (0.00–1.40)	0.35 (0.00–1.70)	0.266
CMT, μm	412.5 (263–861)	333.5 (244–906)	0.009*
Disrupted length of IS/OS, μm	0 (0–2622)	0 (0–3000)	0.087
Disrupted length of ELM, μm	0 (0–2679)	0 (0–2884)	0.075

* Data are shown as medians (ranges).

† Wilcoxon signed rank test.

Table 4. DR Grade, Clinical Data, SD-OCT Parameters and Visual Acuity According to the Level of DKK-3 (Univariate Analysis)

	Univariate Analysis		
Variable	**Estimate**	**SE**	***P* Value**
Age ($n = 44$)	−2.532	1.400	0.078
DM duration ($n = 44$)	2.234	2.192	0.314
logMAR (pre) ($n = 44$)	62.234	56.098	0.274
logMAR (post) ($n = 28$)	18.786	49.609	0.708
HF (pre) ($n = 44$)	2.690	1.688	0.119
CMT (pre) ($n = 44$)	0.237	0.097	0.019*
CMT (post) ($n = 28$)	0.075	0.115	0.523
Change in CMT (pre-post) ($n = 28$)	0.323	0.147	0.038*
Disrupted length of ELM (pre) ($n = 44$)	0.015	0.021	0.466
Disrupted length of ELM (post) ($n = 28$)	0.011	0.027	0.688
Disrupted length of IS/OS (pre) ($n = 44$)	0.008	0.019	0.663
Disrupted length of IS/OS (post) ($n = 28$)	0.002	0.025	0.924
Total retinal layer (vol) ($n = 34$)	33.459	14.477	0.027*
INL (vol) ($n = 34$)	291.867	73.927	0.0004*
OPL/ONL (vol) ($n = 34$)	16.000	25.011	0.527

HF, hyperreflective foci.
* $P < 0.05$.

MIO-M1 cells exposed to a high glucose concentration (25 or 50 mM glucose) compared with a control glucose concentration (5.5 mM glucose) as determined by Western blot and immunofluorescence analysis (Fig. 3). The lack of a significant difference in DKK-3 levels between the low-glucose and osmotic controls was confirmed in advance (Supplementary Fig. S1).

Levels of DKK-3 in Conditioned Medium From MIO-M1 Cells Based on ELISA

As shown in Figure 4, the levels of secreted DKK-3 in conditioned medium from MIO-M1 cells exposed to a high-glucose concentration (50 mM) were higher than those in medium from cells exposed to the control glucose concentration ($P < 0.01$). No significant difference in cell viability or quantity between the control- and 50 mM glucose-treated MIO-M1 cells was observed (Supplementary Fig. S2). Thus, we inferred that the increase in the level of DKK-3 in conditioned media occurred as a result of increased secretion of DKK-3 from the cells.

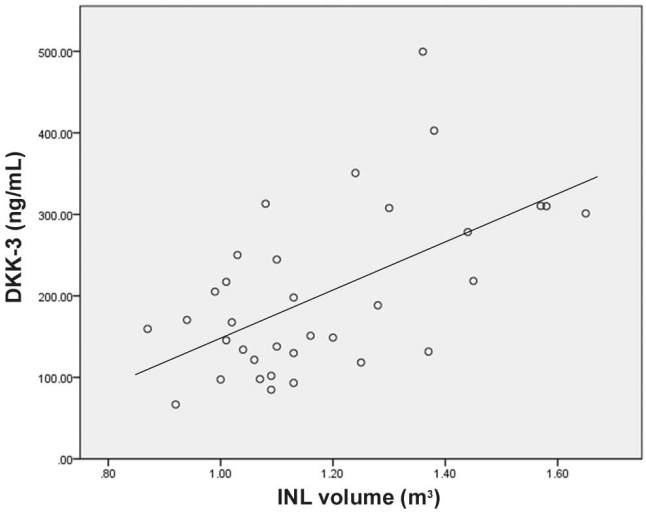

Figure 1. Association between DKK-3 levels and INL volume. Correlation analysis demonstrated that the DKK-3 level is significantly associated with the INL volume (Spearman correlation coefficient = 0.472, $P = 0.005$).

Discussion

In the present study, we demonstrated that the level of DKK-3 was significantly elevated in the AH of eyes from patients with DME compared with those from control subjects. We also showed a strong association between elevated DKK-3 levels and increased edema predominantly in the INL. These results indicate that diffuse leakage resulting from the breakdown of the inner blood–retinal barrier in the INL could induce Müller cells to increase DKK-3 secretion. Moreover, patients with DME whose DKK-3 level was elevated pre-IVB displayed a better response to treatment with anti-VEGF agents, which manifested as a greater decrease in CMT on SD-OCT post-IVB. This finding suggests that this reactive response by Müller cells in the diabetic retina might be a beneficial and protective response, rather than a destructive or damaging response, at least during the early stage of DME. The greater response to anti-VEGF treatment among patients with diffuse or combined type macular edema observed in this study is consistent with previous reports showing that the diffuse type of DME (INL

Table 5. SD-OCT Parameters, Including Segmentation Parameters, According to the Levels of DKK-3 (Multivariate Analysis)

	Multivariate Analysis I		Multivariate Analysis II		Multivariate Analysis III		Multivariate Analysis IV	
Variables	**Estimate**	***P* Value**	**Estimate**	***P* Value**	**Estimate**	***P* Value**	**Estimate**	***P* Value**
Age			–	–	–	–	−1.605	0.310
CMT (pre)			–	–	–	–	–	–
Change in CMT	0.342	0.078						
logMAR (pre)			–	–	−4.458	0.962	–	–
Retina (vol)	–	–	–	–	–	–	–	–
INL (vol)	257.221	0.006*	299.580	0.001*	300.370	0.001*	268.018	0.003*
OPL/ONL (vol)	–	–	−7.624	0.729	−7.698	0.732	−5.459	0.805

Multivariate analysis I: explanatory variables were age, CMT (pre), Change in CMT, retina (vol), INL (vol); multivariate analysis II: explanatory variables were INL (vol) and OPL/ONL (vol); multivariate analysis III: explanatory variables were INL (vol) and OPL/ONL (vol), and logMAR (pre) was adopted for adjustment; multivariate analysis IV: explanatory variables were INL (vol) and OPL/ONL (vol), and age was adopted for adjustment.
* $P < 0.05$.

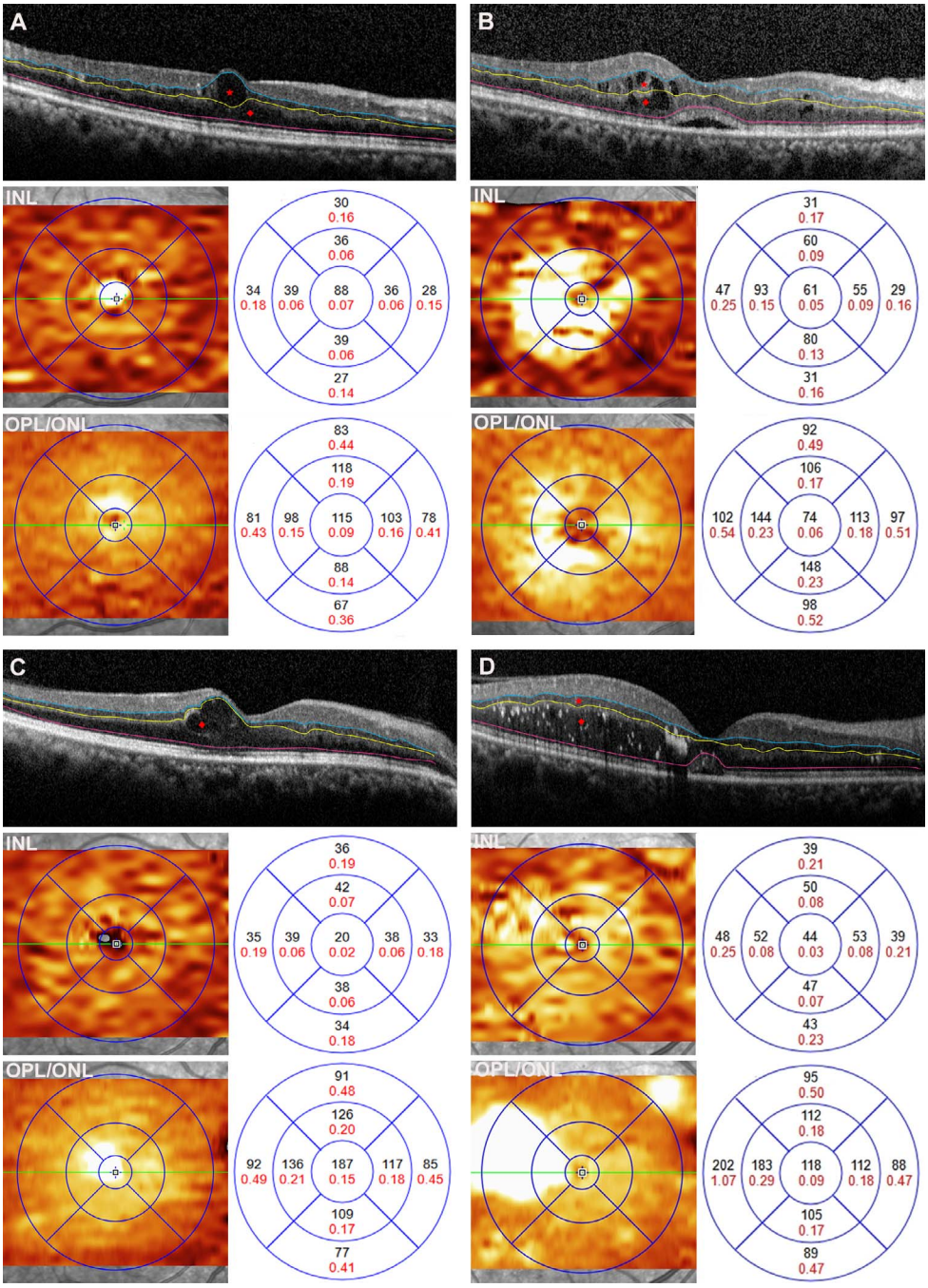

Figure 2. Thickness map of the INL and the OPL/ONL by SD-OCT segmentation. Spectral-domain OCT segmentation (*upper*, *blue ~ yellow line*: INL segmentation, *yellow ~ pink line*: OPL/ONL segmentation, *asterisk*: area of INL edema, *rhombus*: area of OPL/ONL edema), the INL thickness map (*middle*), and the OPL/ONL thickness map (*lower*) before treatment are shown. (**A**) Patient 1: a 59-year-old woman with DME. Her best corrected visual acuity (VA) was logMAR 0.22, and her DKK-3 level was 170.45 ng/mL. Increased INL thickness in the central 1-mm zone (88 μm) was observed. (**B**) Patient 2: a 45-year-old man with DME. His VA was logMAR 0.22, and his DKK-3 level was 350.74 ng/mL. Increased INL thickness in the central 1-mm zone (61 μm) was observed. (**C**) Patient 3: a 59-year-old man with DME. His VA was logMAR 0.15, and his DKK-3 level was 97.36 ng/mL. The INL thickness in the central 1-mm zone of this patient was much lower (20 μm) than that of Patients 1 and 2. (**D**) Patient 4: a 53-year-old man with DME. His VA was logMAR 0.30, and his DKK-3 level was 118.30 ng/mL. The INL thickness in the central 1-mm zone of this patient was much lower (44 μm) than that of Patients 1 and 2.

thickening) responded better to anti-VEGF agents than the focal type of DME due to leakage via microaneurysms.[35,36]

Müller cell activation and hypertrophy have been reported during the early stages of DR.[37,38] Müller cells are known to be extremely susceptible to hyperglycemia and to be responsible for the initiation and progression of retinal damage resulting from hyperglycemia.[37,38] In response to photoreceptor degeneration, the Wnt signaling pathway has been reported to be activated in Müller cells, where this pathway acts to protect the retina from further damage.[20] DKK-3 is known to be localized to the INL, where DKK-3 expression and secretion were increased in Müller cells during photoreceptor injury and where it has been reported to potentiate Wnt signaling.[20,30,31] Thus, we speculate that increased DKK-3 secretion by Müller

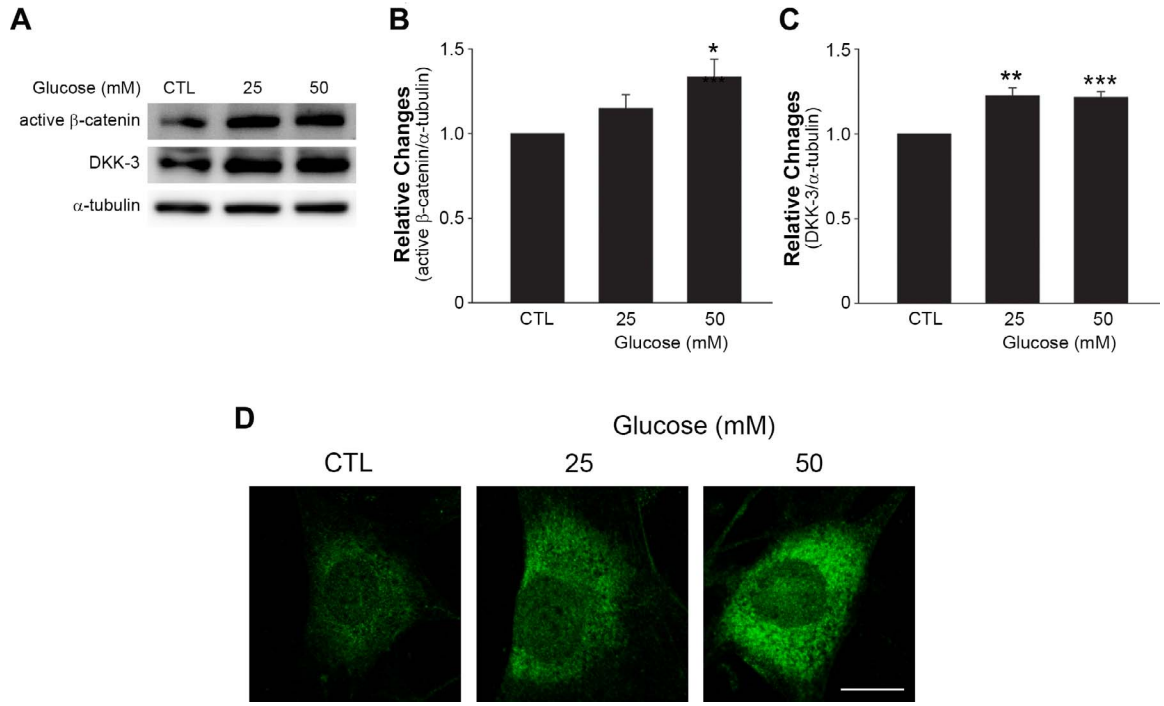

FIGURE 3. Western blot and immunofluorescence analyses of MIO-M1 cells. (**A–C**) DKK-3 expression was significantly increased in MIO-M1 cells treated for 24 hours with either 25 mM (1.22 ± 0.05-fold, $P = 0.001$) or 50 mM glucose (1.21 ± 0.03-fold, $P = 0.000$), compared with the control cells (CTL). The active β-catenin level was increased in cells treated with 50 mM glucose (1.33 ± 0.16-fold, $P = 0.02$) compared with control cells (CTL). The protein expression levels were quantified via densitometry using ImageJ software. (**D**) DKK-3 expression was also increased in glucose-treated cells (25 mM or 50 mM) compared with control cells (CTL) as determined by immunofluorescence (scale bar: 20 μm, original magnification: ×1000). The results are presented as means ± SE of three independent experiments; $n = 5$. Student's *t*-test was used.

cells may augment the protective response to retinal injury in the diabetic retina. However, amplifying Wnt signaling might induce increased expression of VEGF and other inflammatory cytokines; thus, the possibility exists that Wnt signaling may induce neovascularization or further progression of DR. Therefore, modulating or increasing the DKK-3 levels as a possible therapeutic measure may only be effective and safe during the early stage of DR or DME.

We also confirmed that the expression of DKK-3, as well as active β-catenin, is increased in human Müller cells (MIO-M1) exposed to high-glucose concentrations. These results are consistent with a previous report by Yi and associates,[20] who showed that Wnt signaling was activated in rat Müller glial cultures and in MIO-M1 cells following treatment with Wnt3a-conditioned medium.[20] Thus, we speculate that in DME, Wnt signaling is increased in Müller cells in response to retinal injury or dysfunction and is coordinated with the increased secretion of DKK-3 (i.e., the level of DKK-3 in the AH reflects the degree of Wnt activation in the retina). The level of DKK-3 in plasma from patients with DME or controls was extremely low (1–5 ng/mL, data not shown) compared with that of AH, yet there was no significant difference in the plasma DKK-3 levels between the patients and the control subjects. The increase in the DKK-3 level in the AH is most likely due to increased local production of DKK-3 in the retina, primarily by Müller cells. Therefore, the identification of increased DKK-3 expression in Müller glia upon high-glucose treatment, together with the association between increased DKK-3 levels and prominent edema in the INL of patients with DME, indicates that the elevation of the DKK-3 levels in the AH of patients with DME is closely associated with the degree of Wnt signaling activation and Müller cell activity in these patients. Whether the increased level of DKK-3 in patients with DME is a beneficial adaptive or protective response or a damaging response by Müller cells is uncertain. However, considering that most of the patients in this study presented with a relatively recent onset of macular edema that did not result from chronic symptoms and given the greater decrease in CMT (i.e., better treatment responses to anti-VEGF agents) in patients with elevated DKK-3 levels, the former possibility is more likely. We speculate that extracellular edema or fluid accumulation in the INL resulting from diffuse vascular leakage in the deep capillary plexus of the INL induced Müller cell activation and enhanced the secretion of DKK-3, which subsequently increased Wnt signaling in neighboring Müller cells or other cells in the retina.

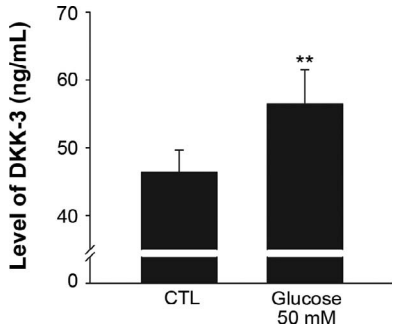

FIGURE 4. ELISA of the DKK-3 levels. The levels of secreted DKK-3 in medium from 50 mM glucose-treated MIO-M1 cells were higher than those in medium from control cells (CTL; 46.37 ± 3.31 vs. 56.50 ± 5.00 ng/mL, $P = 0.005$). Three independent experiments were performed in duplicate; $n = 4$. The results are presented as means ± SE. The paired *t*-test was used.

The current study has several limitations. First, the sample size was relatively small. However, multivariate analysis

showed that edema in the INL was strongly associated with elevated DKK-3 levels, although validation of such a clinical association using biological markers is generally difficult.[38] Second, atrophy of retinal tissue in the INL was not determined. Whether the fluid observed was the result of extracellular fluid accumulation or of Müller cell swelling, including secondary changes such as necrosis, was unclear. However, because we excluded chronic cases from this study, the observed increase in DKK-3 levels likely represents a reactive response by Müller cells that was elicited by edema in the INL rather than Müller cell swelling or severe damage. Third, other cells in the retina, such as astrocytes, could be the source of DKK-3 in the AH. Astrocytes have been reported to play an early role in the neuronal dysfunction that occurs before retinal vessel changes in streptozotocin-induced diabetic rats.[39] However, astrocytes do not appear to contribute significantly to the overall expression of DKK-3 in the AH, as a few studies have described the expression of DKK-1, but not DKK-3, in astrocytes.[40,41] To our knowledge, no report in the literature has described increased DKK-3 expression in astrocytes.

In conclusion, this study is the first to demonstrate that the level of DKK-3 is significantly elevated in the AH of eyes in patients with DME compared with control subjects and that the DKK-3 level in the AH correlates with the severity of macular edema, particularly in the INL. This correlation was confirmed with intraretinal segmentation on SD-OCT. The AH DKK-3 level in patients with DME could serve as a new AH biomarker that may reflect Müller cell status. Whether Müller cell-secreted DKK-3 plays a neuroprotective role in the diabetic human retina remains unclear. Further studies are needed to determine whether the modulation of DKK-3 in Müller cells or the targeting of Müller cells could be a new approach for decreasing macular edema in patients with DME.

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