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Guest Editors: Ahmed M. Abu El-Asrar, Edoardo Midena, Mohamed Al-Shabrawey, and Ghulam Mohammad

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Editorial Board

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Editorial

New Developments in the Pathophysiology and Management of Diabetic Retinopathy

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Diabetes mellitus is a chronic disorder of glucose metabolism with serious multisystem complications including microvascular dysfunction such as retinopathy, nephropathy, and neuropathy. The prevalence of diabetes has been rising in the last few decades and affecting working age population which reflects the socioeconomic burden on different communities and a major challenge to the current health care. Diabetic retinopathy (DR), which is a slow-progressing and multifactorial complication of diabetes, is the most common cause of vision loss in patients with diabetes. According to the World Health Organization, in the world, about more than 371 million people have diabetes and approximately 5 million individuals have diabetic retinopathy, accounting for 5 percent of world blindness. In spite of all advances in the understanding of chronic diabetic complications, DR remains a significant clinical problem partially due to lack of effective therapeutic intervention. The current therapy is mostly invasive intervention and mainly prevents the progression of the disease but does not effectively restore the lost vision. Therefore, there is an urgent need to understand in detail the underlying molecular and cellular mechanisms of the DR and also to advance the diagnostic tools to diagnose the early microvascular and neuronal changes during DR.

The current special issue through a number of experts in the field of DR presents both review and original research articles that provide an overview of the work conducted to date and expand our understanding of the underlying mechanism of DR. The articles include discussion of the mechanism of retinal neovascularization (angiogenesis, vasculogenesis) and neurodegeneration including the role of enhanced inflammatory pathways, cellular signaling, and oxidative stress. Each of the papers in this series is briefly highlighted below.

The improvement of diagnostic technology has revolutionized the clinical approach to eye disorders, mainly retinal diseases. Optical coherence tomography (OCT) has introduced clinicians to a sort of *in vivo* retinal histology, particularly with the use of spectral domain OCT. With spectral domain OCT, we are not yet at cellular level, but we may obtain and discuss individual retinal layers. The clinical use of spectral domain OCT has largely contributed to the understanding of pathophysiology of diabetic retinopathy, both in preclinical and clinical phases. Two papers of this special issue dedicated to diabetic retinopathy clearly show how relevant is the contribution of spectral domain OCT in the quantification of retinal layer changes secondary to diabetes. T. Murakami and N. Yoshimura offer a well-illustrated and updated review of the inner and outer retinal changes in diabetic macular edema, the most important cause of legal blindness among diabetics. They discuss the relevance of any OCT detail to improve our knowledge about the pathophysiology of diabetic macular edema and stress the importance of considering these details as predictive factors of treatment results. They also point to the importance of the still poorly known role of the choroid, which may be measured by OCT, in the pathophysiology of diabetic

retinopathy. S. Vujosevic and E. Midena not only confirm the importance of the study of retinal layers, quantified by OCT, in nonproliferative diabetic retinopathy, but also illustrate the precocious changes of retinal layers in preclinical retinopathy. This means that OCT may contribute to detection of subtle retinal changes before an overt microvasculopathy develops. These changes are visible mainly in the inner retina, with a speculated involvement of the Müller cells, never reported before. In addition, S. Vujosevic and S. Midena demonstrated using spectral domain OCT the presence of retinal hyperreflective spots (HRS) in diabetic eyes even when clinical retinopathy is undetectable. Their number increases with progressing retinopathy. HRS are initially mainly located in the inner retina, where resident microglia is present. With progressing retinopathy, HRS reach the outer retinal layer. HRS may represent a surrogate of microglial activation in diabetic retina.

The paper by A. M. Abu El-Asrar et al. describes levels of angiogenic and endothelial progenitor cell mobilizing (vasculogenic) factors in vitreous fluid from proliferative diabetic retinopathy (PDR) patients and correlates their levels with clinical disease activity. The pathophysiology of DR involves multiple molecular pathways and is characterized by retinal neovascularization. Retinal neovascularization (RNV) in PDR occurs mainly through angiogenesis. However, increasing evidence suggests that vasculogenesis, the de novo formation of blood vessels from circulating bone-marrow-derived endothelial progenitor cells (EPCs), also contributes to retinal neovascularization. Recent studies have shown that circulating bone-marrow-derived EPCs home to the ischemic region, differentiate into mature endothelial cells in situ, and can contribute to the process of neovascularization. Angiogenesis and vasculogenesis are dependent on several cytokines/chemokines and their associated tyrosine kinase receptors. Abu El-Asrar et al.'s findings suggest that the upregulation of VEGF, sVEGFR-2, SCF and s-kit in the vitreous fluid from patients with PDR reflects angiogenesis and vasculogenesis in PDR.

Growing body of evidence supports the hypothesis that damaging effect of elevated glucose in the retina may, in part, be due to its ability to increase mitogen-activated protein kinases (MAPK) signaling pathway in the retina. Extracellular-signal-regulated kinases-1/2 (ERK1/2) are the most extensively characterized members of MAPK family proteins. G. Mohammad et al. describe the role of ERK1/2 signaling pathway in the activation of inflammatory mediators in the diabetic retina. The authors' data provide evidence that ERK1/2 signaling pathway is involved in MMP-9, iNOS, IL-6, and TNF-α induction in diabetic retinas and suggest that ERK1/2 can be a novel therapeutic target in diabetic retinopathy.

Diabetes is known to cause oxidative stress in the retina, and this abnormality results from increased generation of reactive oxygen species (via mitochondria and NADPH oxidase) and decreased activity of antioxidant enzymes. Considering that oxidative stress represents the key factors in the onset and progression of diabetic retinopathy, antioxidant and anti-inflammatory products are expected to produce significant therapeutic advantages.

The paper by G. Bucolo et al. describes that treatment of diabetic rats with the fortified extract of red berries, *Ginkgo biloba*, and white willow bark (containing carnosine and α-lipoic acid) significantly lowered retinal cytokine levels (TNF-α,VEGF) and suppressed diabetes-induced lipid peroxidation.

Diabetes-induced oxidative stress disturbs retinal homeostasis by activating glial cells, reducing neurotrophic support, and increasing proinflammatory cytokines. Recent studies proved that neurotrophins including nerve growth factor (NGF) are emerging as critical mediators of DR. NGF activates two different receptors including the high affinity tropomyosin-related receptor A (TrkA), which is a tyrosine kinase, and the low affinity p75NTR. The paper by S. L. Elshaer et al. demonstrated that the angiogenic response of NGF was mediated via activation of TrkA suggesting that the proNGF can contribute to PDR at least in part via activation of TrkA.

We sincerely hope that the present special issue may provide useful information to understand the mechanisms, the clinical effects, and the novel treatments in the development of DR.

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Clinical Study

Hyperreflective Intraretinal Spots in Diabetics without and with Nonproliferative Diabetic Retinopathy: An *In Vivo* Study Using Spectral Domain OCT

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Purpose. To evaluate the presence of hyperreflective spots (HRS) in diabetic patients without clinically detectable retinopathy (no DR) or with nonproliferative mild to moderate retinopathy (DR) without macular edema, and compare the results to controls. *Methods*. 36 subjects were enrolled: 12 with no DR, 12 with DR, and 12 normal subjects who served as controls. All studied subjects underwent full ophthalmologic examination and spectral domain optical coherence tomography (SD-OCT). SD-OCT images were analyzed to measure and localize HRS. Each image was analyzed by two independent, masked examiners. *Results*. The number of HRS was significantly higher in both diabetics without and with retinopathy versus controls ($P < 0.05$) and in diabetics with retinopathy versus diabetics without retinopathy ($P < 0.05$). The HRS were mainly located in the inner retina layers (inner limiting membrane, ganglion cell layer, and inner nuclear layer). The intraobserver and interobserver agreement was almost perfect ($\kappa > 0.9$). *Conclusions*. SD-OCT hyperreflective spots are present in diabetic eyes even when clinical retinopathy is undetectable. Their number increases with progressing retinopathy. Initially, HRS are mainly located in the inner retina, where the resident microglia is present. With progressing retinopathy, HRS reach the outer retinal layer. HRS may represent a surrogate of microglial activation in diabetic retina.

1. Introduction

An increasing body of evidence suggests that retinal neurodegeneration and inflammation occur in human diabetes even before the development of clinical signs of diabetic retinopathy (DR) [1]. Retinal neural cell loss (neurodegeneration) has already been demonstrated *in vivo* (as thinning of retinal nerve fiber and ganglion cell layers), both in type 1 and 2 diabetes [2–7].

Retinal microglia activation has been recognized as the main responsible for the initial inflammatory response, even though the exact mechanism through which inflammatory cytokines are released remains poorly known [8]. Some experimental studies have shown that retinal inflammation occurring during the course of diabetes mellitus is a relatively early event and that it precedes both vascular dysfunction and neuronal degeneration [1, 8]. Joussen at al. demonstrated in animal models of diabetes mellitus that ICAM-1- and CD18-mediated leukocyte adhesion is increased in the retinal vasculature and accounts for many of the signature lesions of DR [1]. Ibrahim et al. demonstrated in rats that the accumulation of Amadori-glycated albumin (AGA) within the 8-week diabetic retina elicits microglial activation and secretion of Tumor necrosis factor alpha (TNF-α) [8].

Retinal macroglia and retinal microglia activation have been documented histopathologically and hypothesized *in vivo* [7, 9–11]. The activated microglia secretes cytokines and other proinflammatory molecules used for the phagocytosis and the destruction of damaged cells as well as for the triggering of reparative processes which lead to the formation

of glial scars [8]. If microglia remains in an activated state, continuously released cytokines may damage the neighbouring cells particularly the neuronal and the vascular ones, leading to the onset of different retinal changes [8]. According to this hypothesis, some histopathological studies (performed both in animals and in humans) have confirmed the activation of microglial cells, as well as the presence of different inflammatory molecules secreted by microglia, commonly associated with neuronal and endothelial death [9, 10, 12–14].

Spectral domain optical coherence tomography (SD-OCT) has become a valuable tool for the *in vivo* evaluation of single retinal layers (both the inner retina and the outer retina) in diabetic patients [7, 15, 16]. Moreover, it has been used for the evaluation of hyperreflective retinal spots in age related macular degeneration, diabetic macular edema, and retinal vein occlusion [16–21].

The main purpose of this study was to determine, *in vivo*, by SD-OCT, the presence and location of hyperreflective spots in the retina in diabetic patients without DR or with early stages of DR (mild and moderate nonproliferative DR) without macular edema versus normal subjects.

2. Material and Methods

36 subjects were included in the study: 12 subjects served as controls, 12 patients were affected by diabetes without diabetic retinopathy (no DR), and 12 patients were affected by diabetes and diabetic retinopathy (mild to moderate). One eye of each subject was used for the SD-OCT analysis. The exclusion criteria were proliferative DR, macular edema, any type of previous retinal treatment (macular laser photocoagulation, vitrectomy, intravitreal steroids, and/or antiangiogenic drugs), any intraocular surgery, refractive error >6D, previous diagnosis of glaucoma, ocular hypertension, uveitis or other retinal diseases, and significant media opacities that precluded fundus imaging. All patients underwent SD-OCT using Spectralis (Heidelberg Engineering, Heidelberg, Germany). A single 180° SD-OCT line scan (6 mm length) centered onto the fovea was analyzed for each patient, looking for the presence of hyperreflective spots. Two red vertical lines were traced at 500 μm and 1500 μm from the center of the fovea in the temporal region, thus excluding the foveal avascular zone. A manual count of the hyperreflective spots, defined as small, punctiform, white lesions, was performed between the two markers. The layering was obtained using the automatic layering of the Spectralis SD-OCT with manual refinement for the boundaries of the most critical layers (e.g., inferior boundary of ganglion cell layer where contrast is lower).

The count was performed starting from the inner limiting membrane (ILM) to the outer nuclear layer (ONL), including ILM to ganglion cell layer (GCL); inner nuclear layer (INL) to outer plexiform layer (OPL), and ONL. All measurements were performed by two independent, masked graders (Figure 1).

A written consent form was obtained from all patients as well as the approval from our institutional ethics committee. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

The difference in the number of hyperreflective spots was compared among groups by means of analysis of variance (ANOVA).

Table 1: The percentage of eyes with hyperreflective spots.

Retinal microglial activation	Control	Diabetic	
		No DR	NPDR
ILM-GCL	3%	55%	90%
INL-OPL	1%	35%	75%
ONL	—	10%	25%

No DR: diabetic patients without retinopathy; NPDR: nonproliferative diabetic retinopathy; ILM-GCL: inner limiting membrane-ganglion cell layer; INL-OPL: inner nuclear layer-outer plexiform layer; ONL: outer nuclear layer.

Table 2: The number of hyperreflective spots.

Hyperreflective spots/number (±SD)	Control	Diabetic	
		No DR	NPDR
ILM-GCL	00.5 (0.22)	2.92 (1.88)	6.63 (2.5)
INL-OPL	0.03 (0.48)	2.93 (1.73)	8.03 (3.18)
ONL	0.00 (0.00)	1.58 (1.31)	6.18 (2.89)

SD: standard deviation; No DR: diabetic patients without retinopathy; NPDR: nonproliferative diabetic retinopathy; ILM-GCL: inner limiting membrane-ganglion cell layer; INL-OPL: inner nuclear layer-outer plexiform layer; ONL: outer nuclear layer.

3. Results

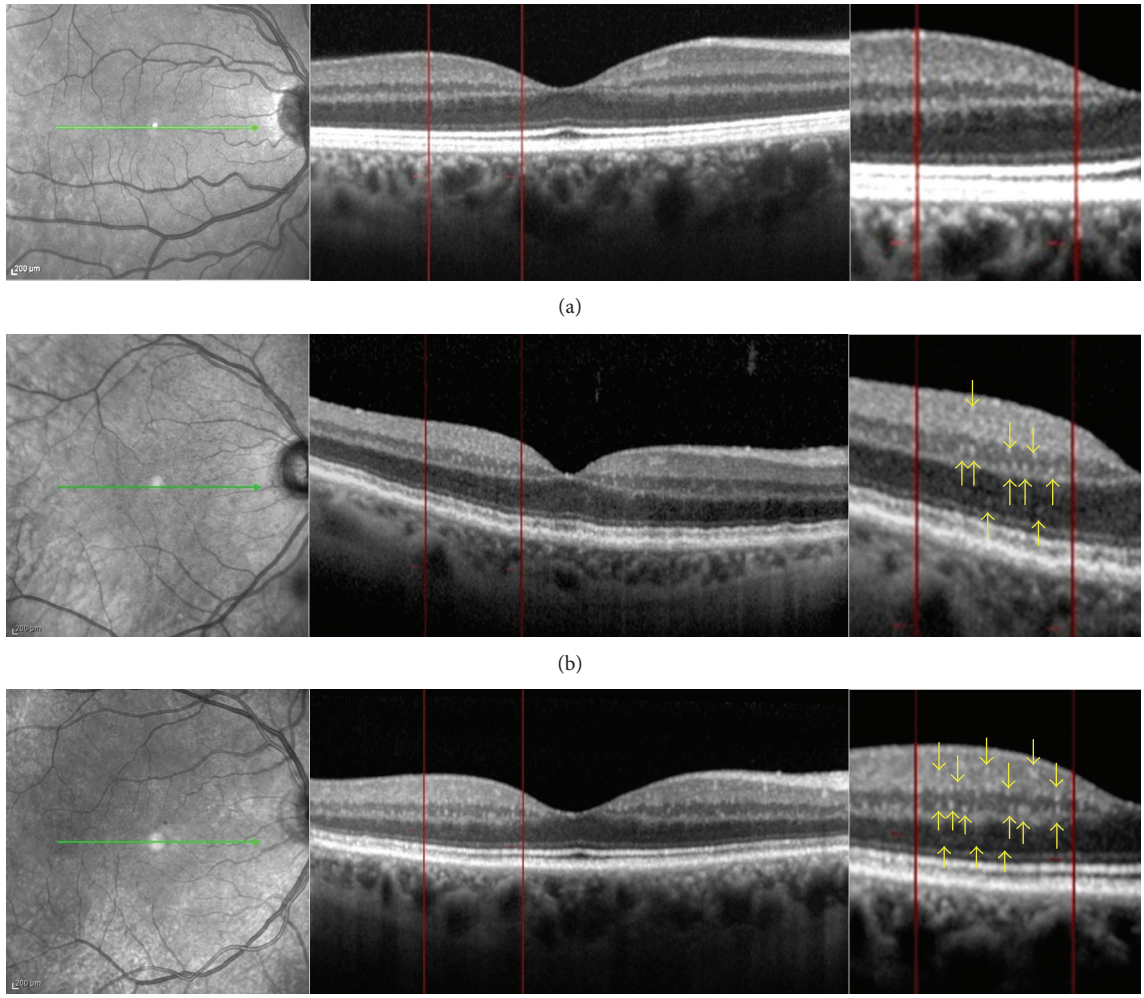
The mean age of the different groups was 55.2 ± 10 years in normal subjects, 56.9 ± 13 years in diabetics without DR (no DR), and 59.3 ± 11.2 years in nonproliferative DR (NPDR). There was no statistically significant difference in the age among the three groups. All diabetics had type 2 diabetes mellitus (DM). Mean HbA1c was 7.6 ± 1.5% in no-DR and 7.8 ± 0.8 in NPDR. Mean diabetes duration was 10.2 ± 4 years.

In the ILM-GCL layer, the hyperreflective spots were present in 3% of control eyes, 55% of no-DR eyes, and 90% of DR eyes. In the INL-OPL layer, the hyperreflective spots were present in 1% of control eyes, 35% of no-DR eyes, and 75% of DR eyes.

In the ONL layer, the hyperreflective spots were present in 0% of control eyes, 10% of no-DR eyes, and 25% of DR eyes (Table 1).

As the number of hyperreflective spots is concerned with the ILM-GCL layer, the mean number was 0.05 ± 0.22 in control eyes, 2.92 ± 1.88 in no-DR eyes, and 6.63 ± 2.5 in DR eyes; in the INL-OPL layer, the mean number was 0.03 ± 0.48 in control eyes, 2.93 ± 1.73 in no-DR eyes, and 8.03 ± 3.18 in DR eyes; in the ONL layer the mean number was 0.0 in control eyes, 1.58 ± 1.31 in no-DR eyes, and 6.18 ± 2.89 in DR eyes (Table 2).

The number of hyperreflective spots was significantly higher in both diabetics with no DR and diabetics with DR versus controls (ANOVA, $P < 0.05$) and in diabetics with DR



(a)

(b)

(c)

Figure 1: Spectral domain OCT linear scans in the macula of (a) normal subject, (b) diabetic patient without retinopathy, and (c) diabetic patient with mild nonproliferative diabetic retinopathy. Two vertical lines were traced at 500 μm and 1500 μm from the center of the fovea in the temporal region where the count of the hyperreflective spots was performed. The count was performed in the following retinal layers: inner limiting membrane (ILM) to ganglion cell layer (GCL), inner nuclear layer (INL) to outer plexiform layer (OPL), and outer nuclear layer (ONL). The yellow arrows indicate the HRS seen on the magnification image. The number of HRS is higher in diabetic eyes with DR versus diabetic eyes without retinopathy.

versus diabetics without retinopathy (ANOVA, $P < 0.05$). The intraobserver and interobserver agreement was almost perfect ($\kappa > 0.9$) for all measurements.

4. Discussion

In this study, we report the presence of hyperreflective spots (HRS), documented by SD-OCT, in the more inner retinal layers (ILM, GCL), in the INL to OPL, and in the ONL in diabetic patients with and without DR. When compared to healthy subjects, these hyperreflective spots were significantly much more numerous in the inner retina of diabetics and completely absent in the outer retina of controls. The HRS have been recently described by some authors, who hypothesized different pathogenetic origin, and who also used two different terms to name these lesions. They named HRS as hyperreflective foci or hyperreflective dots [16–21]. We suggest that the term spots better encompasses the aspect of these lesions, but we do not consider different terms a limitation. Coscas et al. were the first to report the presence of HRS, as small in size, punctiform hyperreflective elements, scattered throughout all retina layers but mainly located in the outer retina layers around fluid accumulation in the intraretinal cystoid spaces in age related macular degeneration, suggesting that they may represent activated microglia cells [18, 19]. Bolz et al. described the HRS distributed throughout all retinal layers (in some cases confluent at the border of the ONL and within the outer plexiform layer) in eyes with different types (diffuse, cystoid) of diabetic macular edema (DME) [17]. Bolz et al. hypothesized that HRS may represent subclinical features of lipoprotein extravasation that act as precursors of hard

exudates, as they were not observed on clinical examination, fundus photography, or fluorescein angiography, due to their small size (approximately 30 microns) [17]. Uji et al. reported the presence of HRS in the outer retina (53.7%) and in the inner retina (99.1%) in eyes with DME [20]. The HRS in the outer retina were closely associated with disrupted external limiting membrane and IS/OS line and decreased visual acuity, suggesting an origin from degenerated photoreceptors or macrophages engulfing them [20]. Ogino et al. reported the presence and distribution of HRS in retinal vein occlusion [21]. The HRS were present in all retinal layers (both inner and outer retinas). In most of the eyes affected by serous retinal detachment, the HRS were attached to the external limiting membrane [21]. The authors did not find any sign of hard exudates. They also suggested that the HRS present in affected areas may explain the leakage of blood constituents, whereas the HRS around the OPL in the unaffected areas in retinal vein occlusion were associated with the absorption of water and solutes [21]. Framme et al. reported the presence of HRS in patients with both focal DME and diffuse DME [16]. After anti-VEGF treatment, the HRS were the first features to disappear or to reduce significantly. Therefore, it has been hypothesized that HRS represent a clinical marker of inflammatory response [16, 19].

In our study, the presence and number of HRS increased in diabetics versus normals, and especially in diabetics with clinical signs of DR. HRS were mainly located, at an early stage, into the inner retinal layers, from ILM to GCL layer. This depends on the fact that the resting retinal microglia is physiologically located in the inner retinal layers, and before migrating toward outer layers, the activation process begins into and expands from the inner retinal layers. When activated, microglial cells undergo significant changes as the shape and size of individual cells are concerned and aggregate among them to form the microglial aggregates [10]. Neither of the patients included in this study had macular edema, hard exudates, or subclinical (OCT) signs of DME. Because all our patients were affected by initial stages of diabetic retinopathy (with normal macula) or even diabetic without retinopathy, we are confident in excluding the hypothesis that in our population, HRS represent lipid exudates or degenerated photoreceptors. HRS in our population are more reasonably secondary to early microglia activation (aggregates of microglial cells), already described in experimental studies in diabetic retina [9, 10, 22]. To confirm our hypothesis, a recent study has documented a significant increase in the thickness of IPL and INL versus a decrease in GCL and RNFL, in patients with nonproliferative DR, as signs of macroglial cell activation [7].

Previous studies also showed that low-level chronic inflammation contributes to retinal dysfunction changes during the course of DM [23–26]. Pathologic levels of neuroretinal inflammatory mediators may contribute to the evolution of diabetic retinopathy, up to the proliferative stage [27]. Moreover, Zeng et al, investigating the microglial activation around retinal capillaries in human eyes, detected signs of the so-called "microglial perivasculitis," a phenomenon secondary to microglial activation. The same group evidenced that this inflammatory local process may secondary affect ganglion cells, suggesting precocious inflammation changes at the level of retinal neuronal cells [10]. These data may be supported by our findings of HRS, which are initially present in most internal retinal layers. The release of inflammatory mediators (including VEGF) provokes the extension of the inflammatory process through the entire retina, increasing both vascular permeability and neuronal damage, thus creating a vicious circle [10]. The spread of activated microglia cells beyond outer retinal layers, through an experimentally documented intracellular mechanism, is also documented by the appearance of HRS up to the level of retinal pigment epithelium [22].

In conclusion, diabetic retinopathy is characterized by retinal microglia activation, previously shown in experimental studies on animals and autoptic eyes. SD-OCT documents discrete reflectivity changes (hyperreflective spots) which may correspond to aggregates of microglia activated cells, as reported in other retinal diseases. The number of SD-OCT hyperreflective spots increases with the clinical progression of DR and shows an inner to outer retina migration. A prospective study on the evolution of DR and HRS location and presence is currently underway in order to strengthen our hypothesis.

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Review Article

Structural Changes in Individual Retinal Layers in Diabetic Macular Edema

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Optical coherence tomography (OCT) has enabled objective measurement of the total retinal thickness in diabetic macular edema (DME). The central retinal thickness is correlated modestly with visual impairment and changes paradoxically after treatments compared to the visual acuity. This suggests the clinical relevance of the central retinal thickness in DME and the presence of other factors that affect visual disturbance. Recent advances in spectral-domain (SD) OCT have provided better delineation of the structural changes and fine lesions in the individual retinal layers. Cystoid spaces in the inner nuclear layer and outer plexiform layer are related to quantitative and qualitative parameters in fluorescein angiography. OCT often shows vitreoretinal interface abnormalities in eyes with sponge-like retinal swelling. Serous retinal detachment is sometimes accompanied by hyperreflective foci in the subretinal fluid, which exacerbates the pathogenesis at the interface of the photoreceptors and retinal pigment epithelium. Photoreceptor damage at the fovea is thought to be represented by disruption of the external limiting membrane or the junction between the inner and outer segment lines and is correlated with visual impairment. Hyperreflective foci in the outer retinal layers on SD-OCT images, another marker of visual disturbance, are associated with foveal photoreceptor damage.

1. Introduction

Diabetic macular edema (DME), a leading cause of visual impairment in individuals of working age, is mediated by multiple and complex mechanisms in its pathogenesis [1–4]. Pathophysiology, that is, vascular hyperpermeability and ischemia, is represented by clinical findings seen on fluorescein angiography (FA) images [5–8]. Basic research has elucidated the molecular mechanisms including vascular endothelial growth factor in DME and proliferative diabetic retinopathy (PDR) [9–18]. Using biomicroscopy, clinical ophthalmologists observe thickening of the sensory retina and vascular lesions in DME. However, it is difficult to objectively evaluate the neuroglial changes in the retina. In contrast, histologic studies have reported that cystoid spaces are present mainly in the inner nuclear layer (INL) and the outer plexiform layer (OPL) and contribute partly to macular thickening [19–21]. Electron microscopy showed that, in addition to the accumulation of the extracellular fluids, intracytoplasmic swelling of the Müller cells might be a pathophysiologic mechanism in DME.

Optical coherence tomography (OCT) provides retinal sectional images as in histology study (Figure 1) and is useful for qualitative and quantitative evaluation of pathological retinal changes [22]. The original instrument, time-domain OCT, has been replaced with spectral-domain OCT (SD-OCT), which has higher resolution and reduced speckle noise. Clinicians can appreciate the improved delineation of the fine pathological lesions and the clearer borders between the individual retinal layers.

This review summarizes the current understanding of the retinal thickness, pathomorphologies, and photoreceptor damage in DME that can be seen on OCT.

2. Retinal Thickening on OCT Images

Biomicroscopy allows ophthalmologists to subjectively evaluate retinal thickening, whereas OCT has enabled objective

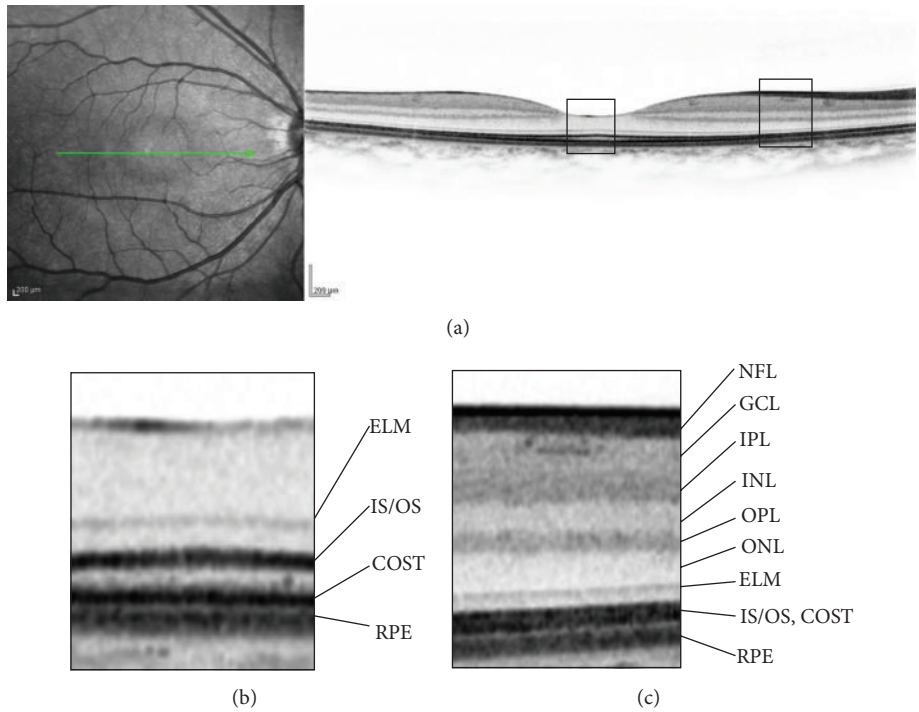


Figure 1: (a) Retinal sections of the physiologic macula dissecting along the green line on fundus photography using SD-OCT. (b) The magnified image at the fovea shows three lines over the retinal pigment epithelium (RPE), that is, the external limiting membrane (ELM), the junction between inner and outer segments (IS/OS), and the cone outer segment tips (COST). (c) A magnified parafoveal image shows the individual retinal layers. NFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.

quantification of the total retinal thickness (Figure 2). The diabetic retinopathy clinical research network (DRCRnet) especially defined an increase in the mean central thickness of 1 mm as center-involved DME [23, 24], which is the new standard for applying treatments and a surrogate marker for evaluating treatment efficacy [25]. It is widely accepted that the central thickness is correlated modestly with visual acuity (VA) in DME [26]. In addition, the DRCRnet reported that paradoxical VA changes are observed after intervention, that is, VA reduction despite resolution of ME or VA improvement with increased retinal thickening [26]. These data suggest the importance of identifying unknown mechanisms and the magnitude of edematous changes. Despite these issues, an increasing number of studies have reported the relevance of measuring the retinal thickness after treatment for DME as a surrogate marker [27–29].

SD-OCT with higher resolution and reduced speckle noises has enabled segmentation of the individual retinal layers, and several kinds of OCT instruments provide automated segmentation. This feature has encouraged quantification of the thickness of the retinal layers and qualitative evaluation of lesions in the individual layers. Interestingly, the thickness in the inner retinal layers is correlated positively with visual impairment, whereas the outer retinal thickness is associated negatively with poor visual prognosis after vitrectomy for DME [30]. This suggests that thinning of the outer retinal layers is related to photoreceptor degeneration (or atrophy) concomitantly contributes to visual disturbance at least partly, and supports the paradoxical VA changes reported by the DRCRnet [26]. Further analyses of retinal thicknesses with segmentation would improve the understanding of the association between clinical findings and pathogenesis in DME.

3. Pathomorphology in Individual Retinal Layers

Numerous pathological mechanisms have been reported regarding diabetic retinopathy (DR), compared to the simple criteria of diabetes per se [1–4]. OCT subjectively shows several types of foveal morphologies in DME, that is, cystoid macular edema (CME), serous retinal detachment (SRD), and sponge-like retinal swelling [31], which might be among other factors that modulate visual function, dependently or independently of retinal thickening. Individual lesions have been delineated in the individual retinal layers. OCT showed cystoid spaces mainly in the INL and OPL, which has been supported by histologic reports [20, 32, 33]. Extracellular fluids pool between the outer segments and retinal pigment epithelium (RPE) in eyes with SRD. Sponge-like retinal swelling at the fovea occurs in the OPL.

Regarding the types of CME, OCT has documented several findings (Figure 3). The cystoid spaces in the INL

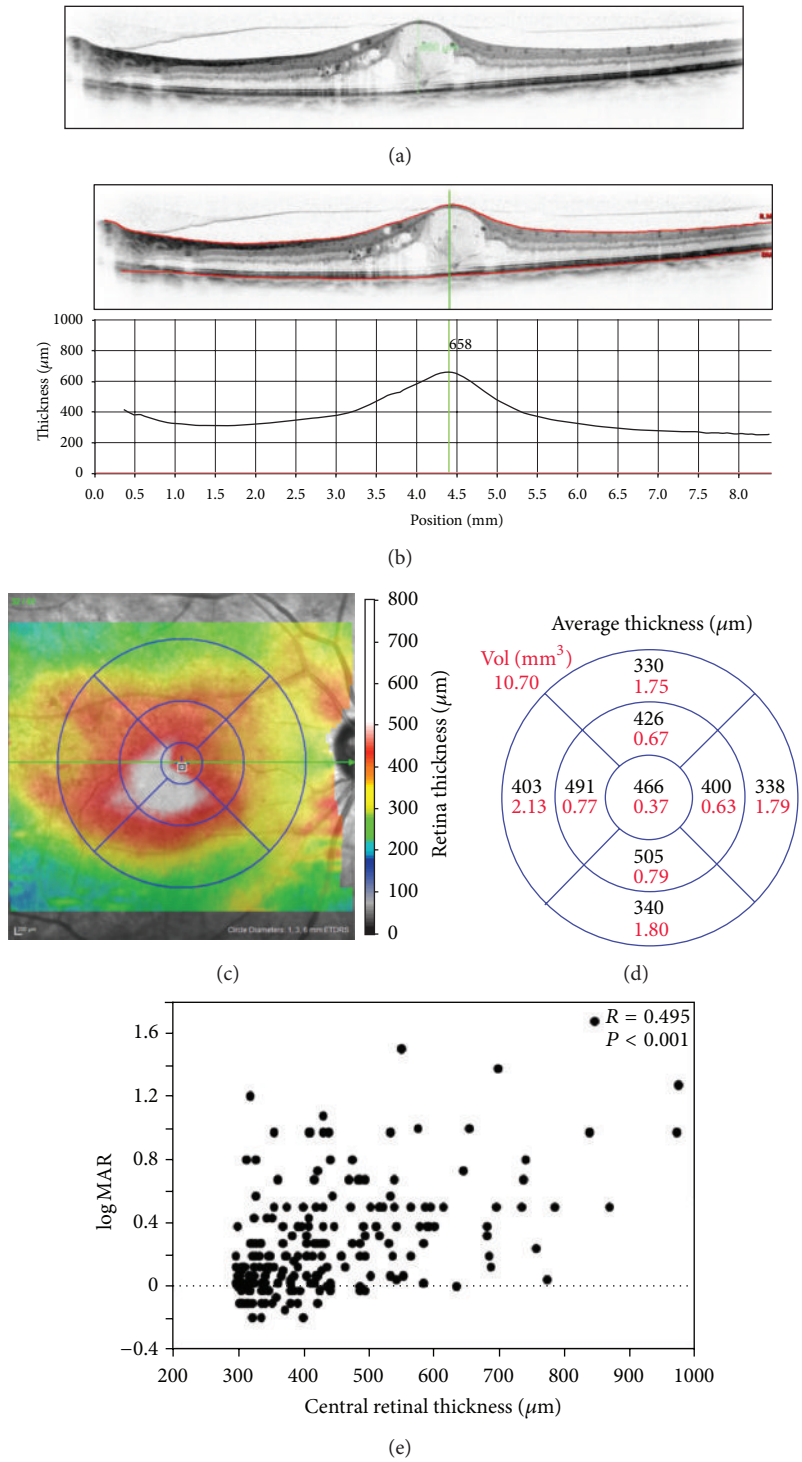


Figure 2: Quantification of the total retinal thickness using OCT. The retinal thickness can be measured semiautomatically (b) or manually using calipers (a). (c) A two-dimensional map of the retinal thickness can be constructed from the automatically measured retinal thickness. (d) The average thickness in each subfield of Early Treatment Diabetic Retinopathy Study grid is shown. (e) The average thickness in the central subfield is correlated modestly with the logarithm of the minimum angle of resolution VA in center-involved DME.

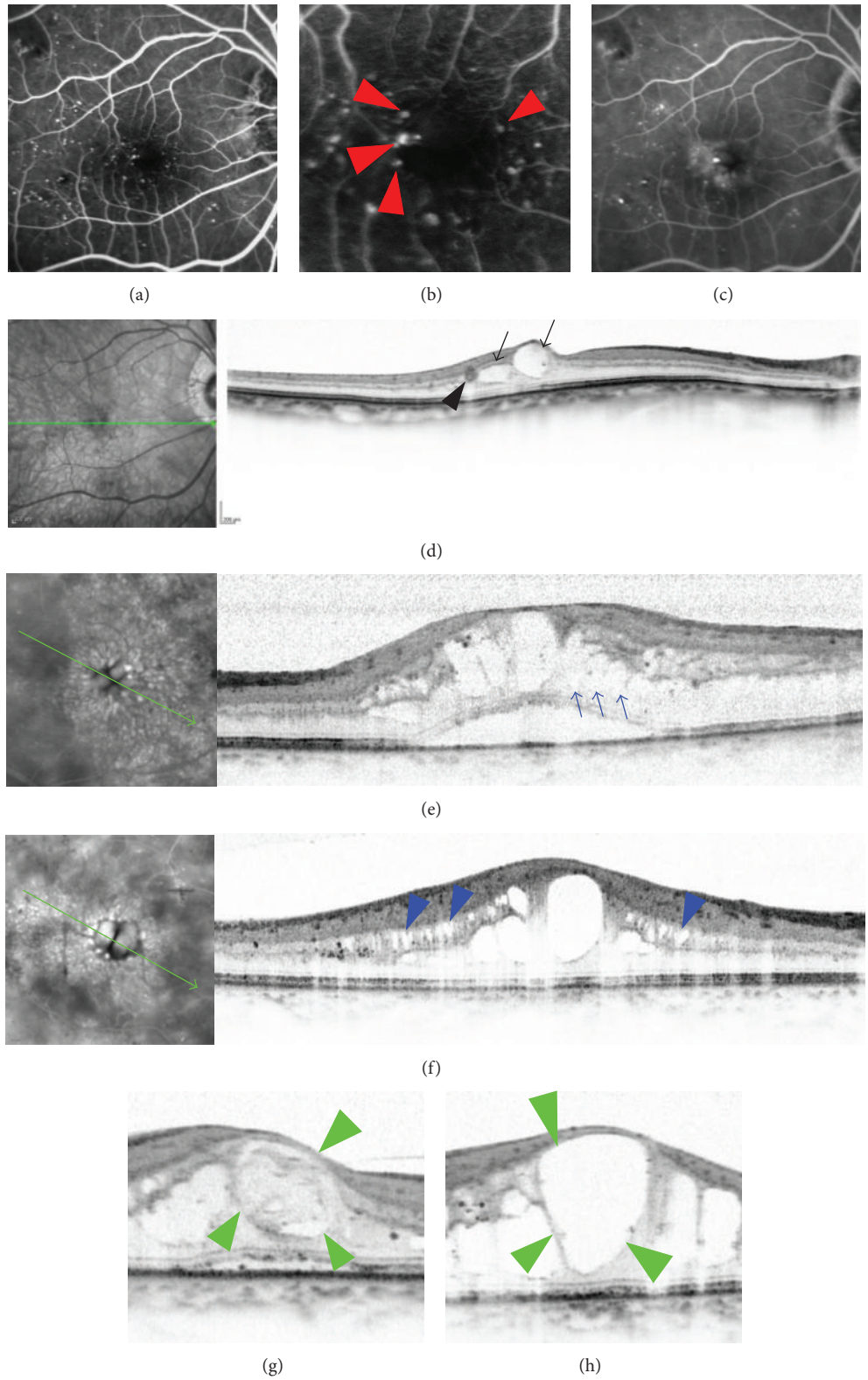


Figure 3: Characteristics of cystoid spaces in DME. ((a), (b)) Early-phase FA shows an enlarged foveal avascular zone and surrounding microaneurysms (arrowheads). (c) A late-phase image shows fluorescein pooling at the fovea. (d) Cystoid spaces (arrows) are accompanied by microaneurysms (arrowhead). (e) A late-phase FA image shows petalloid- (e) or honeycomb-pattern (f) fluorescein pooling, which is considered to correspond to cystoid spaces in OPL (arrow) or INL (arrowhead), respectively, on OCT image. Foveal cystoid spaces (arrowheads) have higher OCT reflectivity and its heterogeneity (g) or lower and homogeneous reflectivity (h).

have a honeycomb pattern of fluorescein pooling, whereas petalloid-shaped pooling corresponds to cystoid spaces in the OPL [32, 33]. Further study of an FA/OCT correlation found that the foveal cystoid spaces on OCT images were associated with enlarged foveal avascular zones and microaneurysms around the perifoveal capillary network [8]. This suggested that ischemia and leakage from microaneurysms contribute to the development or maintenance of cystoid spaces in DME. A recent publication reported that OCT reflectivity in the cystoid spaces was correlated negatively with the intensity of the fluorescein pooling [34] and implicated several types of vascular hyperpermeability regarding the pathogenesis in the cystoid spaces.

SD-OCT has shown the fine structures of the microaneurysms in patients with DR [35–37]. Among the capsular structure patterns, the "incomplete" and "absent" types, in contrast to the "complete" type, often were accompanied by fluorescein leakage and cystoid spaces [36, 37]. Recently, using SD-OCT, clinicians have confirmed that the number of microaneurysms decrease after focal photocoagulation, which suggested that in the future FA would be replaced with noninvasive OCT to evaluate treatment efficacy [38, 39].

Vitreomacular traction sometimes modulates macular thickening in DME [40–43]. Eliminating the vitreoretinal traction during vitrectomy is suggested to be an effective strategy for eyes with DME, and DRCRnet has reported the greater VA improvement in eyes with preoperative ERM and the greater reduction of central subfield thickness in eyes with vitreoretinal abnormalities [44–46]. Ocriplasmin, a recombinant protease with activity against components of the vitreoretinal interface, has recently been reported to be effective for the diseases with vitreomacular interface abnormalities [47]. It remains to be elucidated how ocriplasmin modulates retinal thickening without the removal of the vitreous gel which contains growth factors and cytokines.

Sponge-like retinal swelling especially often is accompanied by pathological findings in the vitreomacular interface on OCT images (Figure 4), and vitreomacular interface abnormalities contribute to thickening of the retinal parenchyma in the OPL at the fovea, as in the case of idiopathic epiretinal membrane (ERM). It also was reported that vitreomacular interface abnormalities also might induce SRD in DME [40], and OCT sometimes shows cystoid spaces with vitreoretinal abnormalities as with idiopathic macular holes [48, 49]. Fibrovascular proliferation in PDR progresses along the posterior hyaloid membrane and induces contraction. Tangential tractional forces increase the retinal thickness and concomitantly contribute to macular edema, and horizontal forces result in macular heterotopia (traction maculopathy) in PDR. Several patterns of vitreomacular interface abnormalities seen on SD-OCT images were reported recently, that is, vitreomacular traction with no or partial posterior vitreous detachment, posterior vitreous separation, ERM, and their combinations. These findings would help surgeons to completely remove the pathological changes of the vitreoretinal interface [43].

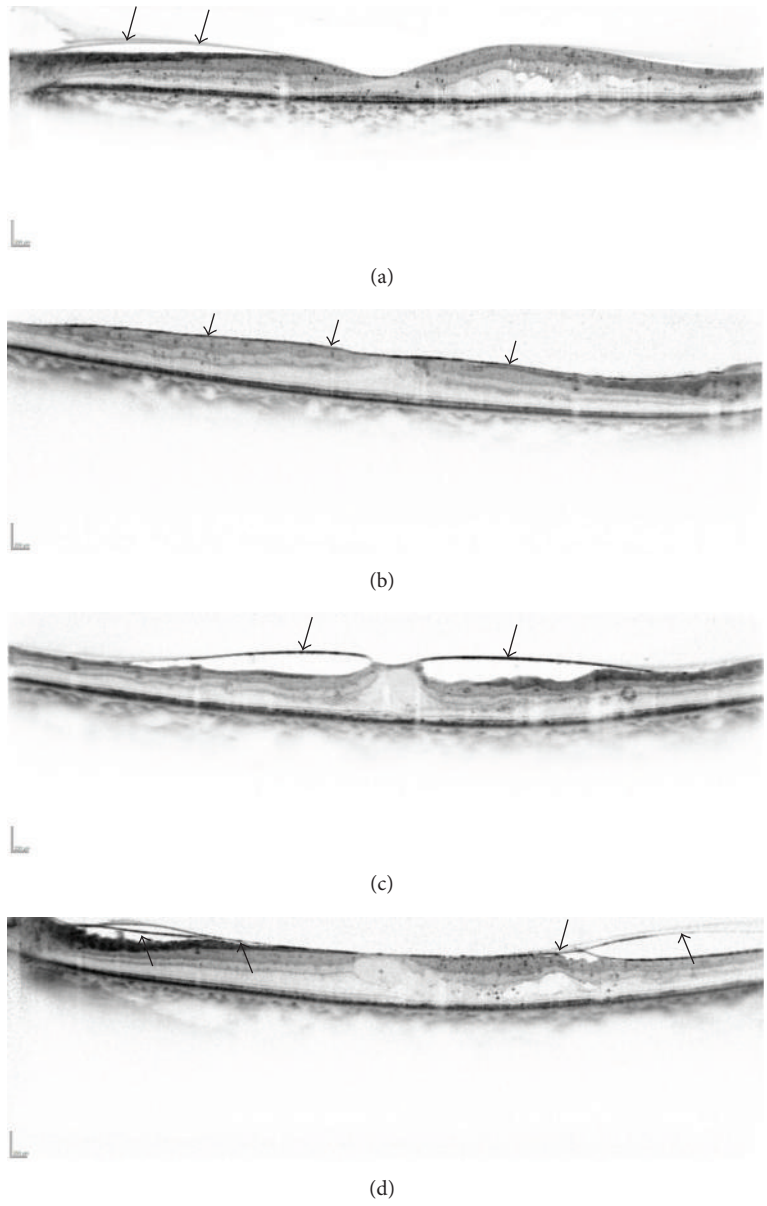
It is not well known how SRD develops in DME compared to the pathogenesis in eyes with CME or sponge-like retinal swelling. Marmor reported numerous clinical and basic data regarding the development of retinal detachment that depended on the osmotic or oncotic pressure of intraocular fluids [50]. In eyes with DME, vascular hyperpermeability might increase such pressures, resulting in SRD. High-resolution OCT has enabled observation of the cystoid spaces in the OPL that sometimes rupture toward the SRD, suggesting that extravasated blood components pour directly into the SRD [51]. Regarding visual function, no association was found between VA and foveal thickness in eyes with foveal SRD [52], whereas these eyes often have a poor prognosis after treatment [51, 53]. OCT often delineates hyperreflective foci in subretinal fluids (Figure 5). In most such cases, subfoveal hard exudates develop after resolution of the macular edema (ME) that correspond to the confluent hyperreflective foci with concomitant impaired visual function [51], as reported in the Early Treatment Diabetic Retinopathy Study [54, 55].

4. Photoreceptor Layers

The superior delineation of the fine structures on SD-OCT images encouraged us to evaluate photoreceptor markers, external limiting membrane (ELM), and the junction between the inner and outer segments (IS/OS) (Figure 6). Sandberg and associates reported that the IS/OS line, to which they referred as the third high-reflectance band in their original manuscript, is associated with visual function in retinitis pigmentosa [56], suggesting that the IS/OS line represents the photoreceptor structure and function *in vivo* [57]. A few years later, disruption of the IS/OS line at the fovea was reported to be associated with a poor visual prognosis in resolved ME associated with branch retinal vein occlusion [58]. Many later cross-sectional or longitudinal studies have shown the clinical relevance of the IS/OS line in DME [59–67]. Histologic publications have reported "cystoid degeneration" in the photoreceptors at the fovea [68], which supports the disruption of the IS/OS line on OCT images. The thickness of the photoreceptor outer segments was quantified and found to be associated with visual function in DME [62]. The transverse length of the disrupted or absent IS/OS line also has been related to visual impairment [63, 66]. In the future, quantification of photoreceptor damage would improve the understanding of visual impairment in DME.

Despite the relevance, it is unknown whether the IS/OS line seen on OCT images truly corresponds to the histologic junction of the inner and outer segments. Spaide and Curcio speculated that this highly reflective band was located at the ellipsoid in the inner segments, considering the correlation between the microstructure on the SD-OCT images and the histologic findings [69]. The OCT reflectivity changed around the line after light exposure, which suggested that the line may represent photoreceptor function per se [70, 71].

The ELM line is another marker of photoreceptor integrity, and its disruption also is associated with visual impairment in DME [52, 66, 67]. Shin and associates reported that ELM disruption predicts poor visual outcomes after treatment with triamcinolone [67]. Since the ELM is an intercellular junction between the Müller cells and photoreceptor



(a)

(b)

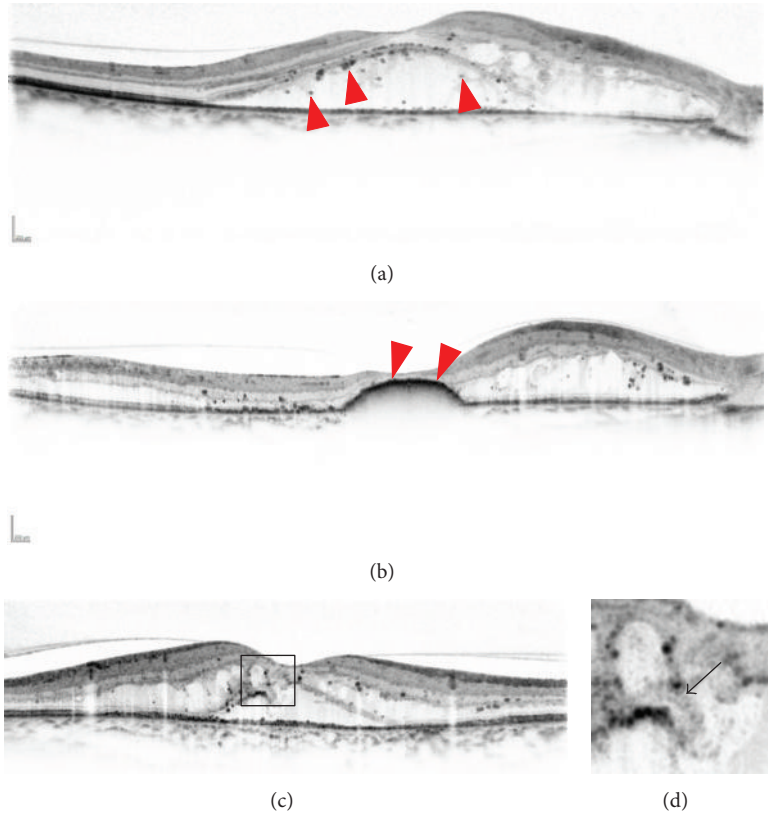
(c)

(d)

Figure 4: Several patterns of vitreomacular interface abnormalities in DME. (a) A posterior hyaloid membrane is sometimes depicted on OCT images (arrows). (b) ERM often is accompanied by sponge-like retinal swelling (arrows). (c) A perifoveal vitreous separation sometimes induces vertical traction, as in idiopathic macular holes (arrows). (d) Some eyes have separation of a posterior vitreous membrane (arrows).

cells and has barrier properties against macromolecules [72], the disrupted ELM might allow blood components to migrate into the outer retinal layers and exacerbate the photoreceptor damage. Although it remains poorly understood how the ELM becomes disrupted, a few possible mechanisms are implicated. Extended cystoid spaces from the INL to the OPL are accompanied by ELM disruption in DME, suggesting disturbance of the Müller cells [66]. A tear in the outer retinal layers also can result in loss of the barrier function in eyes with SRD [51].

SD-OCT shows dot-like lesions, referred to as hyperreflective foci, throughout the retina in DR [35]. Those in the outer layers especially are correlated cross-sectionally with poor visual function in patients with DME without SRD [73]. Hyperreflective foci in subretinal fluids accumulate at the fovea and lead to poor visual prognosis in eyes with SRD [51]. Bolz and associates speculated that the hyperreflective foci in DR corresponded to lipid-laden macrophages and the precursors of hard exudates [35]. The hyperreflective foci also are considered to be degenerated photoreceptors and RPE hyperplasia or metaplasia in other diseases [74, 75]. Although it remains undetermined what the hyperreflective foci are in the outer retinal layers in DME, the disruption of the ELM or IS/OS line is correlated with the hyperreflective foci, suggesting that these lesions reciprocally promote the pathogenesis of photoreceptor degeneration.



(a)

(b)

(c) (d)

Figure 5: A representative case with hyperreflective foci (arrowheads) in subretinal fluids. The preoperative decimal VA is 0.6. (b) DME is improving after focal/grid photocoagulation, although hyperreflective foci have accumulated at the fovea (arrowheads). The postoperative VA is 0.09. (c) OCT sometimes shows that cystoid spaces in the OPL rupture to subretinal fluids (arrow), which might modulate the pathogenesis in SRD.

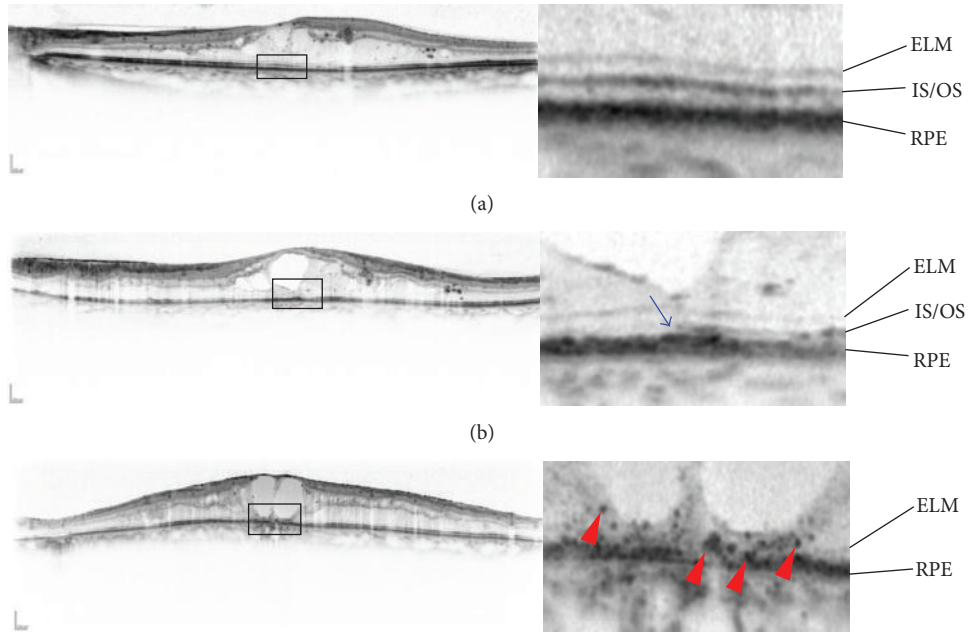


Figure 6: Pathological changes in the photoreceptor layers in DME. (a) Both the ELM and IS/OS lines are continuous. (b) The ELM line seems almost continuous, whereas the IS/OS line is discontinuous on the left (arrow). (c) The ELM line is disrupted, and the IS/OS line is absent at the fovea, accompanied by hyperreflective foci in the outer retinal layers (arrowheads).

5. Ganglion Cells and Nerve Fiber Layers

The nerve fiber layer (NFL) is comprised of axons derived from ganglion cells. The defects in the NFL were clinically reported [76], and basic research showed ganglion cell apoptosis in DR [77]. Since swelling of the NFL occurs in lesions associated with DR, such as cotton-wool spots (soft exudates), it is difficult to evaluate the decrease in the axons from the ganglion cells using OCT. In contrast, thinning of the ganglion cell layer was reported in eyes with ischemic maculopathy with and without DME [78]. Further, glaucoma research has focused on the clinical relevance of the ganglion cell complex (from the inner limiting membrane to the outer boundary of the inner plexiform layer) [79], which should be applied to evaluate neovascular glaucoma in advanced PDR.

It was reported that white spots on fundus photography might correspond to hyperreflective lesions at the level of NFL [80]. Midena and associates have recently demonstrated that hyperreflective spots, which might correspond to activated microglia or Müller cells, were detected in inner retinal layers, as DR progresses. They were suggested to be a novel biomarker of glial activation in DR, and further investigation remains to be planned [81].

6. The Choroid

Disruption of the choroidal circulation in patients with diabetes had been reported as diabetic choroidopathy [82, 83]. Enhanced-depth imaging using SD-OCT or the latest version of OCT, swept-source OCT, recently has enabled measurement of the choroidal thickness. A few publications have reported alteration of the choroidal thickness [84–86] and further investigations will clarify how the pathological choroidal changes contribute to DME.

7. Conclusions

OCT has allowed identification of the morphologic factors in the pathogenesis in DME. The major OCT parameter, the central retinal thickness, is correlated modestly with the VA, and pathomorphologies and photoreceptor damage also cause visual impairment. Further studies of a structural-functional correlation will promote a better understanding of the complex pathogenesis in DME [87].

Abbreviations

CME: Cystoid macular edema
DME: Diabetic macular edema
DR: Diabetic retinopathy
ELM: External limiting membrane
ERM: Epiretinal membrane
FA: Fluorescein angiography
INL: Inner nuclear layer
IS/OS: The junction between inner and outer segments
ME: Macular edema
OPL: Outer plexiform layer
PDR: Proliferative diabetic retinopathy
SD-OCT: Spectral-domain optical coherence tomography
SRD: Serous retinal detachment.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Pronerve Growth Factor Induces Angiogenesis via Activation of TrkA: Possible Role in Proliferative Diabetic Retinopathy

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Proliferative diabetic retinopathy (PDR) is the leading cause of blindness in working age Americans. We demonstrated that diabetes disturbs the homeostasis of nerve growth factor (NGF) resulting in accumulation of its precursor proNGF. Increases in proNGF were positively correlated with progression of diabetic retinopathy, having the highest level in ocular fluids from PDR patients compared to nondiabetic patients. Here, we attempted to evaluate the contribution and the possible mechanism of proNGF to PDR. The angiogenic response of aqueous humor samples from PDR patients was examined in human retinal endothelial cells in the presence or absence of anti-proNGF antibody. Additional cultures were treated with mutant-proNGF in the presence of specific pharmacological inhibitors of TrkA and $p75^{NTR}$ receptors. PDR-aqueous humor samples exerted significant angiogenic response including cell proliferation, migration, and alignment into tube-like structures. These effects were significantly reduced by anti-proNGF antibody but not by IgG. Treatment of retinal endothelial cells with mutant-proNGF activated phosphorylation of TrkA and p38MAPK; however, it did not alter $p75^{NTR}$ expression. Inhibition of TrkA but not $p75^{NTR}$ significantly reduced mutant-proNGF-induced cell proliferation, cell migration, and tube formation. Taken together, these results provide evidence that proNGF can contribute to PDR at least in part via activation of TrkA.

1. Introduction

Diabetic retinopathy (DR) is the leading cause of blindness among working aged adults in the US. It affects 80% of individuals with a 10-year history of diabetes, adding 63,000 new cases of DR each year [1]. DR is characterized by neuro- and vascular degeneration that eventually lead to ischemia and subsequent release of angiogenic growth factors including vascular endothelial growth factor (VEGF) into the vitreous cavity resulting in retinal neovascularization and proliferative diabetic retinopathy (PDR) [2, 3]. PDR is characterized by vitreous hemorrhage, neovascular glaucoma, and tractional retinal detachment, which can result in visual loss [4]. Current treatment options for PDR include laser photocoagulation and anti-VEGF ocular injection, which are invasive and limited by side effects. Repeated injections of anti-VEGF can deprive the retina from the survival actions of VEGF on neurons and vasculature (reviewed in [2, 5]). Therefore, there is a great need to identify contributing factors in PDR other than VEGF; in the hope of devising treatments that will preserve both retina vasculature and neuronal function.

Diabetes-induced oxidative stress disturbs retinal homeostasis by activating glial cells, reducing neurotrophic support, and increasing proinflammatory cytokines including VEGF, IL-1β, and TNF-α [6, 7]. In addition to these known growth factors, recent findings using ocular fluids from diabetic patients and experimental models of diabetes suggest

Table 1: Clinical characteristics of participants providing aqueous humor samples.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
Gender	F	F	F	M	M	F	M
Race	Black	Black	White	White	Black	White	Black
Years of DM	20	26	17	18	16	28	19

that neurotrophins including nerve growth factor (NGF) are emerging as critical mediators of DR [5, 8–11]. NGF is produced by neurons and many nonneuronal cell types such as immune cells, inflammatory cells, and smooth muscle cells [12]. It was originally characterized by its ability to stimulate growth, differentiation, and survival of neurons; however, NGF appears as a pleiotropic modulator of wound healing and reparative angiogenesis [13–15]. NGF activates two different receptors including the high affinity tropomyosin-related receptor A (TrkA), which is a tyrosine kinase, and the low affinity $p75^{NTR}$ neurotrophin receptors ($p75^{NTR}$) [16]. Previous studies demonstrated that the angiogenic response of NGF was mediated via activation of TrkA [15, 17, 18].

NGF is synthesized and secreted by glial cells as the precursor proNGF which is cleaved, by furin intracellularly and by the matrix metalloproteinase-7 (MMP-7) extracellularly, to generate mature NGF [19]. Our studies showed that diabetes-induced peroxynitrite formation impairs maturation of NGF, leading to accumulation of its precursor proNGF both in experimental models and in clinical diabetes [10, 11]. In these studies, we used specific antibodies to detect NGF (13 kDa) and proNGF (32 kDa) rather than ELISA assays that detect both NGF and proNGF. Our results showed that increases in proNGF positively correlated with progression of the disease where ocular fluids from PDR patients showed the higher level of proNGF (5-fold) and lower level of NGF (65% less) compared to nondiabetic samples [10]. Interestingly, earlier studies utilizing ELISA showed higher NGF levels in PDR patients than in controls and nonproliferative diabetic retinopathy (NPDR) patients [9]. Because many NGF antibodies can detect both NGF and proNGF, these increases may reflect the combined presence of both NGF and proNGF. Based on these observations, it appears that proNGF may contribute to development and progression of proliferative diabetic retinopathy clinically. Here, we attempted to evaluate the specific contribution of proNGF to angiogenic response of ocular fluids from PDR patients within retinal endothelial cells and to elucidate the possible role of TrkA and $p75^{NTR}$ in mediating the angiogenic signal.

2. Materials and Methods

2.1. Human Aqueous Humor Samples. Human specimens were obtained with the Institutional Review Board approval from the Human Assurance Committee at Georgia Regents University. Aqueous humor samples were collected from Eye Clinic at Georgia Regents University from patients undergoing intravitreal injections and were identified as being from patients with PDR. Table 1 shows the clinical characteristics of participants providing aqueous humor samples.

2.2. Cell Culture. Human retinal endothelial (HRE) cells and cell culture medium were purchased from Cell Systems Corporations (Kirkland, WA, USA) and VEC Technologies (Rensselaer, NY, USA), respectively. Experiments were performed using cells between passages (4–6) at 37°C in a humidified atmosphere of 5% CO_2. Cells were switched to serum free medium containing 50% of MCDB131 complete medium (VEC Technology, Rensselaer NY) overnight prior to stimulation with aqueous humor samples (10 μL/mL) from various patients in the presence or absence of either anti-proNGF antibody or isotope control rabbit IgG (1 μg/mL). Mutant (cleavage-resistant) proNGF protein and anti-proNGF antibody were purchased from Alomone Labs (Jerusalem, Israel) and IgG was purchased from Cell Signaling Technology (Danvers, MA). For proNGF studies, bovine retinal endothelial (BRE) cells were cultured as described previously [20]. Cells from passages 4 to 8 were used in all experiments. Cells were maintained in M199 supplemented with 10% fetal bovine serum, 10% CS-C complete medium, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified CO_2 incubator. Cells were stimulated with proNGF (50 ng/mL) in the presence or absence of either TrkA antagonist, K252a (0.1 μM) from Calbiochem/EMD Bioscience (La Jolla, CA) or $p75^{NTR}$ selective p75 antagonist A (C30–35, 20 μM), a kind of gift from Uri Saragovi, McGill University, Canada [21].

2.3. Endothelial Cell Migration. HRECs and BRECs were grown to confluence and then were wounded with a single sterile cell scraper of constant diameter as described previously [22]. Images of wounded areas were taken immediately after adding the treatment and after 18 h and % cell migration was calculated. Each condition was verified in triplicate and was repeated using independent cultures.

2.4. Tube Formation. Tube formation assay was performed using growth factor-reduced Matrigel (BD Biosciences) as described previously [23, 24]. HRE cells and BRE cells were counted and plated at 2×10^4 cells/mL with Matrigel in a 96-well plate. Eighteen hours later, images of the tube-like structures were captured and analyzed using Zeiss Axiovert microscope software. Each condition was verified in triplicate and was repeated using independent cultures.

2.5. Endothelial Cell Proliferation. Cells were seeded at a density of 0.5×10^5/mL, switched to medium containing 0.5% FBS, and incubated overnight. Cells were incubated with and without various treatments in medium containing 0.2% FBS for 24 h. After trypsinization, the cell number was determined

using a hemocytometer [23, 24]. Each condition was verified in triplicate and was repeated using independent cultures.

2.6. Western Blot Analysis. For analysis of protein, bovine retinal endothelial cells were homogenized in a modified RIPA buffer from (Millipore, Billerica MA), in the presence of Protease inhibitor cocktail (Sigma Aldrich, St. Louise MO), and Halt Phosphatase inhibitor (Thermo Scientific, Rockford IL). Total protein concentrations were measured using Bio-Rad protein assay. Protein samples (20 μg) were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with the following antibodies: TrkA (Calbiochem/EMD Bioscience (La Jolla, CA)), phospho-TrkA (Santa Cruz Biotechnology, Santa Cruz Biotechnology, Dallas TX), p38MAPK and phospho-p38 MAPK (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-$p75^{NTR}$ provided by Dr. Bruce Carter Vanderbilt University School of Medicine, Nashville, TN, USA) and tubulin (abcam, Cambridge, MA, USA) followed by secondary horseradish peroxidase-conjugated sheep anti-rabbit antibody and enhanced chemiluminescence (Pierce/Thermo Scientific, Rockford IL). The films were subsequently scanned and band intensity was quantified using densitometry software (fluorchem FC2) and expressed as relative optical density to controls.

2.7. Data Analysis. The results are expressed as mean ± SEM. Differences between experimental groups were evaluated by ANOVA and the significance of differences between groups was assessed by the post hoc test (Fisher's protected least significant difference) when indicated. Significance was defined as $P < 0.05$.

3. Results

3.1. PDR-Aqueous Humor Stimulates Cell Migration in a ProNGF-Dependent Manner. Our previous studies have shown that diabetes-induced oxidative stress disturbs the homeostasis of nerve growth factor (NGF) resulting in accumulation of its precursor proNGF at the expense of the mature NGF in diabetic rat [11] and ocular fluids from diabetic patients [10]. Interestingly, the accumulation of proNGF was positively correlated with severity of diabetic retinopathy, where patients identified with proliferative diabetic retinopathy (PDR) showed higher levels (5-fold) of proNGF compared to nondiabetic samples [10]. Here, we examine the angiogenic response of aqueous humor samples from PDR patients using human retinal endothelial (HRE) cells in the presence or absence of anti-proNGF antibody (1 μg/mL). Each aqueous humor sample (total of 100 μL) was tested at least in duplicates on HRE cell culture ($n = 7$). As shown in Figure 1, treatment of HRE cells with PDR-aqueous humor significantly stimulated cell migration by 1.7-fold compared to the control group. Prior treatment of aqueous humor samples with anti-proNGF antibody significantly reduced the stimulatory effect of untreated-aqueous humor on cell migration to 1.2-fold of the control level. Whereas prior treatment with the isotope IgG maintained stimulatory effect

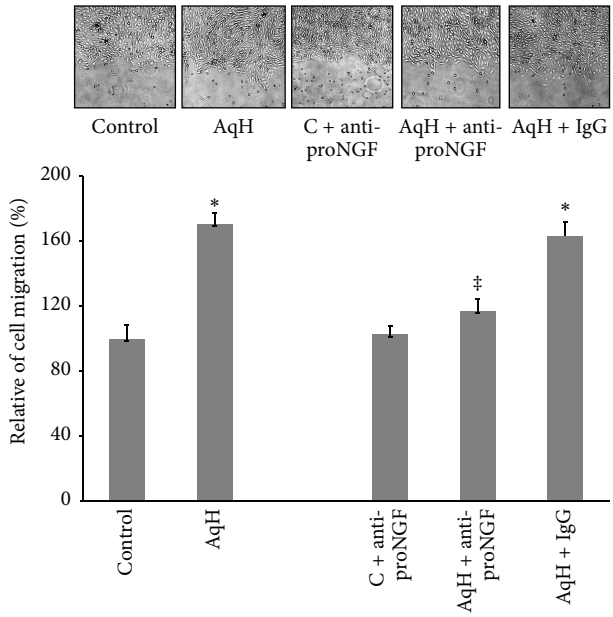

Figure 1: PDR-aqueous humor stimulates cell migration in a proNGF-dependent manner. HRE cells were grown to confluence and then scratched using a standard cell scrapper. Cells were switched to serum free medium and treated with aqueous humor samples (10 μL/mL) in the presence or absence of either anti-proNGF antibody or rabbit IgG (1 μg/mL). Representative micrographs for wounded HME cells are shown after 18 hours of various treatments. Statistical analysis showed that aqueous humor increased mean cell migration by 1.8-fold compared to the control group. Addition of anti-proNGF antibody to aqueous humor samples significantly reduced the mean percent of cell migration to the level of the control group whereas IgG did not significantly impact stimulatory effect of aqueous humor samples. Addition of anti-ProNGF antibody to control HRE cells did not significantly affect percent cell migration compared to control group (*,‡statistically significant compared to control and aqueous humor groups, resp., ($P < 0.05$), n of aqueous humor samples = 7, n of cell cultures = 14–16).

(1.6-fold) of aqueous humor on cell migration. Treatment of control HRE cells with anti-proNGF antibody did not significantly impact cell migration compared to untreated control group.

3.2. PDR-Aqueous Humor Stimulates Tube-Like Structures in a ProNGF-Dependent Manner. We next examined the effects of PDR-aqueous humor on alignment of endothelial cells to tube-like structures. As shown in Figure 2, aqueous humor from PDR patients increased the relative mean tube length by 1.75-fold compared to the control group ($n = 7$). Prior treatment of aqueous humor samples with anti-proNGF antibody (1 μg/mL) blunted the stimulatory effect of aqueous humor on inducing tube formation whereas IgG did not significantly affect tube formation. Meanwhile, treatment of control cells with anti-proNGF antibody did not markedly reduce tube formation compared to untreated control group.

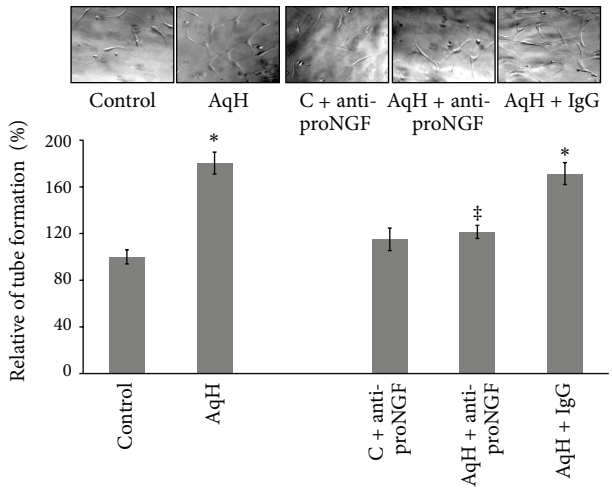

Figure 2: PDR-aqueous humor stimulates tube-like structures in a proNGF-dependent manner. HRE cells were grown into confluence then trypsinized and mixed with reduced-growth factor Matrigel and treated with aqueous humor samples (10 μL/mL) in the presence or absence of either anti-proNGF antibody or rabbit IgG (1 μg/mL). Representative micrographs for alignment of HRE into tube-like structures are shown after 18 hrs of incubation. Statistical analysis of tube length showed that aqueous humor increased mean tube formation 1.7-fold compared to the control group. Addition of anti-proNGF antibodies to aqueous humor samples significantly reduced the relative mean tube length but did not affect control group. Prior treatment of humor samples with rabbit IgG did not significantly reduce relative mean length when compared to the untreated aq. humor group (*,‡statistically significant compared to control and aqueous humor groups, resp., ($P < 0.05$), n of aqueous humor samples = 7, n of cell cultures = 14–16).

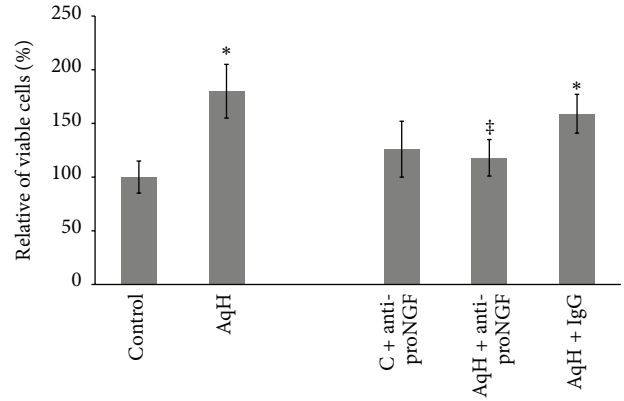

Figure 3: ProNGF and aqueous humor from PDR patients stimulated cell proliferation. HRE cells were grown into confluence then trypsinized and plated as described in method section. Cells were switched to serum free medium and treated with aqueous humor samples (10 μL/mL) in the presence or absence of either anti-proNGF antibody or rabbit IgG (1 μg/mL) for 24 hours then cells were trypsinized and counted. Statistical analysis showed that aqueous humor from PDR patients stimulated cell proliferation by 1.8-fold compared to the control group. Adding anti-proNGF antibody to aqueous humor samples significantly reduced the relative number of proliferating cells while IgG did not. Addition of anti-proNGF antibody to HRE cells did not affect number of proliferating cells (*,‡statistically significant compared to control and aqueous humor groups, resp., ($P < 0.05$), n of aqueous humor samples = 6, n of cell cultures = 12–14).

3.3. PDR-Aqueous Humor Stimulates Cell Proliferation in a ProNGF-Dependent Manner. We next examined the effect of aqueous humor on HRE cell proliferation. As shown in Figure 3, PDR-aqueous humor stimulated cell proliferation by 1.8 compared to the control group ($n = 7$). Prior treatment of aqueous humor samples with anti-proNGF antibody (1 μg/mL) blunted the stimulatory effect of aqueous humor on cell proliferation, whereas prior treatment with IgG did not markedly reduce cell proliferation. Treatment of control cells with anti-proNGF antibody did not affect relative number of proliferating cells compared to untreated control.

3.4. ProNGF Activates TrkA/p38 MAPK in Retinal Endothelial Cells. Previous studies showed that Trk receptors play a key role in mediating the mitogenic and angiogenic response of neurotrophins including NGF and BDNF [17, 25, 26]. Our previous work demonstrated significant upregulation of $p75^{NTR}$ receptor expression in clinical and experimental diabetes [11, 27]. Therefore, we examined the impact of proNGF on activating TrkA and $p75^{NTR}$ receptors in endothelial cells. As shown in Figure 4(a), there was no significant difference in $p75^{NTR}$ expression among various groups. As shown in Figure 4(b), treatment of BRE cells with proNGF (50 ng/mL) stimulated phosphorylation of TrkA. Prior treatment of BRE cells with the TrkA antagonist K252a (0.1 μM) blocked proNGF-mediated TrkA activation confirming the possibility that proNGF can activate TrkA. Interestingly, inhibition of $p75^{NTR}$ using a selective antagonist modestly increased TrkA activation in both control and proNGF-stimulated cells suggesting mutual regulation of the two receptors TrkA and $p75^{NTR}$. We next examined activation of p38 MAPK, and the results showed that proNGF activated p38 MAPK and this effect was abolished with TrkA antagonist (Figure 4(c)). Prior treatment of BRE cells with $p75^{NTR}$ antagonist (20 μM) significantly reduced proNGF-mediated activation of p38 MAPK (Figure 4(c)). These results suggest that proNGF can activate the mitogenic p38 MAPK signal in retinal endothelial cells.

3.5. Inhibiting TrkA Prevents ProNGF-Mediated Retinal Endothelial Cell Proliferation. We next attempted to examine the effects of inhibiting TrkA on the mitogenic and angiogenic function of proNGF. As shown in Figure 5, treatment with the mutant proNGF (50 ng/mL) induced cell proliferation (1.6-fold) compared to untreated control. This effect was blocked by the specific TrkA receptor antagonist K252a (0.1 μM), meanwhile, it was not reduced by $p75^{NTR}$ inhibitor (20 μM). Inhibition of TrkA in control group did not significantly inhibit cell proliferation compared to untreated controls.

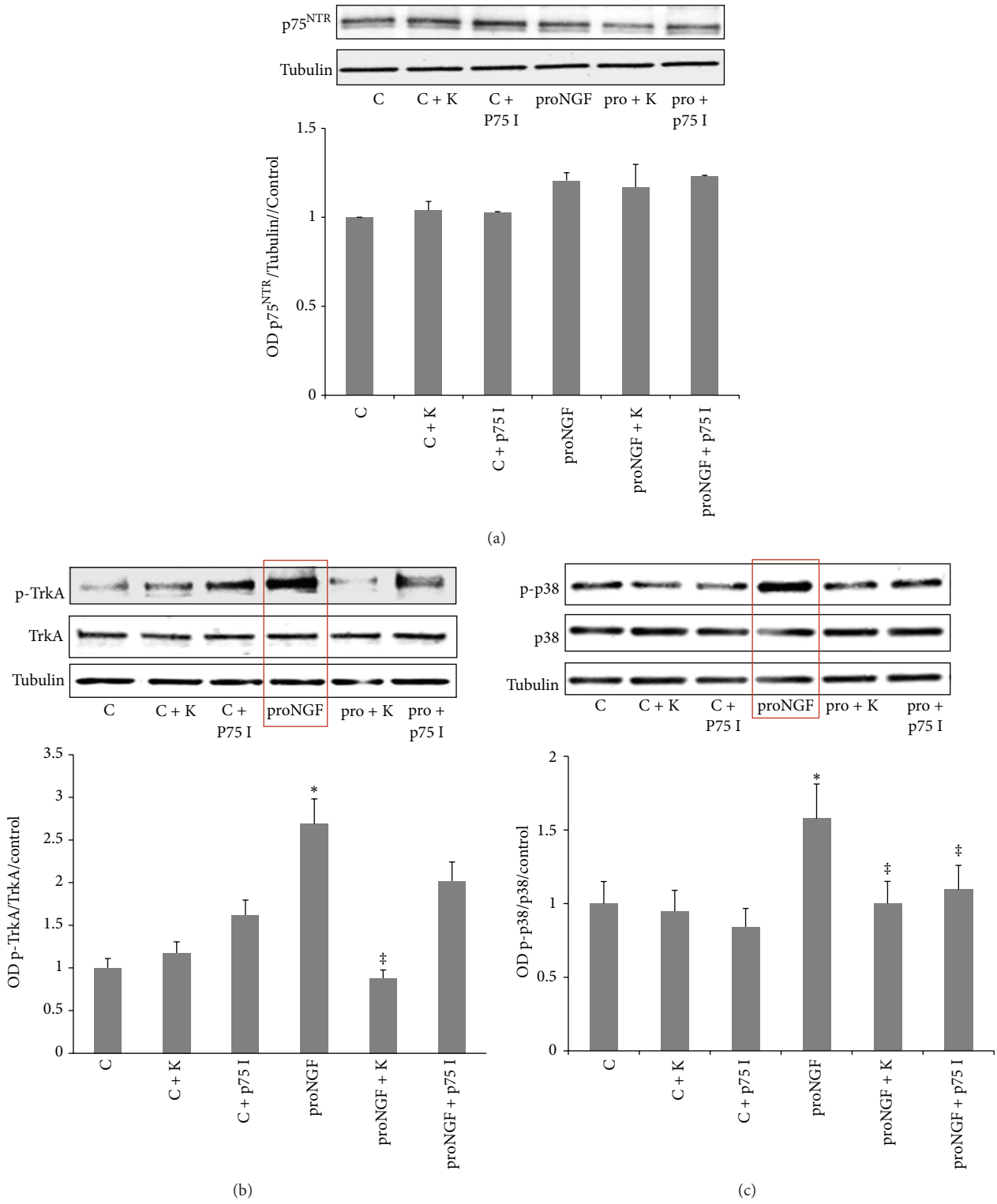

FIGURE 4: ProNGF activates TrkA/p38 MAPK in retinal endothelial cells. BRE cells were grown to subconfluence then switched to serum free medium and treated with mutant proNGF (50 ng/mL). Cells were harvested after 4 hours and subjected to western blot. (a) Representative image of $p75^{NTR}$ and tubulin showing no significant change in $p75^{NTR}$ expression among various groups. (b) Representative image and statistical analysis showed that proNGF is capable of activating TrkA in BRE cells compared to control cells. Treatment of BRE cells with the TrkA antagonist K252a abolished the ability of proNGF to activate TrkA while pharmacological inhibition of $p75^{NTR}$ modestly increased TrkA activation. (c) Representative image and statistical analysis showed that proNGF activates p38 MAPK compared to controls. Inhibiting TrkA or $p75^{NTR}$ abolished the ability of proNGF to activate p38 MAPK (*,‡statistically significant compared to control and proNGF groups, resp., ($P < 0.05$), $n = 3$–5).

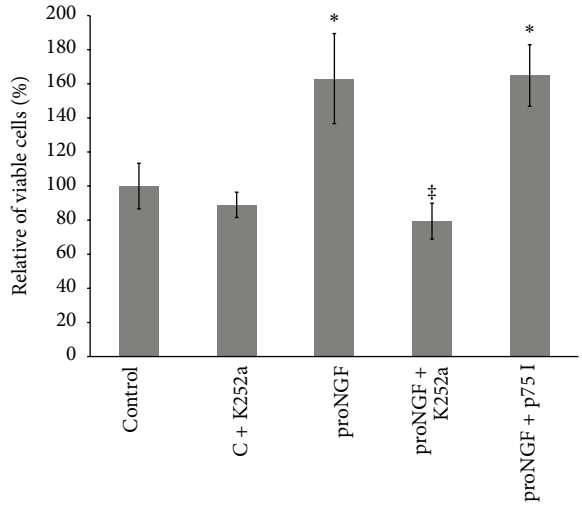

Figure 5: Inhibiting TrkA prevents proNGF-mediated retinal endothelial cell proliferation. BRE cells were grown into confluence and then trypsinized and plated as described in method section. Cells were switched to serum free medium and treated with mutant proNGF (50 ng/mL) in the presence or absence of K252a, TrkA inhibitor (0.1 μM), or p75^{NTR} inhibitor (20 μM) for 24 hours and then cells were trypsinized and counted. Statistical analysis showed that proNGF increased the percentage of proliferated cells by 1.6-fold compared to the control group. This effect was blocked by the specific TrkA receptor antagonist (K252a) but not with p75 inhibitor (*,‡statistically significant compared to control and proNGF groups, resp. ($P < 0.05$), $n = 5$–7).

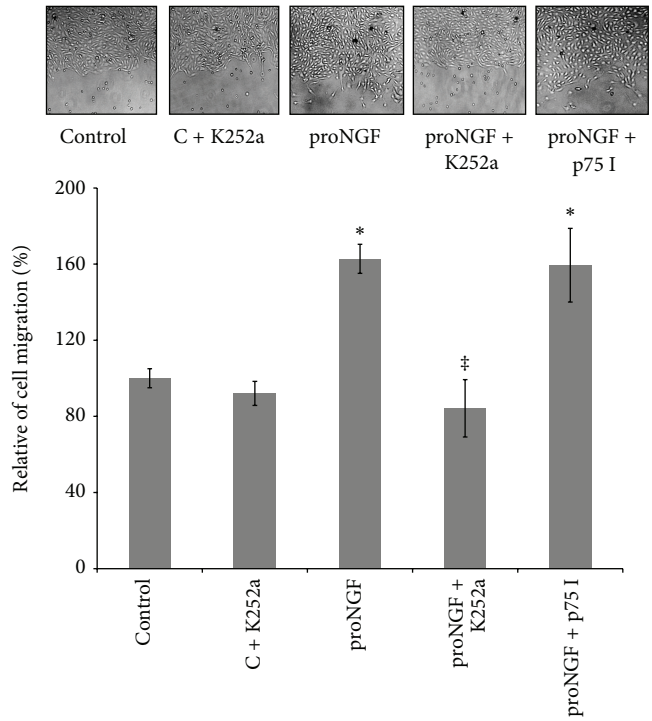

Figure 6: Inhibiting TrkA prevents proNGF-mediated retinal endothelial cell migration. BRE cells were grown to confluence and then scratched using a standard cell scrapper. Cells were switched to serum free medium and treated with mutant proNGF (50 ng/mL) in the presence or absence of either TrkA receptor antagonist, K252a (0.1 μM), or p75 inhibitor (20 μM). Representative micrographs for wounded BRE cells are shown after 18 hours of various treatments. Statistical analysis showed that proNGF increased mean cell migration by 1.98-fold compared to the control group. This effect was blocked by the specific TrkA receptor antagonist (K252a) but not with p75 inhibitor (*,‡statistically significant compared to control and proNGF groups, resp., ($P < 0.05$), $n = 6$).

3.6. Inhibiting TrkA Prevents ProNGF-Mediated Retinal Endothelial Cell Migration. As shown in Figure 6, proNGF (50 ng/mL) increased the relative percentage of BRE cell migration by 1.8-fold compared to the control group. These effects were blocked with the specific TrkA receptor antagonist K252a (0.1 μM), but not with p75^{NTR} inhibitor (20 μM). Inhibition of TrkA in control group did not significantly inhibit cell migration compared to untreated controls.

3.7. Inhibiting TrkA Prevents ProNGF-Mediated Retinal Endothelial Tube Formation. As shown in Figure 7, proNGF (50 ng/mL) stimulated alignment of BRE cells into tube-like structures and tube length by 1.5-fold compared to the control group. This effect was blocked by the specific TrkA receptor antagonist K252a (0.1 μM), but not with p75^{NTR} inhibitor (20 μM). Inhibition of TrkA in control group did not significantly inhibit tube formation compared to untreated controls.

4. Discussion

Although increases in cytokines and growth factors including VEGF, TNF-α, IL-1β, and IL-6 have been well documented in vitreous from diabetic patients [28–30], little is known about the role of proNGF in PDR. The current study was conducted to evaluate the contribution of proNGF to the angiogenic response and to identify the possible underlying mechanisms. The main findings of the current study are that aqueous humor samples from PDR patients stimulate the angiogenic response in HRE cells in a proNGF-dependent manner and that exogenous proNGF mediates proangiogenic action via activation of TrkA/p38 MAPK pathway in retinal endothelial cells. We believe that this study is the first one to demonstrate evidence that proNGF can contribute to PDR and to provide insight into the possible mechanism. Future studies are warranted to further elucidate the complex role of proNGF in angiogenesis.

Angiogenesis/neovascularization can be detrimental in pathological diseases, including diabetic retinopathy, arthritis, and tumor growth, as well as beneficial during wound healing and postischemic repair (reviewed in [31, 32]). Under diabetic conditions, prooxidative stress and pro-inflammatory milieu stimulate apoptosis of retinal vascular endothelial cells and capillary drop out leading to ischemia [6]. Normally to counteract the ischemic condition and salvage injured ischemic tissue, growth of collateral arteries from preexisting arterioles (reparative angiogenesis) is initiated [33]. This reparative mechanism appears to be

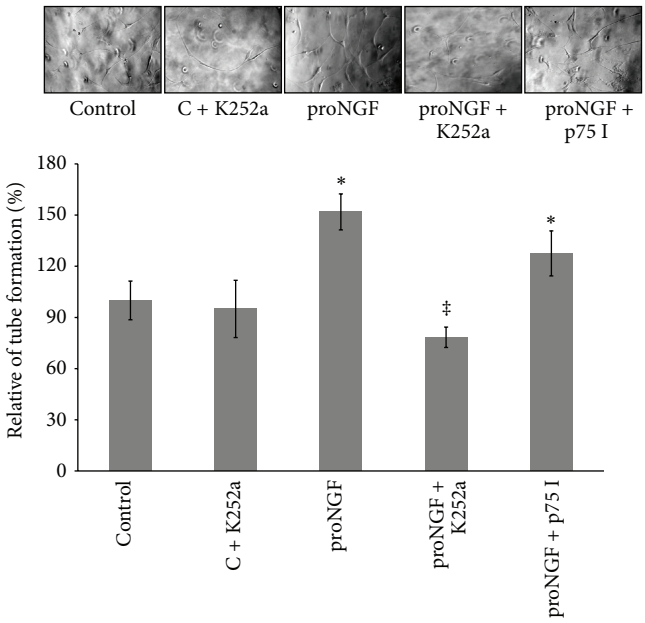

Figure 7: Inhibiting TrkA prevents proNGF-mediated retinal endothelial cell tube formation. BRE cells were grown into confluence and then trypsinized and mixed with reduced-growth factor Matrigel and treated with mutant proNGF (50 ng/mL) in the presence or absence of either TrkA receptor antagonist, K252a (0.1 μM), or p75^{NTR} inhibitor (20 μM). Representative micrographs for alignment of BRE into tube-like structures are shown after 18 hrs of incubation. Statistical analysis of tube length showed that proNGF increased mean tube formation 1.5-fold compared to the control group. This effect was blocked by the specific TrkA receptor antagonist (K252a) but not with p75 inhibitor (*,‡statistically significant compared to control and proNGF groups, resp., ($P < 0.05$), $n = 6$).

impaired in the diabetic retina; however, in an effort to meet the metabolic demand of the retina, sprouting of capillaries and pathological neovascularization is triggered eventually leading to PDR. In response to ischemic stress, several growth factors including NGF are secreted to induce reparative angiogenesis via activation of TrkA receptor, promoting endothelial cell survival and angiogenesis [34]. Prior studies detected NGF at mRNA level or utilized ELISA assays, both of which cannot distinguish NGF from its precursor, and showed a positive correlation of NGF with progression of PDR in human [9, 35] or experimental retinal neovascularization models [25]. NGF is secreted as precursor form (proNGF) that gets cleaved to the mature NGF. Our previous analyses showed that diabetes-induced oxidative stress disturbs the homeostasis of NGF by hampering the cleavage of proNGF resulting in accumulation of proNGF and reducing NGF levels in experimental [11] and ocular fluids from PDR patients [10]. Therefore, it is conceivable that the previously reported increases in NGF are mixed proNGF/NGF rather than NGF alone. So far, researchers have focused on studying angiogenic response of NGF in retinal endothelial cells [18, 25, 36–38]; however, until now no studies have evaluated the possible angiogenic action of proNGF. Therefore, we tested the hypothesis that accumulated proNGF contributes to angiogenic response elicited by ocular fluids from PDR patients. Treatment of HRE cells with aqueous humor samples from diabetic patients stimulated endothelial cell migration, cell proliferation, and tube formation, all of which were inhibited by prior treatment with anti-proNGF antibody but not with rabbit IgG, confirming that proNGF directly contributes to angiogenesis. These results are consistent with the concept that diabetes deprives the retina from the neurotrophic support of NGF and favors accumulation of pro-inflammatory proNGF that can contribute to pathological neovascularization and PDR.

Neurotrophins including NGF, BDNF, and neurotrophin-3 (NT-3) have been extensively studied for their actions on the nervous system. However, recent studies demonstrated the effects of neurotrophins as pleiotropic modulators of wound healing and angiogenesis [13–15, 39, 40]. The angiogenic response was either mediated through direct activation of the corresponding tropomyosin kinase receptor such as TrkA and TrkB in endothelial cells or indirectly via paracrine effects from the release of angiogenic factors from other cells [14, 34, 40]. Our results clearly show that proNGF can induce early activation (within 4 hours) of TrkA in retinal endothelial cells without significant effect on p75^{NTR} expression (Figure 4). Our results also show that inhibiting TrkA activation blocked proNGF-induced angiogenic response in retinal endothelial cells (Figures 5–7). Our results lend further support to a recent study showing that the angiogenic effect of proNGF in cancer cells is exerted mainly via TrkA rather than p75^{NTR} receptor [41]. The inhibitory effect of K252a, staurosporine-related compound [42], on angiogenic response have been demonstrated in several studies [14, 15, 18, 25, 36–38], nevertheless, we believe that our results are the first to demonstrate involvement of TrkA activation in response to proNGF in retinal endothelial cells.

Activation of TrkA leads to its phosphorylation at Tyr^{490}, which recruits the adaptor proteins GRB2-associated binding protein-1 and SH2-containing protein, activating MAPK/ERK kinase, and promotes neurite and endothelial growth [43]. Our results showing that proNGF activates TrkA/p38 MAPK and that inhibition of TrkA significantly inhibited proNGF-mediated cell proliferation, migration, and tube formation lend further support to other studies of the role of TrkA/p38 MAPK promoting cell growth, migration, and invasion of cancer cells [44, 45]. A study in smooth muscle cells showed also that activation of p38 MAPK and ERK was necessary for TrkA-mediated cell proliferation [45].

The p75^{NTR} receptor, a member of the tumor necrosis factor (TNF) receptor superfamily [46], has multiple and cell-specific functions dependent on availability of ligands and coreceptors (reviewed in [47–49]). In the retina, p75^{NTR} is expressed predominately by Müller cells; however, stress can induce expression of p75^{NTR} in other retina cell types including retinal ganglion cells [50] and endothelial cells [26, 51]. ProNGF has great affinity to bind and activate p75^{NTR} along with the sortilin receptor to mediate cell death [52]. We and others have shown that upregulation of proNGF induces p75^{NTR}-mediated retinal neurodegeneration [10, 11, 53, 54];

and inflammation [54, 55] as well as endothelial cell death [26, 51, 56]. Interestingly, in the present study, results showed that inhibition of $p75^{NTR}$ modestly activated TrkA (Figure 4(a)) and did not significantly alter proNGF-induced angiogenic response in retinal endothelial cells (Figures 5–7). These results lend further support to previous work demonstrating that inhibition of $p75^{NTR}$ contributes to endothelial cell survival and inhibition of apoptosis rather than angiogenic function [26, 56]. Recent studies showed that $p75^{NTR}$ played critical role in guiding migration of neuronal precursor cells and repair of vasculature in ischemic stroke model [57, 58]. Another study showed that $p75^{NTR}$ is required for nitric oxide production in pulmonary endothelial cells [59]. As such, the proNGF/$p75^{NTR}$ pathway is more likely involved in paracrine effects of other retina cell types in the diabetic retina rather than direct angiogenic process within endothelial cells. Further studies warrant characterization of the complex signaling pathway of proNGF/$p75^{NTR}$ using in vivo models of retinal angiogenesis.

Conflict of Interests

The authors do not have any commercial associations that might create a conflict of interests in connection with their paper.

Authors' Contribution

Sally L. Elshaer, Mohammed A. Abdelsaid, and Ahmad Al-Azayzih equally contributed to this paper.

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Clinical Study

Retinal Layers Changes in Human Preclinical and Early Clinical Diabetic Retinopathy Support Early Retinal Neuronal and Müller Cells Alterations

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Purpose. To evaluate the changes in thickness of individual inner and outer macular and peripapillary retinal layers in diabetes. *Methods*. 124 subjects (124 eyes) were enrolled: 74 diabetics and 50 controls. Macular edema, proliferative diabetic retinopathy (DR), any intraocular treatment and refractive error > 6 diopters were the main exclusion criteria. Full ophthalmic examination, stereoscopic fundus photography, and spectral domain-OCT were performed. After automatic retinal segmentation (layering) in 5 layers, the thickness of each layer was calculated, and values compared among groups. *Results*. Thirty patients had no DR, 44 patients had non proliferative DR. A significant increase of inner plexiform and nuclear layers was found in DR eyes versus controls ($P < 0.001$). A significant decrease ($P < 0.01$) of retinal nerve fiber layer (RNFL) and at specific sites of retinal ganglion cell layer ($P = 0.02$) was documented in the macula. In the peripapillary area there were no differences between diabetics and controls. *Conclusions*. Decreased RNFL thickness and increased INL/OPL thickness in diabetics without DR or with initial DR suggest early alterations in the inner retina. On the contrary, the outer retina seems not to be affected at early stages of DM. Automatic intraretinal layering by SD-OCT may be a useful tool to diagnose and monitor early intraretinal changes in DR.

1. Introduction

Diabetic retinopathy (DR) is the first cause of visual impairment and blindness in the adult working-age population [1]. For a long period of time, DR has been considered primarily a retinal microvascular disorder caused by the direct effects of hyperglycemia and by the metabolic pathways it activates [2]. Nevertheless, some recent studies have demonstrated that retinal neurodegeneration (the result of a negative balance between neurotoxic and neuroprotective factors) is present even before the development of clinically detectable microvascular damage. Retinal neurodegeneration may therefore represent an early event in the pathophysiology of DR and may anticipate the onset of microvascular changes [2–4]. The term neurodegeneration used in this paper encompasses pathologic phenomena affecting both the pure neuronal component and the glial one. The hypothesis according to which neurodegeneration precedes the vascular one is confirmed by some electrophysiological and psychophysical studies, which show that the alterations are present even before the microvascular damage becomes ophthalmoscopically or angiographically visible. Such retinal function alterations mainly consist in contrast sensitivity loss, altered color perception, and failure of retinal recovery time [5, 6]. Moreover, it has been observed that in diabetic mice the oscillatory potentials of the electroretinogram (ERG) have increased peak latencies and/or reduced amplitudes, suggesting a compromised inner retinal function secondary to neuronal transmission alterations or to the combined loss of amacrine and ganglion cells [7].

During the course of DR, apoptotic cells have been observed in all retinal layers, suggesting the involvement of different types of neurons [8]. Numerous studies have evidenced that diabetes, through the alteration of different

metabolic pathways, induces functional deficits and even the loss of different types of retinal cells which cover from the inner to the outer retinal cells: ganglion cells, bipolar cells, amacrine cells, horizontal cells, and eventually photoreceptors [9].

Different authors reported a decrease in retinal thickness in diabetic eyes with or without clinical signs of DR compared to normal subjects [10–13]. Biallosterski et al. found a significant reduction in pericentral macular thickness in 53 diabetic patients with mild nonproliferative DR [10]. Van Dijk et al. have demonstrated by spectral domain optical coherence tomography (SD-OCT) a decrease in the inner retinal thickness in the macula in diabetics with mild DR, suggesting that this phenomenon might be firstly due to ganglion cells loss in the pericentral areas and secondly to retinal nerve fiber layer (RNFL) thinning in the peripheral macula [14, 15].

The main purpose of this study was to identify in vivo, by SD-OCT, the changes in thickness of selected retinal layers both in the macula and the peripapillary area in diabetic patients without DR or with early stages of DR (mild and moderate nonproliferative DR) versus normal subjects.

2. Material and Methods

One hundred twenty-four subjects (74 diabetic patients and 50 normal subjects) were included in this study. One eye of each subject was used for the spectral domain optical coherence tomography (SD-OCT) analysis. The exclusion criteria were as follows: proliferative DR, macular edema, any type of previous retinal treatment (macular laser photocoagulation, vitrectomy, intravitreal steroids, and/or antiangiogenic drugs), any intraocular surgery, refractive error > 6D, previous diagnosis of glaucoma, ocular hypertension, uveitis, other retinal diseases, neurodegenerative disease (e.g., Alzheimer's, Parkinson's, and dementia), and significant media opacities that precluded fundus examination or imaging.

A written consent form was obtained from all patients as well as the approval from our institutional ethics committee. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

Each subject underwent a complete ophthalmic examination, with determination of best corrected visual acuity, anterior segment examination, Goldman applanation tonometry, indirect ophthalmoscopy, and 90D lens biomicroscopy. Then, SD-OCT and fundus photography were obtained.

2.1. Study Procedures

2.1.1. Visual Acuity. Best corrected distance visual acuity (BCVA) for each eye was measured by a trained examiner using standard Early Treatment Diabetic Retinopathy Study (ETDRS) protocol at 4-meter distance with a modified ETDRS distance chart transilluminated with a chart illuminator (Precision Vision) [16]. Visual acuity was scored as the total number of letters read correctly and converted to the logarithm of the minimum angle of resolution (logMar).

2.1.2. Fundus Photography. Color stereoscopic fundus photographs (7 ETDRS fields) were taken after an adequate dilatation by a trained photographer using the same TOPCON TRC 50IA 35 degree fundus camera (TOPCON, Tokyo, Japan). Diabetic retinopathy was graded as no DR and as nonproliferative DR mild or moderate DR (NPDR) by two independent graders experienced in grading DR.

2.1.3. Spectral Domain OCT. All eyes were examined with spectral domain optical coherence tomography (SD-OCT, RS-3000, NIDEK, Gamagori, Japan). This instrument has a light source of 880 nm wavelength. Each eye was examined, after pupillary dilation, both in the macula and the peripapillary area. The following scanning protocols were used: "Macula Map" in the macula and "Disc Circle" in the peripapillary area.

The Macula Map scan pattern evaluates 6×6 mm area centered on the fovea with 64 horizontal B-scan lines, each consisting of 1024 A-scans per line. For each SD-OCT linear scan, an automatic algorithm has individuated 5 different retinal layers based on the different shades of gray corresponding to the reflectivity indexes of each layer, which include from inside to outside the following: inner limiting membrane + nerve fiber layer (ILM + RNFL); ganglion cell layer + inner plexiform layer (GCL + IPL); inner nuclear layer + outer plexiform layer (INL + OPL); outer nuclear layer + external limiting membrane (ONL + ELM); and inner segment/outer segment photoreceptor layer + retinal pigment epithelium (IS/OS + RPE). Retinal thickness was automatically calculated in the 9 ETDRS areas (consisting in a central circular zone with a 1-mm diameter, representing the foveal area and inner and outer rings of 3 and 6 mm diameter, resp.). The inner and the outer rings are divided into four quadrants: superior, nasal, inferior, and temporal. Mean retinal thickness and mean thickness of each of the five retinal layers in each of the nine ETDRS subfields were recorded (Figures 1(a) and 1(b)).

In the peripapillary area, a circle scan centered on the optic disc (3.46 mm diameter, "Disc Circle" option) was used. Peripapillary retinal thickness was automatically measured by the instrument in the temporal, superior, nasal, and inferior quadrants (Figure 1(c)).

If any instrument error in the automatic segmentation of retinal layers was documented, the manual correction consisted in the repositioning into proper place of the incorrectly placed points (using high magnification images), in order to redefine the retinal profile. Each grader was blinded to clinical data of all examined eyes.

2.1.4. Statistics. Age, spherical equivalent, IOP, and visual acuity were compared among groups by means of analysis of variance (ANOVA); The mean values of retinal layers' thickness in each group, both in macula and peripapillary area, were confronted using the repeated measures analysis of variances (ANOVA-RM). In cases of significant results ($P < 0.05$), the ANOVA-RM was followed by the Bonferroni multiple comparisons post hoc test. All statistical analyses

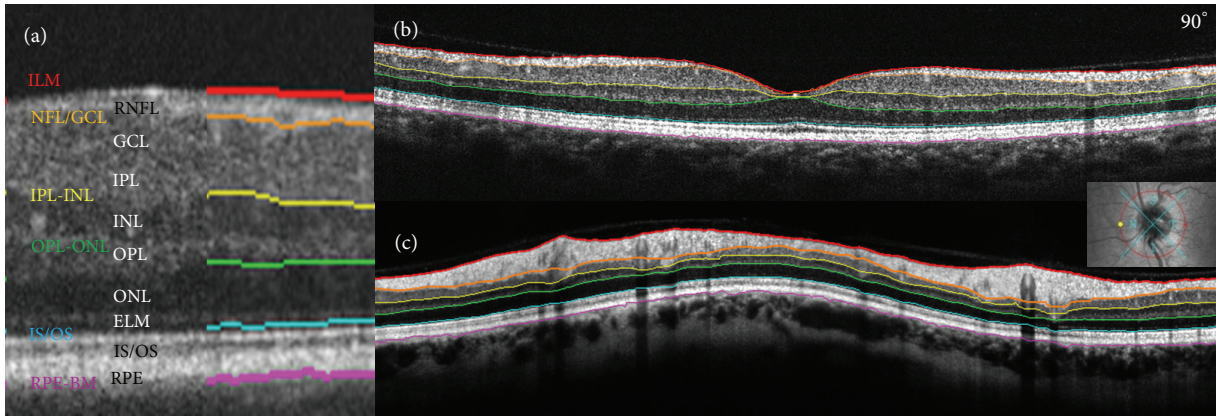


Figure 1: Spectral domain OCT automatic segmentation of retinal layers in the macula ((a) and (b)) and in the peripapillary area (c). In the macula the segmentation is performed on the linear scan (b) and in the peripapillary area on the circular scan around the optic disc (c). Six lines determine 5 retinal layers which from inside out are as follows: inner limiting membrane + nerve fibre layer (ILM + RNFL); ganglion cell layer + inner plexiform layer (GCL + IPL); inner nuclear layer + outer plexiform layer (INL + OPL); outer nuclear layer + external limiting membrane (ONL + ELM); and inner segment/outer segment photoreceptor layer + retinal pigment epithelium (IS/OS + RPE) (a).

Table 1: Demographic characteristics of the patients.

	Control	Diabetic	Grade of DR	
			No DR	NPDR
Patients, number	50	74	30	44
Mean age, yrs (SD)	55.8 (13.0)	56.4 (12.7)	56.1 (12.8)	55.9 (12.6)
Mean diabetes duration, yrs (SD)	—		5.9 (4.1)	18.6 (10.3)
Mean HbA1c % (SD)	—	8.1 (1.4)	7.8 (0.8)	8.2 (1.5)
Visual acuity, logMAR (SD)	0.003 (0.020)		0.011 (0.039)	0.024 (0.066)
IOP, mmHg (SD)	15.3 (1.9)		16.1 (3.4)	16.8 (3.2)
SE, (SD)	−0.04 (1.5)		0.46 (1.1)	0.42 (1.0)

SD: standard deviation; No DR: diabetic patients without retinopathy; NPDR: non proliferative diabetic retinopathy; IOP: intraocular pressure; SE: spherical equivalent.

were performed with SAS 9.2 for Windows, SAS (Cary, NC, USA).

3. Results

Of 124 enrolled subjects 74 were diabetics, (49 males and 25 females). Of 50 normal subjects, 21 were males and 29 females. Mean age of diabetics was 56.4 ± 12.7 years (range: 31–83 years); mean age of controls was 55.8 ± 13 years (range: 25–80 years). Thirty eyes were graded as no DR and 44 eyes as nonproliferative DR (NPDR). Eighteen patients (24.32%) had type 1 DM and 56 (75.68%) had type 2 DM. Mean HbA1c was 8.1% (range: 5.3%–11%). There was no significant difference in age (ANOVA, $P = 0.98$), spherical equivalent (ANOVA, $P = 0.12$), IOP (ANOVA, $P = 0.4$), and visual acuity (ANOVA, $P = 0.5$) among controls, no DR, and NPDR groups (Table 1).

In the macula, ILM + RNFL thickness was significantly decreased in the superior outer quadrant (SOM, $P < 0.0001$), inferior outer quadrant (IOM, $P < 0.0001$), temporal outer quadrant (TOM, $P = 0.01$), nasal outer quadrant (NOM, $P = 0.0003$), superior inner quadrant (SIM, $P = 0.0003$), and inferior inner quadrant (IIM, $P = 0.01$) in no DR group versus controls. RNFL thickness was significantly decreased in the SOM ($P < 0.0001$), NOM ($P = 0.0003$), IOM ($P = 0.001$), TOM ($P = 0.01$), and SIM ($P = 0.003$), in the NPDR group versus controls (Figure 2(a)).

GCL/IPL thickness was not statistically significantly different between diabetics and controls, although there was a trend toward decreasing thickness in no DR group versus controls in the inner and outer rings' quadrants. GCL/IPL thickness was significantly decreased only in the NOM and SOM ($P = 0.02$, for both) in diabetics with no DR versus NPDR group (Figure 2(b)).

INL/OPL thickness was significantly increased in the central OCT subfield (CSF, $P = 0.004$), SIM ($P = 0.003$), NIM ($P = 0.04$), TIM ($P = 0.0018$), SOM ($P = 0.002$), IOM ($P = 0.04$), and TOM ($P = 0.001$) in the NPDR group versus controls (Figure 2(c)).

There was no difference in the ONL/ELM and IS/OS – RPE thickness between diabetics with and without DR and controls (Figures 2(d) and 2(e)).

In the peripapillary area, retinal thickness was significantly decreased with increasing age ($P = 0.0021$) and in

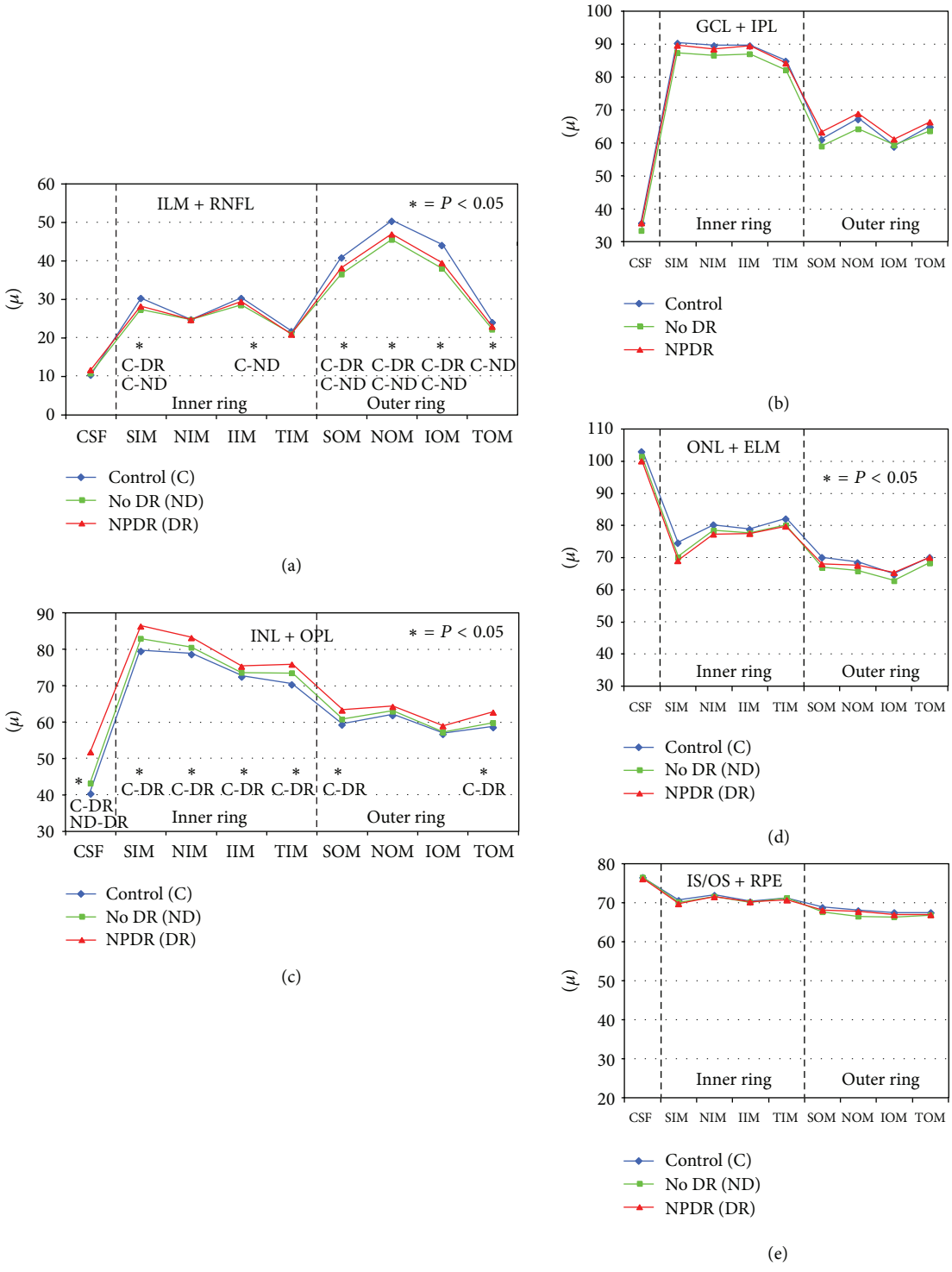


Figure 2: Graphs showing specific retinal layer thickness in normal subjects (control), diabetics without retinopathy (no DR), and diabetics with nonproliferative diabetic retinopathy (NPDR) determined automatically by spectral domain OCT in 9 ETDRS areas in the macula. (a) Inner limiting membrane + nerve fibre layer (ILM + RNFL); (b) ganglion cell layer + inner plexiform layer (GCL + IPL); (c) inner nuclear layer + outer plexiform layer (INL + OPL); (d) outer nuclear layer + external limiting membrane (ONL + ELM); and (e) inner segment/outer segment photoreceptor layer + retinal pigment epithelium (IS/OS + RPE). (∗) indicates statistically significant values; CSF: central subfield thickness; SIM: superior inner quadrant in the macula; NIM: nasal inner quadrant; IIM: inferior inner quadrant; TIM: temporal inner quadrant; SOM: superior outer quadrant in the macula; NOM: nasal outer quadrant; IOM: inferior outer quadrant; TOM: temporal outer quadrant.

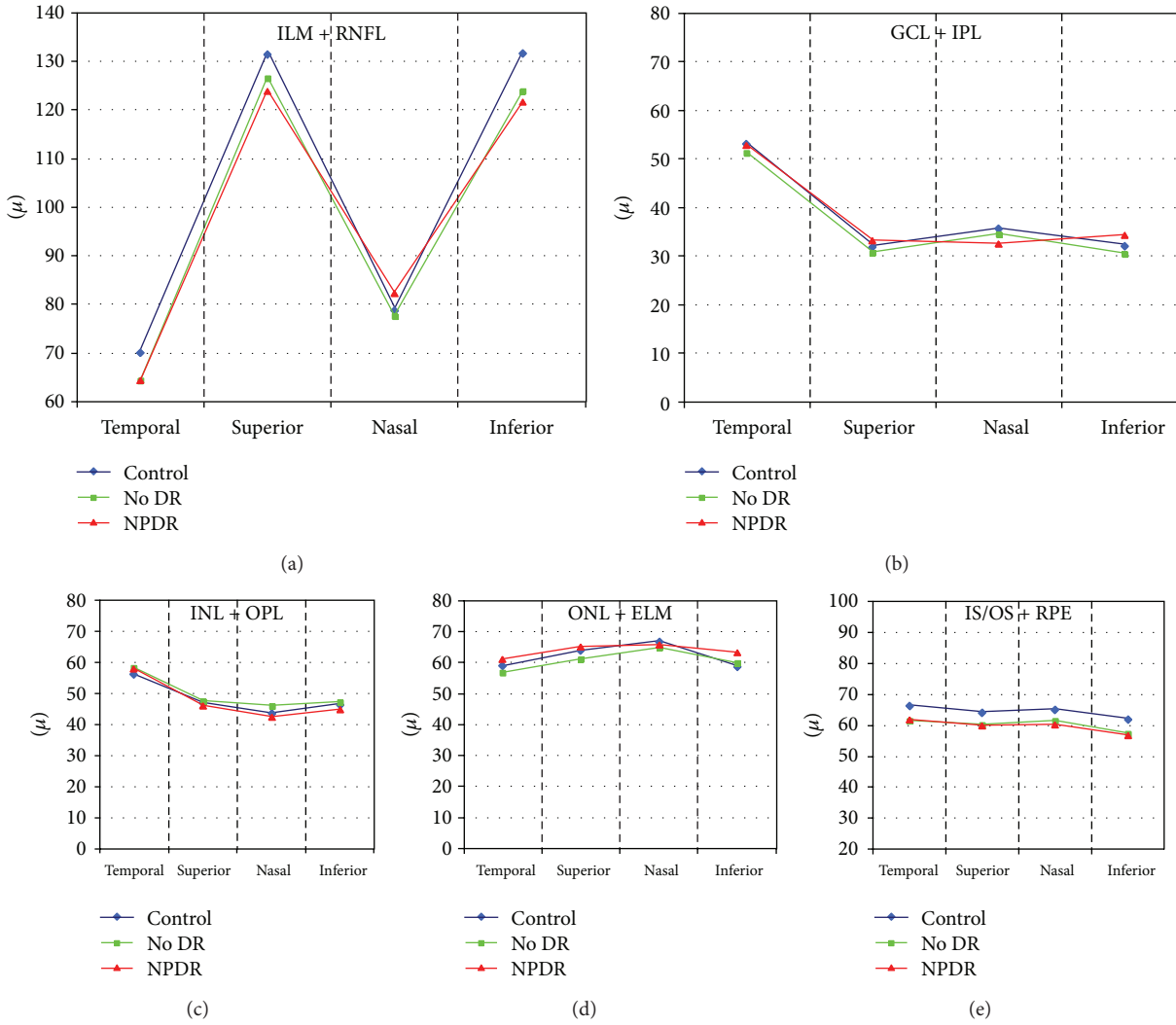


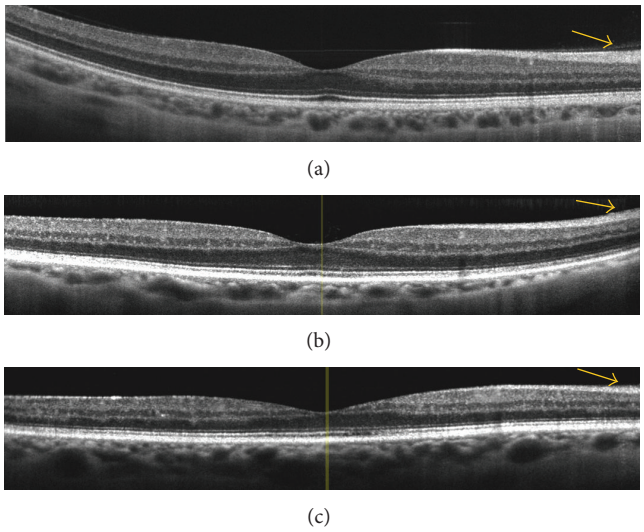
Figure 3: Graphs showing specific retinal layer thickness in normal subjects (control), diabetics without retinopathy (no DR), and diabetics with nonproliferative diabetic retinopathy (NPDR) determined automatically by spectral domain OCT in 4 peripapillary areas (temporal, superior, nasal, and inferior). (a) Inner limiting membrane + nerve fibre layer (ILM + RNFL); (b) ganglion cell layer + inner plexiform layer (GCL + IPL); (c) inner nuclear layer + outer plexiform layer (INL + OPL); (d) outer nuclear layer + external limiting membrane (ONL + ELM); and (e) inner segment/outer segment photoreceptor layer + retinal pigment epithelium (IS/OS + RPE). There is no significant difference in the retinal layer thickness among the controls, no DR, and NPDR groups.

males versus females ($P = 0.0004$) in both controls and diabetics.

ILM + RNFL thickness was significantly different in 4 quadrants, thicker in the superior and inner quadrants, and thinner in the nasal and temporal quadrants. There was no significant difference in the ILM + RNFL, GCL/IPL, INL/OPL, ONL/ELM, and IS/OS – RPE thickness between controls and diabetics (Figure 3).

4. Discussion

In this study we report a decrease in RNFL thickness in the macula of diabetic eyes even without any clinical sign of retinopathy (Figure 4). Reduced RNFL thickness may be explained by progressive ganglion cells and astrocytes loss induced by diabetes. It may depend on a direct toxicity of hyperglycemia or on Müller cells dysfunction, which are unable to maintain an adequate osmotic equilibrium between the intra- and the extracellular matrices with consequent apoptosis of neuronal cells and progressive axonal degeneration [2, 9, 17–20]. Different authors have reported the thinning of RNFL and, in some cases, of the GCL + IPL complex, suggesting that retinal neurodegeneration is an early event in diabetes mellitus, representing a preclinical stage of DR [15, 21, 22]. In fact, the decrease of RNFL thickness in the superior macular region in diabetics without DR or with minimal signs of DR has been documented in vivo [23–26]. Lonneville et al. have demonstrated that RNFL thickness decreases with poor metabolic control in diabetics with or



(a)

(b)

(c)

Figure 4: Spectral domain OCT linear scans in the macula of (a) normal subject, (b) diabetic patient without retinopathy, and (c) diabetic patient with mild nonproliferative diabetic retinopathy. The arrows indicate a progressive thinning of the retinal nerve fiber layer in diabetics (without and with retinopathy) versus normal subjects.

without clinically detectable DR [27]. In this study, we did not find significant difference in RNFL thickness between diabetics without DR and with NPDR. This is probably due to the fact that all our patients were at early stages of DR and had quite good metabolic control. In this study we did not find statistically significant difference in the thickness of GCL/IPL between diabetics and controls, although there was a trend of decreasing GCL/IPL thickness in diabetics without DR versus controls in the pericentral macula. This may be due to the small differences in the specific layer thickness, thus suggesting a more numerous study population.

The INL and the OPL showed increased thickness in diabetic patients with NPDR versus controls in this study. The INL is mainly formed by the nuclei of bipolar and Müller cells and by the association of horizontal and amacrine cells. Different experimental studies have reported an activation of Müller cells with consequent hypertrophy in the earlier stages of diabetic retinopathy [28–32]. No histopathologic studies have reported changes in OPL thickness in the early stages of diabetes mellitus. Therefore, the INL/OPL thickening would be mostly due to the changes in INL thickness. INL thickening, never previously reported in vivo, may represent a sign of Müller cells activation which is represented by significant hypertrophy of these cells. Müller cells are particularly susceptible to hyperglycemia and are recognized as key elements in the onset and the progression of retinal damage induced by hyperglycemia [33]. Diabetes induces hypertrophy (swelling) of Müller cells with a limited impact on the apoptotic cascade [34, 35]. Metabolic and morphological alterations of Müller cells induce secondary progressive neuronal loss, due to the crucial role of Müller cells in mediating relationship between retinal vessels and neurons [36–38]. Carrasco et al. have proven that both apoptosis and glial activation precede microvascular lesions, although it is still not known which one of these two events appears first [39, 40]. Müller cells become hyperplastic in DM, with an increasing number of nuclei, as histopathologically demonstrated. In fact, the number of cell nuclei is increased in the INL and reaches a multiplication factor of 1.6 times, at 20 weeks of DM [28].

There was not a significant difference in ONL/ELM thickness between diabetics without DR and normal subjects. In diabetics with retinopathy the ONL/ELM was reduced just in the superior macular quadrants. The photoreceptor/RPE layer was not different in thickness between diabetics and normal subjects. Therefore, it seems that outer retina is not significantly influenced by diabetes at least in the early stages of disease, whereas the inner retina is precociously affected.

In the peripapillary area, although RNFL thickness was reduced in diabetics versus controls, it did not reach statistical and clinical significance, probably due to the fact that in this area small changes are more difficult to be clinically detected because of the high density of retinal nerve fibers [41]. The automatic segmentation of SD-OCT used in this study, although not able to identify any single retinal layer, but rather layers by couple, can be easily used in both the macula and the peripapillary region for the inner and outer retinal thickness analysis in normal subjects and in diabetic eyes. Its use in a more advanced cases of diabetic retinopathy, mostly in macular edema, needs to be further validated. A detection of retina layer thickness changes in diabetic patients without retinopathy or at early stages of retinopathy may also help in the early diagnosis of retinal tissue loss in DM and to better elucidate the pathophysiology of this severe chronic disease. Moreover, as inner and outer retinas appear differently affected, it seems crucial to have the possibility to evaluate the different retinal layers separately.

In conclusion, the thinning of the inner neural retina in diabetic patients without clinically detectable retinopathy and with mild and moderate nonproliferative retinopathy without macular edema is confirmed in vivo using SD-OCT. Retinal thinning is mainly due to the selective thinning of inner retinal layers in the central retina, strongly suggesting an early neuronal loss in DR. The neuronal loss is accompanied (or induced) by Müller cells activation, with increasing thickness in the INL. Automatic intraretinal layering by SD-OCT may be a useful tool to diagnose and monitor early intraretinal changes in diabetic retinopathy.

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Research Article

Fortified Extract of Red Berry, *Ginkgo biloba*, and White Willow Bark in Experimental Early Diabetic Retinopathy

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Diabetic retinopathy is a complex condition where inflammation and oxidative stress represent crucial pathways in the pathogenesis of the disease. Aim of the study was to investigate the effects of a fortified extract of red berries, *Ginkgo biloba* and white willow bark containing carnosine and α-lipoic acid in early retinal and plasma changes of streptozotocin-induced diabetic rats. Diabetes was induced by a single streptozotocin injection in Sprague Dawley rats. Diabetics and nondiabetic (control) rats were treated daily with the fortified extract for the ten days. Retina samples were collected and analyzed for their TNF-α and VEGF content. Moreover, plasma oxidative stress was evaluated by thiobarbituric acid reacting substances (TBARS). Increased TNF-α and VEGF levels were observed in the retina of diabetic rats. Treatment with the fortified extract significantly lowered retinal cytokine levels and suppressed diabetes-related lipid peroxidation. These data demonstrate that the fortified extract attenuates the degree of retinal inflammation and plasma lipid peroxidation preserving the retina in early diabetic rats.

1. Introduction

Diabetic retinopathy, a diabetes-related complication, is the leading cause of blindness and visual impairment in working-age individuals [1]. Diabetic retinopathy is a chronic disease that develops in stages and is rarely detected in the first few years of diabetes. The incidence of the disease increases to 50% by 10 years and to 90% by 25 years of diabetes [1].

Oxidative stress appears to be an important feature of the diabetic complications such as retinopathy. Apart from the well-known increase in lipid peroxide, diabetics have lower concentrations of erythrocyte glutathione and have higher concentrations of dehydroascorbate in their plasma and lower levels of vitamin E in their platelets. Oxidative stress causes a production of chemically reactive molecules, which induce a variety of proinflammatory mediators such as VEGF and TNF-α [2, 3]. The earliest changes detectable in diabetic retinopathy are loss of pericytes, capillary basement membrane thickening, edema, and formation of microaneurysms. These structural and functional changes are followed by microvascular occlusion, neovascularization, and neurodegeneration [4].

Considering that oxidative stress and inflammation represent the key factors in the onset and progression of diabetic retinopathy, antioxidant and anti-inflammatory products are expected to produce significant therapeutic advantages.

Current treatments associated with antidiabetic drugs are mostly intended to regulate vascular changes, inflammation, and the increased oxidative stress. Dietary supplements have been shown to play an important role in ameliorating clinical signs of diabetes [5]. Many studies have identified flavonoids that are associated with a reduction in the risk of advanced retinal degeneration. Recently [6], it has been demonstrated that eriodictyol, a strong antioxidative flavonoid extracted from *Eriodictyon californicum*, significantly reduces the retinal levels of VEGF, ICAM-1, TNF-α, and eNOS in diabetic rats.

Much evidence supports the roles of berry extracts, α-lipoic acid, and L-carnosine as antioxidant and anti-inflammatory products [5, 7, 8]. It has been demonstrated

that supplementation with these compounds can inhibit retinal diabetes-induced abnormalities [5]. In particular, α-lipoic acid can scavenge free radicals and can act as an antioxidant [9]. Alpha-lipoic acid acts both directly, by radical quenching and metal chelation, and indirectly through the recycling of other antioxidants such as ascorbic acid, vitamin E, and glutathione [9]. Alpha-lipoic acid has been shown to protect against cataract formation [10, 11], a diabetic complication resulting from polyol accumulation, and it is beneficial also in a rat model of diabetic retinopathy [12]. Alpha-lipoic acid attenuates apoptosis in the retinal capillary endothelial cells of rats and decreases the levels of oxidative stress markers such as 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine [13]. Furthermore, Kowluru et al. [14, 15] demonstrated that its supplementation completely prevents the diabetes-induced increase in nitrotyrosine and activation of NF-κB, while decreasing the levels of VEGF in the rat retina [14, 15]. Multiple biochemical pathways that are known to increase the production of reactive oxygen species (ROS), advanced glycosylation end products (AGEs), and reactive nitrogen species (RNS) have been linked to hyperglycemia/diabetes-induced vascular injury [16].

L-Carnosine, a dipeptide primarily produced in skeletal muscle and the central nervous system, acts both as a scavenger of ROS and as an inhibitor of AGE production [8]. L-carnosine is one of the most abundant antioxidants in the brain and retina [8]. Carnosine is synthesized from β-alanine and histidine and is specifically degraded by selective enzymes such as carnosinase-1 (CN-1). It has been suggested that the carnosine/carnosinase system plays an important role in the pathogenesis of diabetic complications [8]. Red berries are rich in phenolic compounds as well as many other essential nutritional components, such as flavonoids and phenolic acids, which have a wide range of beneficial properties, including retinal protection [17]. Epidemiologic evidence suggests that a high consumption of flavonoids may be useful against coronary heart disease, stroke, and neurodegenerative disorders [18, 19]. The high level of scavenging activity of red berry extracts toward chemically generated reactive oxygen species has been described in several studies [18]. *Salix alba* (white willow bark) extract is used for anti-inflammatory medical treatments due to its ability to suppress prostaglandin synthesis. The main component of *Salix alba* is salicin, an analogue of the widely used acetyl salicylic acid [20]. Two trials investigating the effects of *Salix alba* found evidence that daily doses standardized to 120 mg or 240 mg of salicin were better than placebo for short-term improvements in pain and rescue medication [20, 21].

Ginkgo biloba leaf extract (GBE) contains many different flavone glycosides and terpenoids [22]. It is well known that GBE has an antioxidant action as a free radical scavenger, and an anti-inflammatory effect suppressing the production of active oxygen and nitrogen species [22]. GBE inhibits the increase in the products of the oxidative decomposition of low-density lipoprotein (LDL), reduces the cell death in various types of neuropathy, and prevents the oxidative damage to mitochondria, suggesting that its beneficial effects on neurodegenerative diseases are related to prevention of chronic oxidative damage [23].

In the present study, we investigated the effect of systemic treatment with a fortified extract (FE) on proinflammatory mediators (TNF-α and VEGF), in the diabetic rat retina. Moreover, we evaluated plasma oxidative stress by measuring the thiobarbituric acid reacting substances (TBARS) [24].

2. Materials and Methods

2.1. Animals and Reagents. Male Sprague Dawley rats (approximately 200 g) were obtained from Charles River (Calco, Italy). All the animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Directive 2010/63/EU of the European Parliament and of the Council. The animals were fed on standard laboratory food and were allowed free access to water in an air-conditioned room with a 12 h light/12 h dark cycle. Final group sizes for all measurements were $n = 8$–10. STZ was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were purchased from standard commercial suppliers unless otherwise noted.

2.2. Induction of Diabetes and Treatment Schedule. STZ acts by producing concentrations of peroxides greater than can be tolerated by the islets of Langerhans, since these are poor in glutathione peroxidase. The induction of diabetes was performed as previously described [24]. Briefly, the animals received a single injection (iv) of STZ (60 mg/kg). Control (nondiabetic) animals received the vehicle alone. After 24 h, animals with blood glucose levels greater than 250 mg/dL were considered diabetic and randomly divided into groups. All experiments were carried out 10 days after induction of diabetes. We confirmed the diabetic state by evaluating glycemia daily using a blood glucose meter (Accu-Check Active; Roche Diagnostic, Milan, Italy). A group of rats were treated with the FE extract intraperitoneally (i.p.) starting from 30 min after STZ administration. Treatment consisted of daily injections with the blend suspension containing the following: 300 mg/kg α-lipoic acid, 150 mg/kg *Salix alba* extract (containing 15% salicin), 100 mg/kg berry extract (35% polyphenols; 6% anthocyanins), and 65 mg/kg *Ginkgo biloba* extract (22.0–27.0% *Ginkgo* flavonoids; 5.0–7.0% terpene lactones; ginkgolic acid content <5 ppm), and 50 mg/kg L-carnosine (Tiomax, Sooft Italia SpA, Montegiorgio, Italy). We chose these concentrations because they represent the dose recommended by an ophthalmologist in clinical practice [25]. After 10 days animals were killed, and retina and blood samples were collected to assess cytokines (TNF-α and VEGF) and TBARS, respectively.

2.3. Measurements of TNF-α and VEGF. After 10 days from the STZ injection, the eyes were enucleated and retinal samples were collected; each retina was handled as previously described [24]. Briefly, the retinal samples were homogenized in 100 μL of cocktail solution supplemented with protease inhibitors before use. Samples were centrifuged, and protein levels were measured (Mini BCA Kit; Pierce Scientific, CA, USA). TNF-α and VEGF protein levels were estimated with commercial ELISA kits. The tissue sample concentration was

calculated from a standard curve and corrected for protein concentration.

2.4. Lipid Peroxidation Assay. Lipid peroxidation is defined as "the oxidative deterioration of polyunsaturated lipids," that is, lipids that contain more than two carbon-carbon double covalent bonds. As previously described [6], plasma lipid peroxidation was assessed by the thiobarbituric acid reacting substances method. The thiobarbituric acid test is one of the most frequently used tests for measuring the peroxidation of fatty acids. Briefly, plasma was mixed with hydrochloric acid and thiobarbituric acid, incubated and heated for 20 min, and then deproteinated with trichloroacetic acid. The absorbance of the malonaldehyde and thiobarbituric acid pink product was detected at 532 nm. The results are expressed in nmol MDA per mL of plasma.

3. Results

3.1. Glycemia and Body Weight. Ten days after onset of diabetes, blood glucose values in diabetic rats treated with FE were significantly higher than corresponding values in nondiabetic rats (Table 1). FE does not interfere with glycemia values in nondiabetic rats (data not shown). Body weights of diabetic rats treated with FE were significantly less than those of nondiabetic rats but were not different compared with the diabetic group (Table 1).

3.2. TNF-α and VEGF. Figure 1 shows the retinal TNF-α and VEGF levels. Experimental diabetes significantly increases the TNF-α level (from 3.8 ± 0.5 pg/mg to 9.7 ± 1.0 pg/mg; $P < 0.001$). FE treatment significantly reduced the retinal levels of TNF-α in the STZ-treated group (from 9.7 ± 1.0 pg/mg to 4.5 ± 0.3 pg/mg; $P < 0.001$). We also assessed the effect of FE treatment on VEGF content. As shown in Figure 1, STZ-treated animals showed a 2-fold increase in VEGF levels (from 7.5 ± 2.5 pg/mg to 14.9 ± 2.0 pg/mg; $P < 0.001$). FE treatment in diabetic rats significantly lowered VEGF levels compared with control animals (from 14.9 ± 2.0 pg/mg to 8.7 ± 1.5 pg/mg; $P < 0.001$). The FE did not significantly affect cytokine levels in normal nondiabetic rats (data not shown).

3.3. Lipid Peroxidation. A significant increase in plasma lipid peroxidation was observed after 10 days of diabetes (Figure 2). FE treatment significantly ($P < 0.001$) suppressed diabetes-related lipid peroxidation (1.9 ± 0.2 MDA nmol/mL and 3.8 ± 0.1 MDA nmol/mL, resp.). The FE did not significantly affect lipid peroxidation in normal nondiabetic rats (data not shown).

4. Discussion

The present results show that FE of red berry, *Ginkgo biloba* and white willow bark, containing α-lipoic acid and L-carnosine, may blunt some of the negative effects due to hyperglycemia, such as oxidation, inflammation and VEGF expression, which are the main causes of diabetic retinopathy.

Table 1: Effects of STZ-induced diabetes on body weight and blood glucose levels after 10 days. Control (nondiabetic) group is rats injected with only the vehicle used to dissolve STZ. FE was given intraperitoneally for 10 days. Diabetes was induced by 60 mg/kg (i.v.) injection of STZ.

Groups	Body weight (g)	Blood glucose (mg/dL)
Control	215 ± 15	98 ± 10
Diabetic	$170 \pm 12^{*}$	$360 \pm 30^{**}$
Diabetic + FE	$180 \pm 20^{*}$	$380 \pm 15^{**}$

Data are expressed as mean ± SD.
$^{*}P < 0.01$, $^{**}P < 0.0001$ versus control; ($n = 8$–10).

Diabetes is characterized by a progressive vascular impairment mediated by pericyte loss that leads to an increase of retinal leakage and macular edema and the formation of new vessels [26]. Many studies demonstrated that VEGF and TNF-α contribute to the progression of diabetic retinopathy and that their expression is increased in the diabetic retina. The role of VEGF in the development of diabetic complications in the eye is well established, whereas the role of TNF-α is still under investigation. TNF-α has been implicated in the pathogenesis of diabetic retinopathy, this proinflammatory cytokine induces expression of endothelial adhesion molecules via activation of NF-κB. This latter is able to increase the expression of other inflammatory mediators such as cyclooxygenase enzyme-2 (COX-2). This enzyme may also be activated by glycosylation products [27]. In the present study, we observed that TNF-α levels in the diabetic rat retina were significantly higher in comparison with retina obtained from the control group (Figure 1(a)). According to our data, other groups demonstrated that retinal TNF-α is significantly elevated in diabetic rats [28, 29]. TNF-α is a proinflammatory cytokine mainly generated by inflammatory cells and activated endothelial cells. This cytokine is a potent inducer of the leukostasis elicited by other actors such as VEGF, IL-1β, and PAF in the retinal vasculature [30]. As reported by Joussen et al. [31] TNF-α is one of the most important cytokines in diabetic retinopathy both involved in leukocyte activation and in endothelial cell apoptosis. Further, as showed by the same authors [29, 31], TNF-α receptor inhibitor and nonsteroidal anti-inflammatory drugs attenuated leukocyte adhesion in diabetic retinal vessels as well as the retinal leakage, suggesting a key role of TNF-α.

Our results, beside confirming that TNF-α levels are increased in the retina of STZ-induced diabetic rats, show that FE treatment prevents the increase in TNF-α levels (Figure 1(a)), suggesting that the antioxidant treatment improved the inflammatory milieu of the diabetic retina.

To further investigate whether this FE had any effect on VEGF levels in our model of early diabetic retinopathy, we also measured VEGF levels in retina.

VEGF is a growth factor, which stimulates angiogenesis, promotes vascular permeability, and induces dissociation of tight junctions. Production of VEGF is elicited by high glucose levels, AGEs, IGF-I, angiotensin II, and hypoxia; all these factors increase in the retinal diabetic microvascular bed [32]. Moreover, VEGF levels have been found to be

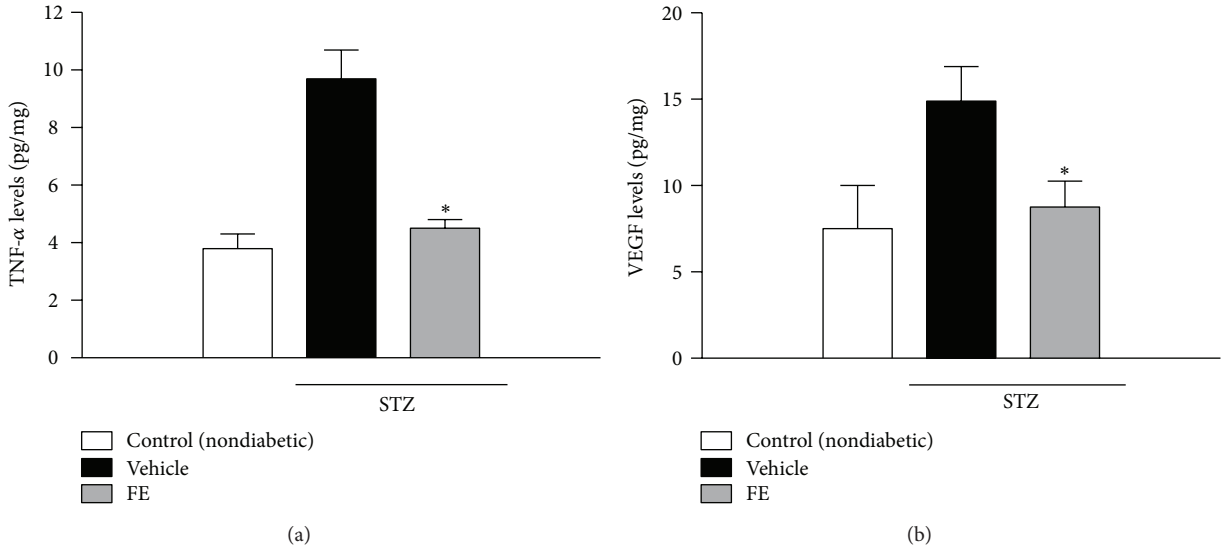

Figure 1: Retinal levels of TNF-α (a) and VEGF (b) 10 days after STZ injection with or without FE treatment. Data are expressed as the mean ± SD. $^{*}P < 0.001$ versus vehicle ($n = 8$–10).

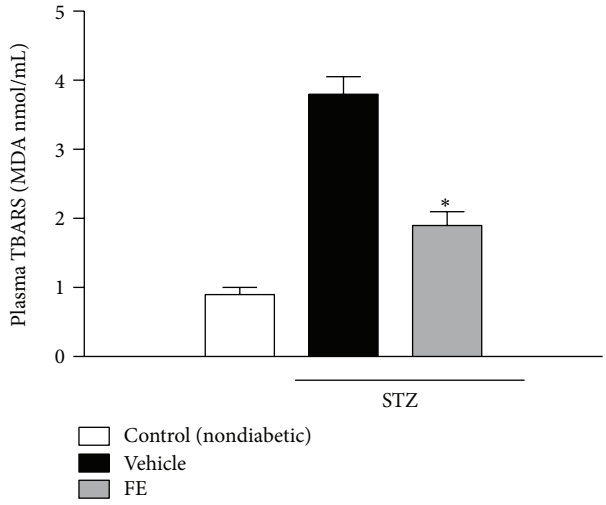

Figure 2: Lipid peroxidation. Effects of FE treatment in STZ-induced diabetic rats on the formation of plasma thiobarbituric acid reactive substances (TBARS). Data are expressed as the mean ± SD. $^{*}P < 0.001$ versus vehicle ($n = 8$–10).

significantly elevated in the ocular fluids such as humor vitreous and humor aqueous of patients with proliferative diabetic retinopathy [33], and many studies confirmed its primary role in the neovascularization and in the breakdown of the blood-retinal barrier [6, 34]. We found that retinal VEGF levels increase in STZ rats and can be blunted by FE treatment (Figure 1(b)).

Oxidative stress elicited by diabetes might play a role in the development of diabetic complications [1]. Many studies have demonstrated both the crucial role of oxidative stress in the retina of diabetic animals [35] and the correlation between increased serum lipid hydroperoxides and the prevalence of retinopathy in diabetic patients [36]. Lipid peroxidation is a free radical-induced process leading to oxidative deterioration of polyunsaturated fatty acids (PUFAs). Under physiological conditions, the concentrations of plasma lipid peroxides are low. Our results show that in diabetic animals the levels of TBARS were high in the plasma and were restored to normal values after the treatment with FE.

Kowluru et al. [12] investigated the effect of long-term administration of different antioxidants on the development of retinal capillary lesions in two animal models of the early stages of diabetic retinopathy. They demonstrated in their animal model that a multiantioxidant diet that significantly inhibited (by 55–65%) the formation of both pericyte ghosts and acellular capillaries, and significantly blunted oxidative stress. Previous studies demonstrated an increase of lipid peroxide levels in the vitreous of patients suffering from proliferative diabetic retinopathy [37]. Increased serum lipid peroxides indicate that an increased free radical activity is associated with retinopathy, with pathogenetic implications [37]. It is interesting to note that, in the present study, treatment with the complex extract mixture inhibited such oxidative stress induced by the diabetic state.

5. Statistical Analysis

All values are expressed as mean ± SD. The results were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. Differences were considered statistically significant when P values were less than 0.05.

6. Conclusions

These data suggest that FE of red berry, GBE, and white willow bark, along with α-lipoic acid and L-carnosine may be useful in the treatment of diabetic retinopathy and that clinical studies to evaluate this possibility are warranted.

List of Abbreviations

VEGF: Vascular endothelial growth factor
TNF-α: Tumor necrosis factor-alpha
ICAM-1: Intercellular adhesion molecule-1
eNOS: Endothelial nitric oxide synthase
8-OHdG: 8-Hydroxy-2′-deoxyguanosine
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
ROS: Reactive oxygen species
AGE: Advanced glycation end-product
RNS: Reactive nitrogen species
CN-1: Carnosinase
GBE: *Ginkgo biloba* leaf extract
TBARS: Thiobarbituric acid reactive substances
LDL: Low-density lipoprotein
STZ: Streptozotocin
ELISA: Enzyme-linked immunoSorbent assay
FE: Fortified extracts
COX-2: Cyclooxygenase enzyme-2
MDA: malonaldehyde.

Conflict of Interests

All authors have no conflict of interest or a direct financial relation with the commercial identities mentioned in the present paper.

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Research Article

The $ERK_{1/2}$ Inhibitor U0126 Attenuates Diabetes-Induced Upregulation of MMP-9 and Biomarkers of Inflammation in the Retina

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This study was conducted to determine the expression of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in a time-dependent manner and the effect of extracellular-signal-regulated kinases-1/2 ($ERK_{1/2}$) inhibition on the expressions of MMP-9, TIMP-1, and inflammatory biomarkers in the retinas of diabetic rats. The expression of MMP-9 was quantified by zymography, and the mRNA level of MMP-9 and TIMP-1 was quantified by RT-PCR. The expression of inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) was examined by Western blot analysis. MMP-9 expression was significantly higher in diabetic rat retinas compared to controls at all time points.TIMP-1 expression was nonsignificantly upregulated at 1week of diabetes and was significantly downregulated at 4 and 12 weeks of diabetes. Intravitreal administration of the $ERK_{1/2}$ inhibitor U0126 prior to induction of diabetes decreased $ERK_{1/2}$ activation, attenuated diabetes-induced upregulation of MMP-9, iNOS, IL-6, and TNF-α and upregulated TIMP-1 expression. In MMP-9 knockout mice, diabetes had no effect on retinal iNOS expression and its level remained unchanged. These data provide evidence that $ERK_{1/2}$ signaling pathway is involved in MMP-9, iNOS, IL-6, and TNF-α induction in diabetic retinas and suggest that $ERK_{1/2}$ can be a novel therapeutic target in diabetic retinopathy.

1. Introduction

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and remains one of the leading causes of blindness worldwide. DR is characterized by gradual progressive alterations in the retinal microvasculature, leading to loss of retinal capillary cells, disruption of vascular barrier, retinal nonperfusion, and preretinal neovascularization [1–4]. However, the exact molecular mechanisms, which mediate such response, remain largely unknown. In recent years, it has become evident that inflammatory mechanisms play an important role in the pathogenesis of DR, and proinflammatory mediators contribute significantly to the development and progression of DR [5–13]. Inflammation is a multistep process where proteases, growth factors, cytokines, and chemokines are released from retinal cells and interact with each other to promote inflammation in the diabetic retinal microenviroment. In the retina, it was shown that diabetes activates induction of proinflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1) [8], interleukin-6 (IL-6) [9], intercellular adhesion molecule-1 (ICAM-1) [10], inducible nitric oxide synthase (iNOS) [11], tumor necrosis factor-alpha (TNF-α) [12], and matrix metalloproteinase-9 (MMP-9) [13]. Recently, much research has focused on MMP-9 because it acts as a potent proinflammatory, proangiogenic and pro-apoptotic factor, and in diabetes, latent MMP-9 is activated in the retina and facilitates retinal capillary cell apoptosis, which is a pathological hallmark of DR development [13–17].

Matrix metalloproteinases (MMPs) are a large family of proteinases that remodel extracellular matrix components and play an important role in the regulation of numerous physiological processes including vascular remodeling and angiogenesis. Altered MMPs activities have been implicated

in many diseases, including diabetes. Diabetes is believed to stimulate the secretion of several MMPs which participate in both macrovascular and microvascular diseases such as coronary artery disease, peripheral arterial disease, stroke, nephropathy, neuropathy, and retinopathy [18–20]. MMP-9, the largest and most complex member of the MMP family, regulates a variety of cellular functions, including proliferation, differentiation, and angiogenesis. Endogenous tissue inhibitors of MMPs (TIMPs) regulate their activation, and TIMP-1 shows greater preference for MMP-9 than any other MMP [21]. The published literature and our previously published data demonstrated elevated levels of MMP-9 in the epiretinal membranes and vitreous fluid from patients with proliferative diabetic retinopathy (PDR) and increased MMP-9 expression in the retinas of diabetic rodents [13, 15, 16, 22–24]. In addition, our previous data also suggested a pro-apoptotic role of MMP-9 in the pathogenesis of DR [13, 15]. Recently, several studies documented that MMP-9 expression is regulated by extracellular-signal-regulated kinases- (ERK-) dependent pathways [24–27] and in the retina RAF protooncogene serine/threonine-protein kinase- (Raf-) mitogen-activated protein kinase kinase (MEK)-ERK cascade is activated by diabetes [13, 24]. Therefore, inhibitors of Raf-MEK-ERK pathway represent a unique opportunity to prevent MMP-9 induction in the retina induced by diabetes. Previously, it was shown that 1 week after diabetes was induced, the retinal $ERK_{1/2}$, vascular endothelial growth factor (VEGF), ICAM-1, leukostasis, and retinal vascular permeability are significantly upregulated [28–30]. Based on these observations, this study was designed to investigate the potential therapeutic role of $ERK_{1/2}$-specific inhibitor U0126 on the retinas at one week of diabetes in rats. We, therefore, investigated the time course change in the expression of MMP-9 and TIMP-1 in the retinas of diabetic rats and examined the effect of intravitreal administration of U0126 on the expression of MMP-9, TIMP-1, iNOS, IL-6, and TNF-α in the diabetic retinas.

2. Methods

2.1. Animals. Rats: Diabetes was induced in rats (male Sprague Dawley, 200–220 g) with streptozotocin (55 mg/kg body weight; Sigma Aldrich, MO, USA). Rats were considered diabetic if their blood glucose was greater than 250 mg/dL. Age-matched normal rats served as control. Blood glucose and weight of animals were measured once a week during the study period. At 1, 4, 8, and 12 weeks after the onset of diabetes, the rats were sacrificed by pentobarbital overdose and the retinas were removed, snap frozen in liquid nitrogen for biochemical measurements. Each group had eight or more rats. The same methods were used for the normal control group. All experiments were performed in accordance to the Association of Research in Vision and Ophthalmology on treatment of Animals in Research and the King Saud University's Animal Care and Use Committee Guidelines.

2.2. Intravitreal Injection of the ERK Inhibitor U0126. Sprague Dawley rats (210–225 g) were kept under deep anesthesia, and sterilized solution of U0126 (0.1 mM in phosphate buffer saline (PBS) with a concentration of 5% of dimethyl sulfoxide (DMSO)/5 μL; Santa Cruz Biotechnology, CA, USA) was injected into the vitreous of the right eye. The left eye received 5 μL of sterile solution without U0126 (DMSO to PBS −1 : 19) as previously described [28]. After 24 hours, diabetes was induced with STZ as mentioned above. The animals were sacrificed one week after diabetes was induced, and the retinas were carefully dissected, snap frozen in liquid nitrogen, and stored at −80°C to be analyzed by PCR and western blotting.

2.3. Mice. Wild-type (WT) and MMP-9 knockout (MMP-9 KO) mice [31] (a generous gift from Professor Ghislain Opdenakker, Rega Institute for Medical Research, university of Leuven, Belgium) were backcrossed 13 times into C57BL6 background to maximally reduce confounding small but additive genetic influences. To illustrate the latter, only in the 13th backcross generation, the brown fur coat of the knockout mice became black. The WT and KO mice were made diabetic by intraperitoneal injection of streptozotocin for five consecutive days. Mice with blood glucose above 200 mg/dL, 3 days after the last injection of streptozotocin, were considered as diabetic. Age-matched normal WT mice served as controls. To investigate long-term effects on the retina, 3 months after induction of diabetes, mice were sacrificed by pentobarbital overdose, and the retinas were removed from one eye, snap frozen in liquid nitrogen, and stored at −80°C for RNA isolation. All experiments were performed in accordance to the Association of Research in Vision and Ophthalmology (ARVO) and King Saud University's Animal Care and Use Committee Guidelines on handling and treatment of animals for basic science research.

2.4. Zymography. Gelatinolytic levels of MMP-9 were estimated in the retina by zymography technique. The samples (50–100 μg protein) were electrophoresed under nonreducing conditions onto 10% SDS-PAGE gels polymerized with 1 mg/mL gelatin. After washing the gel with 2.5% Triton X-100, it was incubated overnight at 37°C in substrate buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, and 0.02% NaN_3. The gel was stained with Coomassie blue stain (0.5% Coomassie blue R-250, 5% methanol, and 10% acetic acid), and this was followed by destaining (5% methanol, 10% acetic acid). The image was taken with the GeneSys (version 1.2.0.0) software on a G:BOX (Syngene, Cambridge, UK) and signal intensity of bands (~92 kDa) quantified using the Gene Tools software (Syngene).

2.5. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from retina using TRI reagent (Ambion, TX, USA), according to manufacturer's protocol. cDNA were synthesized from 1 μg RNA, using an high capacity cDNA reverse transcription kit (Applied Biosystem, CA, USA) following manufacturer's instruction. Real-time RT-PCR was performed using a SYBR green PCR master mix. The PCR primers for rats were MMP-9 forward 5′-GCAACGGAGACGGCAAACC-3′ reverse

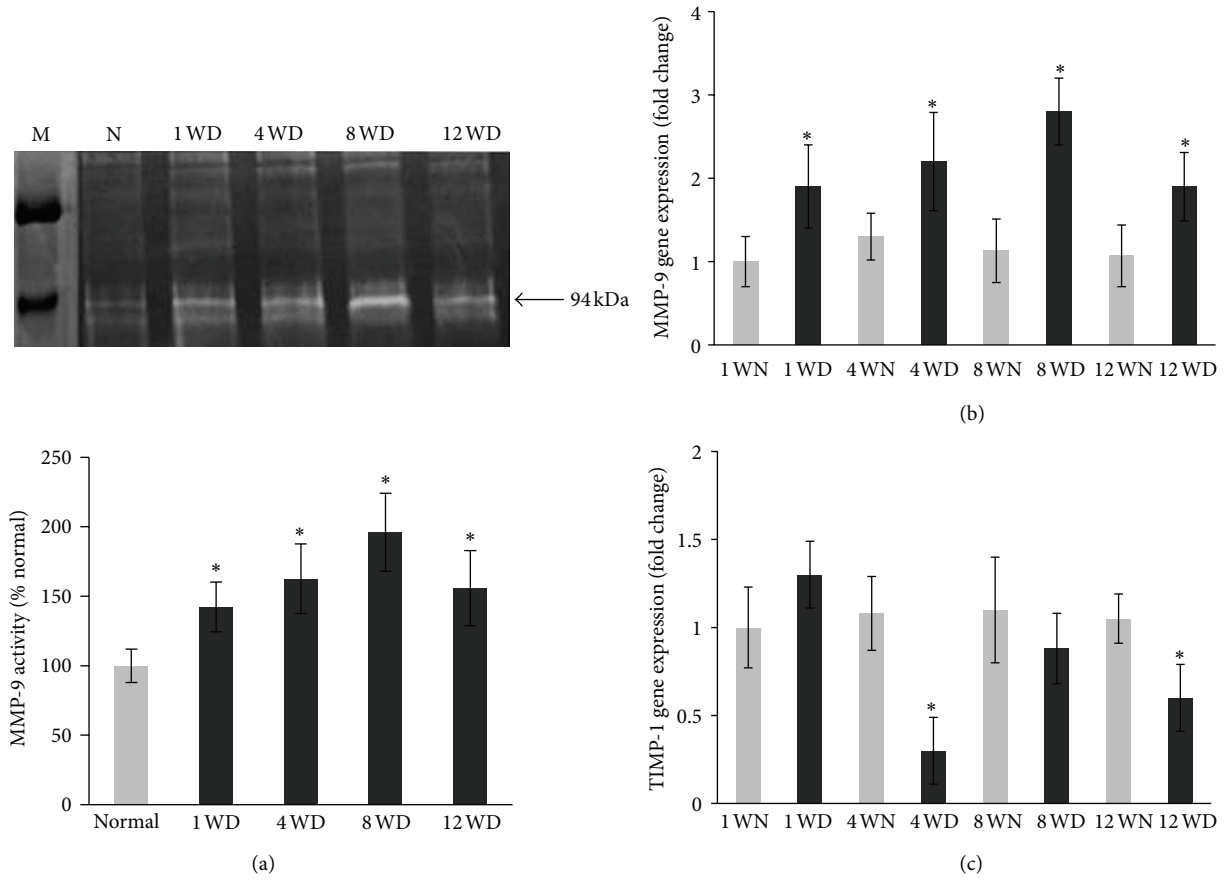

Figure 1: Effect of 1, 4, 8, and 12 weeks of diabetes on retinal MMP-9 and TIMP-1 expression. (a) The gelatinase level of MMP-9 was determined in the retinal homogenate by zymography technique. (b) Gene expressions of MMP-9 and (c) TMP-1 were quantified by RT-PCR using primers given in the Materials and Methods and were adjusted to the mRNA levels of β-actin in each sample. Each measurement was performed at least three times. Results are expressed as mean ± s.d. of at least six rats in each group. $^{*}P < 0.05$ compared with normal rats. M = molecular weight marker; 1 WN, 4 WN, 8 WN, and 12 WN = 1 week normal, 4 weeks normal, 8 weeks normal, and 12 weeks normal rat; 1 WD, 4 WD, 8 WD, and 12 WD = 1 week diabetic, 4 weeks diabetic, 8 weeks diabetic, and 12 weeks diabetic rat.

5′-GACGAAGGGGAAGACGCA-3′; TIMP1 forward 5′-CTGGCATCCTCTTGTTGCT-3′ reverse 5′-CACAGCCAGCACTATAGGTCTTT-3′ and β-actin forward 5′-CCTCTATGCCAACACAGTGC-3′ reverse 5′-CATCGTACTCCTGCTTGCTG-3′. The PCR primers for mice were iNOS forward 5′-CACCTTGGAGTTCACCCAGT-3′ reverse 5′-ACCACTCGTACTTGGGATGC-3′ and β-actin forward 5′-CCTCTATGCCAACACAGTGC-3′ reverse 5′-CAT CGT ACT CCT GCT TGC TG-3′. The standard PCR conditions included 2 minutes at 50°C and 10 min at 95°C followed by 40 cycles of extension at 95°C for 15 seconds and one minute at 60°C. Threshold lines were automatically adjusted to intersect amplification lines in the linear portion of the amplification curves, and cycles to threshold (Ct) were recorded automatically. Data were normalized with β-actin mRNA level (housekeeping gene), and the fold change in gene expression relative to normal was calculated using the ddCt method as previously described [19].

2.6. Western Blot Analysis. Retinas were homogenized in a western lysis buffer (30 mM Tris-HCL pH 7.5, 5 mM EDTA, 1% Triton X-100, 250 mM sucrose, 1 mM Sodium vanadate, and protease inhibitor cocktail). The lysate was centrifuged at 14,000 ×g for 15 min at 4°C and the supernatants were collected, and equal amounts of protein (25–40 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Immunodetection was performed using antibodies against p-ERK$_{1/2}$, iNOS (1 : 1000; Abcam, MA), TNF-α (1 : 500; Santa Cruz Biotechnology), and IL-6 (1 : 500; R&D Systems, MN). Membranes were stripped and reprobed with β-actin to evaluate the lane-loading control. Bands were visualized using high-performance chemiluminescence machine (G: Box Chemi-XX8 from Syngene, Synoptic Ltd. Cambridge, UK), and the intensities were quantified by using GeneTools software (Syngene by Synoptic Ltd.).

2.7. Statistical Analysis. Each measurement was made in duplicate, and the assays were repeated three or more times. Data are expressed as mean±SD. The Mann-Whitney U test was used to compare means from two independent groups. A P value less than ≤0.05 indicated statistical significance. SPSS version 12.0 was used for the statistical analyses.

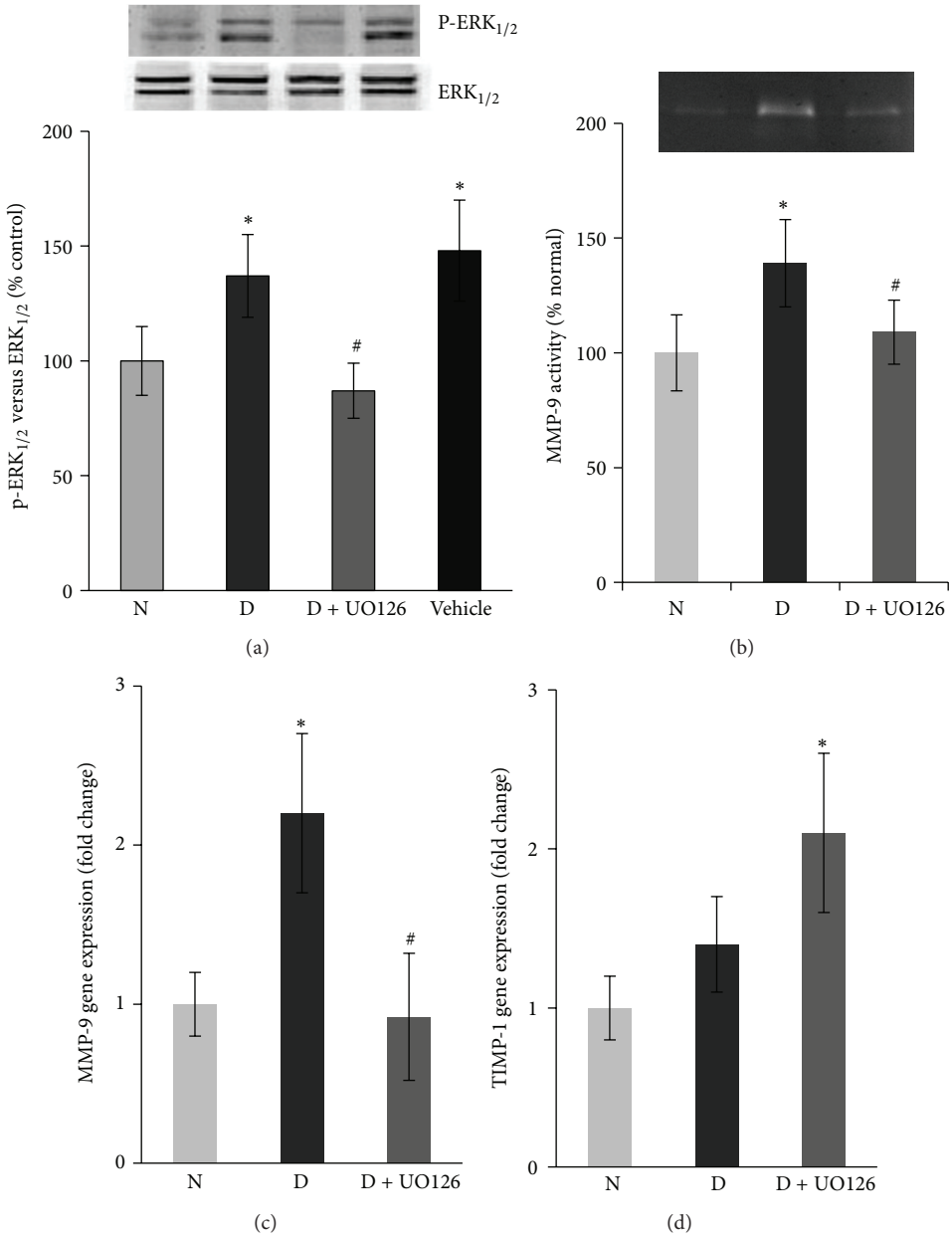

Figure 2: Effect of $ERK_{1/2}$ inhibitor (U0126) on diabetes induced retinal $ERK_{1/2}$ activation and MMP-9 and TIMP-1 expressions. Protein expressions of $ERK_{1/2}$ activation (phosphorylation) were quantified by western blotting using phosphospecific antibody and were adjusted to the protein levels of unphosphorylated antibody in each sample. The expression of (b) MMP-9 gelatinase activity was quantified by using zymography technique. Gene expressions of (c) MMP-9 and (d) TIMP-1 were quantified by RT-PCR using the specific primers and were adjusted to the mRNA levels of β-actin in each sample. Each measurement was performed at least three times. Results are expressed as mean ± s.d. of at least six rats in each group. $^{*}P < 0.05$ compared with normal rats and $^{\#}P < 0.05$ compared to diabetic rats. N, D, and D + U0126 = normal, 1-week diabetic and U0126 pretreated diabetic rat.

3. Results

3.1. Time-Dependent Changes in MMP-9 Expression in Diabetic Retinas. The MMP-9 gelatinase levels in the retinas of diabetic rats were increased by about 40%, 55%, 85%, and 50% at 1, 4, 8, and 12 weeks, respectively, after the onset of diabetes compared to nondiabetic rats (Figure 1(a)). The relative mRNA levels of MMP-9 were detected by real-time RT-PCR analysis at 1, 4, 8, and 12 weeks after the onset of diabetes. The MMP-9 mRNA levels of the control groups at all time points remained at very constant levels (1 ± 0.3-fold). However, a significant increase ($P < 0.05$) in MMP-9 mRNA levels by about 0.5- to 2-fold was detected in the retinas from 1, 4, 8, and 12 week diabetic rats compared with nondiabetic rats (Figure 1(b)).

3.2. Time-Dependent Changes in TIMP-1 Expression in Diabetic Retinas. As shown in Figure 1(c), the TIMP-1 mRNA levels of the diabetic group was significantly decreased by

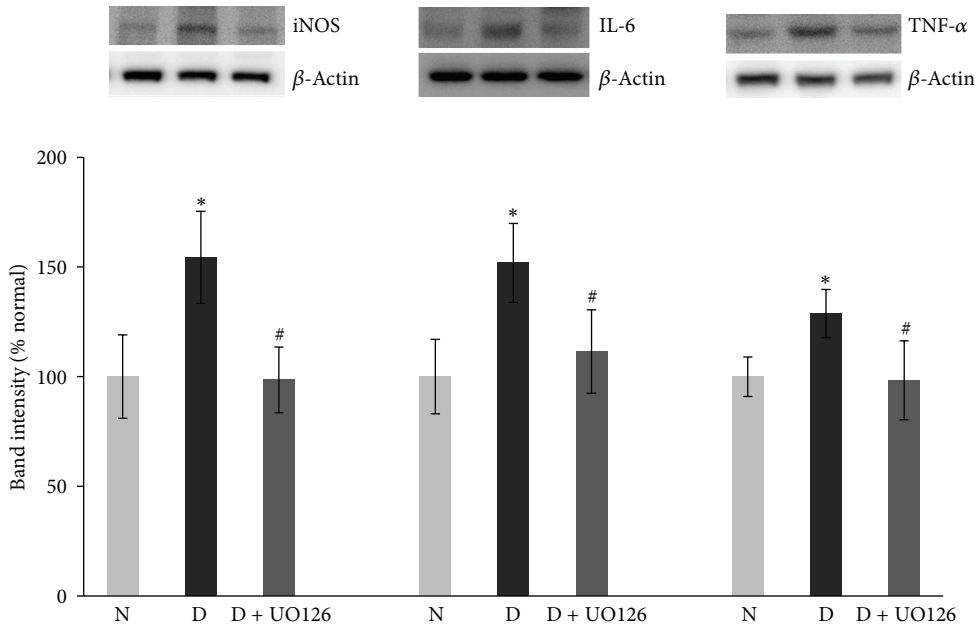

Figure 3: Effect of $ERK_{1/2}$ inhibitor (U0126) on diabetes induced iNOS, IL-6, and TNF-α upregulation in diabetic retina. (a) Protein expression of iNOS, (b) IL-6, and (c) TNF-α was measured by western blot and β-actin was used as housekeeping control. Each measurement was performed at least three times. Results are expressed as mean ± s.d. of at least six rats in each group. $^{*}P < 0.05$ compared with normal rats and $^{\#}P < 0.05$ compared to diabetic rats. N, D, and D + U0126 = normal, 1 week diabetic and U0126 pretreated diabetic rat.

about 70% and 46% ($P < 0.01$) in the retinas from 4- and 12-week diabetic rats compared to the control group, but there was no significant difference between the control and diabetic animals at week 8. Interestingly, there was a nonsignificant increase in TIMP-1 mRNA in the retinas from 1-week diabetic rats compared to the nondiabetic rats.

3.3. U0126 Attenuates Diabetes-Induced $ERK_{1/2}$ Phosphorylation in Diabetic Retinas. U0126 is a potent $ERK_{1/2}$ antagonist and decreases $ERK_{1/2}$ activity in diabetic retina [28]. We employed U0126 to investigate the anti-inflammatory function in the retinas of diabetic rats. Rats that were pretreated with U0126 followed by induction of diabetes showed significant ($P < 0.05$) attenuation of $ERK_{1/2}$ activation as compared to untreated diabetic rats (Figure 2(a)).

3.4. Effect of U0126 on the Expression of MMP-9 and TIMP-1. The expression of MMP-9 was significantly attenuated in the U0126-treated diabetic rat retinas as compared to untreated diabetic rats (Figures 2(b) and 2(c)). U0126 pretreatment of the diabetic rats significantly upregulated TIMP-1 expression compared to nondiabetic rats (Figure 2(d)).

3.5. Effect of U0126 on the Expression of the Inflammatory Biomarkers, iNOS, IL-6, and TNF-α. Diabetes significantly increased the retinal expressions of iNOS by 40%, IL-6 by 60%, and TNF-α by 35% as compared to nondiabetic rat retinas. The results as in Figure 3 showed that pretreatment with U0126 significantly attenuated diabetes-induced increase in the expressions of iNOS, IL-6, and TNF-α as compared to untreated diabetic rats.

3.6. Effect of MMP-9 Inhibition on the mRNA Level of iNOS. Diabetes at ~3 months in WT mice, as expected, increased the expressions of iNOS in the retina by about twofold compared to the WT normal mice. In contrast, in MMP-9 KO mice diabetes had no effect on retinal iNOS expression; the values obtained from diabetic MMP-9 KO mice retina were significantly lower compared to those obtained from WT-diabetic mice (Figure 4).

4. Discussion

Inflammation represents a highly coordinated set of events that allow tissues to respond to injury, and it requires the participation of various cell types expressing and reacting to diverse mediators in a sequential manner. In the development of DR, biomarkers of inflammation such as IL-6, iNOS, TNF-α, and MMP-9 are elevated in the retina [9, 11–13]. One of the specific objectives of this current study was to determine the role of U0126, an $ERK_{1/2}$ inhibitor, in the regulation of MMP-9 and inflammatory mediators in the diabetic retinas. Salient features of the current study are as follows: (i) in the retina, diabetes upregulates MMP-9 and downregulates TIMP-1 expression in a time-dependent manner; (ii) U0126, a specific inhibitor of $ERK_{1/2}$, attenuates early diabetes-induced upregulation of MMP-9 and enhanced TIMP-1 expression; (iii) U0126 attenuates diabetes-induced upregulation of iNOS, IL6, and TNF-α in the retina; (iv) knockdown of MMP-9 prevents diabetes-induced upregulation of iNOS level in the retina.

The mitogen-activated protein kinases (MAPKs) play a critical role in the regulation of cell growth and differentiation

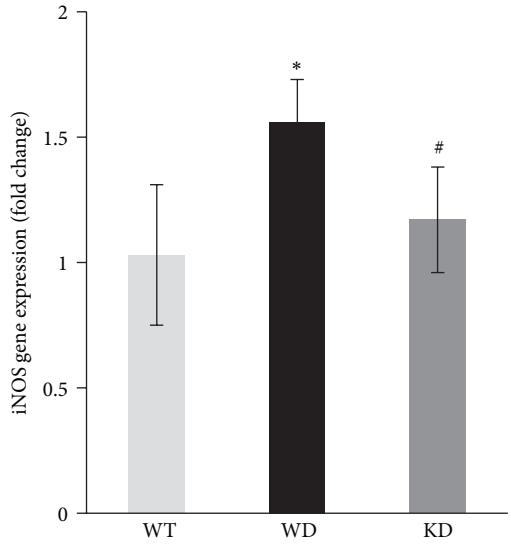

Figure 4: Diabetes does not alter retinal iNOS expression in the mouse lacking MMP-9. Gene expression of iNOS was quantified in the retina of wild-type (WT) mice and MMP-9 KO (KO) mice diabetic for 3 months by real-time quantitative PCR. The mRNA levels of the targeted proteins were adjusted to the levels of β-actin in each sample. Results are expressed as mean ± s.d. of at least five to six mice in each group. $^{*}P < 0.05$ compared with WT nondiabetic mice and $^{\#}P < 0.05$ compared to WT diabetic mice. WT and WD = wild-type nondiabetic and diabetic, respectively, and KD = MMP-9 KO diabetic mice.

and in the control of cellular responses to cytokines and stressors. MAPK cascades are a series of cytosolic enzymes that can transmit extracellular signals to the nucleus [32]. These cascades consist of at least three protein kinases that are activated sequentially: a MAPK kinase kinase such as Raf-1 activates a MAPK kinase such as MEK1, which in turn activates a MAPK such as ERK. The activated ERK can translocate to the nucleus [33, 34], where it can phosphorylate or induce transcription factors leading to the activation of genes and the expression of proteins needed for differentiation or proliferation. Growing body of evidence supports the hypothesis that damaging effect of elevated glucose in the retina may, in part, be due to its ability to increase MAPK signaling pathway in the retina [35–38]. $ERK_{1/2}$ is the most extensively characterized member of MAPK family proteins. It plays an important role in cell growth and differentiation, but recent several reports suggested that $ERK_{1/2}$ pathway can also be related to inflammation, apoptosis, and cell injury [39, 40]. A strong relation between $ERK_{1/2}$ activation and MMP-9 is observed in various pathological conditions including diabetes. Our previous work has indicated that H-ras/Raf MEK/$ERK_{1/2}$ mechanisms play a crucial role in the development of diabetic retinopathy [13, 38]. In the present study, we observed that MMP-9 level increases at all time points; however, TIMP-1 expression was downregulated on a time-dependent manner in the retinas of diabetic rats compared to nondiabetic rats. This observation is consistent with previous studies that demonstrated the upregulation of MMP-9 in the retina of diabetic rodents as well as in vitreous of PDR patients [15, 16, 23, 24]. Our findings are also consistent with a previous study that demonstrated decreased TIMP-1 expression in the retina and its microvasculature at both 2 months and 12 months of diabetes [24]. These data suggest a role of TIMP-1-free MMP-9 in the development and progression of DR. Previously, it was demonstrated that the TIMP-1-free MMP-9, readily available for rapid release by neutrophils upon their influx into target tissue, is the major factor determining the high levels of its angiogenic response [41, 42]. We showed here that in the retina, diabetes activates $ERK_{1/2}$ and that UO126 attenuates diabetes-induced activation of $ERK_{1/2}$. Our results are in agreement with a recent report showing increased $ERK_{1/2}$ activity in the retina of one-week diabetic rats compared to normal rats and pretreatment of the retina with U0126 attenuating diabetes-induced $ERK_{1/2}$ activation [28]. Recent studies demonstrated that the activation of $ERK_{1/2}$ plays an essential role in induction of inflammation [40]. $ERK_{1/2}$ activation is required for cytokine signaling, and inhibition of $ERK_{1/2}$ prevents nuclear transcription factor Kappa B (NF-κB) activation [43]. NF-κB has been localized to the inner nuclear layer and ganglion cells of the retina [44], and NF-κB-regulated inflammatory gene products are reported to be upregulated in the retinas during diabetes such as cyclooxygenase-2 [45, 46], iNOS (45,47), ICAM-1 [47, 48], and TNF-α [49]. We demonstrated that the therapies that inhibit $ERK_{1/2}$ activation significantly attenuated MMP-9 expression and upregulated TIMP-1 in the retinas of one-week diabetic rats. Similarly, a previous report demonstrated that $ERK_{1/2}$ activation makes a significant contribution to induction of MMP-9 in the rat cortical astrocytes via NF-κB activation [50]. Moreover, selective inhibition of the $ERK_{1/2}$ pathways by U0126 significantly attenuated the recombinant human erythropoietin-induced MMP-9 secretion in mouse brain endothelial cells and neural progenitor cells [51]. iNOS, IL-6, and TNF-α are known to be important inflammatory mediators [52].

Strong evidence indicates that chronic low-grade inflammation is implicated in the pathogenesis of diabetic retinopathy. Diabetic retinal vascular leakage, capillary nonperfusion, and endothelial cell damage are associated with leukocyte recruitment and adhesion to the retinal vasculature, findings that correlate with the increased expression of ICAM-1 and the leukocyte integrin CD18. Inhibition of ICAM-1 activity in animals deficient in the gene encoding for ICAM-1 or by a neutralizing antibody suppresses both retinal leukostasis and vascular leakage [10, 53]. The causal relationship between inflammation and angiogenesis is now widely accepted [54]. Previously, various studies have documented that diabetes enhanced the production of inflammatory mediators such as iNOS, IL6, and TNF-α in the retina [9, 11, 45, 49, 55]. In agreement with these studies, we found a significant upregulation of iNOS, IL6, and TNF-α in one-week diabetic rat retinas. In addition, we also demonstrated here that inhibition of $ERK_{1/2}$ by U0126 significantly ameliorates diabetes-induced upregulation of iNOS, IL6, and TNF-α in the retina. Recent several reports demonstrated the beneficial effect of $ERK_{1/2}$ inhibition on various inflammatory parameters and on the production of inflammatory cytokines [56, 57]. Maddahi and Edvinsson demonstrated that U0126 significantly inhibits the

iNOS, IL-6, and TNF-α secretion in rat model of cerebral ischemia [58]. The expression of iNOS in the retina of diabetic mice with manipulated MMP-9 gene remains normal. Similarly, a previous report demonstrated that inhibition of MMP-9 suppresses lipopolysaccharide-induced expression of proinflammatory cytokines and iNOS in microglia [59].

In conclusion, these results indicate that $ERK_{1/2}$ pathway is an upstream signal for MMP-9 production and induction of inflammation in the diabetic retina, and targeting $ERK_{1/2}$ pathway can be a novel therapeutic strategy for the treatment of DR.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

Angiogenic and Vasculogenic Factors in the Vitreous from Patients with Proliferative Diabetic Retinopathy

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This study was conducted to determine levels of angiogenic and endothelial progenitor cell mobilizing (vasculogenic) factors in vitreous fluid from proliferative diabetic retinopathy (PDR) patients and correlate their levels with clinical disease activity. Vascular endothelial growth factor (VEGF), soluble vascular endothelial growth factor receptor-2 (sVEGFR-2), stem cell factor (SCF), soluble c-kit (s-kit), endothelial nitric oxide synthase (eNOS), and prostaglandin E_2 (PGE_2) levels were measured by ELISA in vitreous samples from 34 PDR and 15 nondiabetic patients. eNOS was not detected. VEGF, sVEGFR-2, SCF, and s-kit levels were significantly higher in PDR with active neovascularization compared with quiescent PDR and nondiabetic patients ($P < 0.001$; 0.007; 0.001; < 0.001, resp.). In contrast, PGE_2 levels were significantly higher in nondiabetic patients compared with PDR patients ($P < 0.001$). There were significant correlations between levels of sVEGFR-2 versus SCF ($r = 0.950$, $P < 0.001$), sVEGFR-2 versus s-kit ($r = 0.941$, $P < 0.001$), and SCF versus s-kit ($r = 0.970$, $P < 0.001$). Our findings suggest that upregulation of VEGF, sVEGFR-2, SCF, and s-kit supports the contributions of angiogenesis and vasculogenesis in pathogenesis of PDR.

1. Introduction

Angiogenesis, the process by which new vascular networks develop from preexisting vessels, is a hallmark feature of proliferative diabetic retinopathy (PDR). In addition, increasing evidence suggests that vasculogenesis, the de novo formation of blood vessels from circulating bone marrow-derived endothelial progenitor cells (EPCs), can contribute to neovascularization. Recent studies have shown that circulating bone marrow-derived EPCs home to the ischemic region, differentiate into mature endothelial cells in situ, and can contribute to the process of neovascularization [1, 2]. In previous studies, we demonstrated that bone marrow-derived $CD133^+$ EPCs and c-kit^+ cells contribute to the new vessel formation in PDR fibrovascular epiretinal membranes [3, 4].

Angiogenesis and vasculogenesis are dependent on several cytokines/chemokines and their associated tyrosine kinase receptors. A key player of both these processes is vascular endothelial growth factor (VEGF), also called vascular permeability factor [5, 6]. VEGF binds with high affinity and activates two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR in humans/Flk-1 in mice). These receptors regulate physiological as well as pathological angiogenesis. From the postnatal to adult stage, VEGFR-2 is expressed mostly on vascular endothelial cells [7]. VEGFR-2 is also expressed by bone marrow-derived circulating EPCs. EPCs are characterized by the expression of markers like CD133, CD34, and VEGFR-2 [1, 2]. VEGFR-2 has strong tyrosine kinase activity and is the major positive signal transducer for pathological angiogenesis including cancer and diabetic retinopathy as well as microvascular permeability [7]. Activation of VEGFR-2 stimulates endothelial cell proliferation, migration, and survival, as well as angiogenesis and microvascular permeability [7]. VEGFR-2 has a truncated soluble form (sVEGFR-2) that can be detected in mouse and human plasma. However, it is unknown whether the sVEGFR-2 is a product of ectodomain shedding from cell-surface VEGFR-2 or a product of alternative mRNA splice variation [8, 9].

Stem cell factor (SCF) or kit ligand is a peptide growth factor that exists as a membrane-bound protein but may be cleaved by proteases such as matrix metalloproteinase-9 (MMP-9), to produce the soluble form [10–12]. SCF is important for the survival, proliferation, and differentiation of hematopoietic stem cells. The receptor for SCF, the proto-oncogene c-kit is a tyrosine kinase that is expressed by bone marrow-derived endothelial stem/progenitor cells that can give rise to endothelial cells [13, 14]. SCF ligand binding leads to phosphorylation and activation of the c-kit receptor and its downstream signaling proteins which have been implicated in cell proliferation, cell adhesion, cell survival, chemotaxis, and mobilization of EPCs required for neovascularization [11, 12, 15]. SCF/c-kit signaling has been implicated in the regulation of angiogenesis [10, 13, 15–18]. A soluble form of c-kit (s-kit), consisting of only the extracellular ligand-binding domain, that can be generated by proteolytic cleavage from the surface of hematopoietic cells, mast cells, and endothelial cells or by alternative splicing has been identified [19].

Several studies reported that endothelial nitric oxide synthase (eNOS) is crucial for the recruitment of EPCs in the circulation from the bone marrow and for firm c-kit$^+$ cell adhesion to the vascular endothelium. eNOS is also required for neovascularization in ischemic tissue [20–23]. Recently, it was reported that prostaglandin E_2 (PGE_2), one of the major products of cyclooxygenase, plays an essential role in EPCs homeostasis [24]. In addition, PGE_2 directly stimulates angiogenesis, apart from VEGF signaling, and further induces VEGF expression in endothelial cells [25].

We hypothesized that the vitreous levels of these biomarkers directly reflects angiogenesis and vasculogenesis in PDR. To elucidate the role of angiogenic and EPC mobilizing factors in PDR progression, we measured the levels of VEGF, sVEGFR-2, SCF, s-kit, eNOS, and PGE_2 in the vitreous fluid from patients with PDR and patients without diabetes and correlated their levels with clinical disease activity.

2. Materials and Methods

2.1. Vitreous Samples Collection and Preparation. Undiluted vitreous fluid samples (0.3–0.6 mL) were obtained from individual eyes of from 34 patients with PDR during pars plana vitrectomy. The indications for vitrectomy were traction retinal detachment and/or nonclearing vitreous hemorrhage. The severity of retinal neovascular activity was graded clinically at the time of vitrectomy using previously published criteria [26]. Neovascularization was considered active if there were visible perfused new vessels on the retina or optic disc present within tractional epiretinal membranes. Neovascularization was considered inactive (involuted) if only nonvascularized; white fibrotic epiretinal membranes were present. Active PDR was present in 21 patients, and inactive PDR was present in 13 patients. Traction retinal detachment was present in 23 patients and vitreous hemorrhage in 16 patients. Vitreous hemorrhage was present in 12 patients with active PDR and in 4 patients with inactive PDR. The diabetic patients were 24 males and 10 females, whose ages ranged from 27 to 75 years with a mean of 53.3 ± 11.7 years. The duration of diabetes ranged from 7 to 32 years with a mean of 16.4 ± 5.6 years. Twenty-two patients had insulin-dependent diabetes mellitus, and 12 patients had noninsulin-dependent diabetes mellitus. At presentation, the fasting blood glucose was uncontrolled in 22 patients and controlled in 12 patients. Twenty-four patients were receiving treatment for hypertension, 3 patients had diabetic nephropathy, and 3 patients had cardiovascular disease. The control group consisted of 15 patients who had undergone vitrectomy for the treatment of rhegmatogenous retinal detachment (RD) with no proliferative vitreoretinopathy. Controls were free from systemic disease and were 10 males and 5 females whose ages ranged from 26 to 78 years with a mean of 52.6 ± 15.2 years. None of the control patients had vitreous hemorrhage (Table 1). Vitreous samples were collected undiluted by manual suction into a syringe through the aspiration line of vitrectomy, before opening the infusion line. The samples were centrifuged (5000 rpm for 10 min, 4°C), and the supernatants were aliquoted and frozen at −80°C until assay. The study was conducted according to the tenets of the Declaration of Helsinki, and informed consent was obtained from all patients. The study was approved by the Research Centre, College of Medicine, King Saud University.

2.2. Enzyme-Linked Immunosorbent Assay Kits. Enzyme-linked immunosorbent assay (ELISA) kits for human VEGF (Quantikine Human Vascular Endothelial Growth Factor, Cat number: SVE00), human sVEGFR-2 (Quantikine Human Vascular Endothelial Growth Factor Receptor 2, Cat number: SVR200), human SCF (Quantikine Human Stem Cell Factor/c-kit ligand, Cat number: DCK00), human s-kit (Quantikine Human Stem Cell Factor soluble receptor, SCF sR**,** Cat number: DSCR00), and eNOS (Quantikine Human Endothelial Nitric Oxide Synthase, Cat number: DEN00) were purchased from R and D Systems, Minneapolis, MN. Whereas, PGE_2 (Quantikine Human Prostaglandin E2, Cat number: 514010) was purchased from Cayman Chemical Company, Ann Arbor, NI. The detection limit of each ELISA kit for VEGF, sVEGFR-2, SCF, s-kit, eNOS, and PGE_2 is 9.0, 4.6, 9.0, 65, 25, and 15 picograms/ mL (pg/mL), respectively. The ELISA plate readings were done using FLUOstar Omega-Miroplate reader from BMG Labtech, Offenburg, Germany.

2.3. Measurement of Human VEGF, sVEGFR-2, SCF, s-Kit, and eNOS. The quantifications of the level of VEGF, sVEGFR-2, SCF, s-kit, and eNOS in the vitreous fluid were determined using specific ELISA kits according to the manufacturer's instruction (R and D Systems).

Vitreous samples were diluted 2-fold, 3-fold, and 2-fold for VEGF, sVEGFR-2 and SCF measurements, respectively. 100 μL of diluted sample was added to each well of the ELISA plate for the analysis. For the measurement of s-kit and eNOS, vitreous was not diluted, and 100 μL of undiluted sample was used in the ELISA assay. Optical density was read at 450 nm in microplate reader. Each assay was performed in duplicate. Using the 4-parameter fit logistic (4-PL) curve equation, the actual concentration for each sample was calculated. For the vitreous samples that have been diluted, the correction read from the standard curve obtained using 4-PL was multiplied

Table 1: Clinical characteristics and levels of angiogenic and vasculogenic factors in 15 control patients.

Age (yr)/sex	Duration of symptoms (days)	Status of lens	Extent of detachment (quadrants)	VEGF (pg/mL) (detection limit: 9.0 pg/mL)	sVEGFR-2 (pg·mL) (detection limit: 4.6 pg/mL)	SCF (pg/mL) (detection limit: 9.0 pg/mL)	s-Kit (pg/mL) (detection limit: 65 pg/mL)	PGE_2 (pg/mL) (detection limit: 25 pg/mL)	eNOS (pg/mL) (detection limit: 15 pg/mL)
78/M	4	Pseudophakic	2	19.3	837.4	ND	87	68.2	ND
60/F	3	Pseudophakic	2	38.8	161.2	ND	ND	78.8	ND
47/M	6	Pseudophakic	4	ND	1126.6	128.4	247	38	ND
48/F	5	Phakic	2	ND	1730.7	ND	ND	42.8	ND
59/M	3	Phakic	2	ND	1016.2	22.2	163	80.2	ND
38/F	14	Phakic	2	33.1	93	ND	ND	28.1	ND
40/M	5	Phakic	3	ND	915.3	59.1	135	20.7	ND
27/F	7	Phakic	2	ND	656.2	ND	224.9	70.8	ND
65/F	10	Pseudophakic	4	27.7	783	ND	173	63.9	ND
55/M	2	Phakic	2	26.4	304.4	ND	ND	45	ND
40/F	4	Aphakic	2	ND	912.5	ND	ND	61.3	ND
70/M	2	Pseudophakic	2	16.5	934.4	37.6	58	67.3	ND
74/M	3	Pseudophakic	1	21	664.4	ND	137	63.2	ND
63/M	2	Pseudophakic	2	30.8	140.7	ND	ND	80.3	ND
50/M	5	Pseudophakic	3	ND	731.2	76.7	ND	61.7	ND

VEGF: vascular endothelial growth factor; sVEGFR-2: soluble vascular endothelial growth factor receptor-2; SCF: stem cell factor; s-kit: soluble c-kit; PGE_2: prostaglandin E_2; eNOS: endothelial nitric oxide synthase; ND: not detected.

by the dilution factors to get the actual reading for each sample.

2.4. Measurement of Human Prostaglandin E_2. The quantification of the level of PGE_2 in the vitreous fluid was determined using a specific ELISA kit according to the manufacturer's instruction (Cayman Chemical Company). 50 μL of 2-fold diluted vitreous fluid was added to each ELISA well followed by the addition of PGE_2 AChE tracer (acetylcholinesterase) and PGE_2 monoclonal antibodies. The antibody-PGE_2 complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate was washed after incubation for 18 hours at 4°C to remove any unbound reagents, and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction gives yellow color, and the corresponding optical density was measured at 405 nm using microplate reader. The intensity of this color is proportional to the amount of PGE_2 tracer bound to the well, which in turn is inversely proportional to the amount of free PGE_2 present in the well. The %B/B0 (%Bound/Maximum Bound) is calculated which is ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B0) well. Using the 4-parameter fit logistic (4-PL) curve equation, the actual concentration for each sample was calculated using standard curve that was plotted using %B/B0 values versus concentration of a series of wells containing series of known amounts of PGE_2. As the vitreous fluid has been 2-fold diluted, the correction read from the standard curve obtained using 4-PL was multiplied by the dilution factors to get the actual reading for each sample.

2.5. Statistical Analysis. The Mann-Whitney test was used to compare means from two independent groups. Pearson correlation coefficients were computed to investigate correlations between variables. One-way ANOVA and post-ANOVA pairwise comparisons of means were conducted using the Kruskal-Wallis test. For three groups, the critical Z value for post-ANOVA pairwise mean comparisons was $Z = 2.39$ at a 5% level of significance. A P value less than 0.05 indicated statistical significance. SPSS version 12.0 and program 3S from the BMDP 2007 Statistical Package were used for the statistical analyses.

3. Results

3.1. Levels of Angiogenic and Vasculogenic Factors in Vitreous Samples. VEGF was detected in all vitreous samples from patients with PDR and in 8 (53.0%) samples from nondiabetic control patients (Table 1). When all patients were considered, mean VEGF level in vitreous samples from PDR patients (711.6 ± 1271.3 pg/mL) was significantly higher than that in nondiabetic control patients (31.6 ± 37.9 pg/mL) ($P < 0.001$; Mann-Whitney test).

sVEGFR-2 was detected in all vitreous samples from patients with PDR and nondiabetic control patients (Table 1). The detected sVEGFR-2 levels in PDR patients (1497.0 ± 1590.5 pg/mL) were significantly higher than that in nondiabetic control patients (678.0 ± 471.5 pg/mL) ($P = 0.002$; Mann-Whitney test).

SCF was detected in all vitreous samples from patients with PDR and in 5 (33.0%) samples from nondiabetic control patients (Table 1). SCF mean level in vitreous samples from PDR patients (176.6 ± 490.1 pg/mL) was significantly higher than that in nondiabetic control patients (21.6 ± 38.5 pg/mL) ($P < 0.001$; Mann-Whitney test).

s-kit was detected in all vitreous samples from patients with PDR and in 8 (53.0%) samples from nondiabetic control patients (Table 1). s-kit mean level in vitreous samples from PDR patients (493.8 ± 1183.1 pg/mL) was significantly higher than that in nondiabetic control patients (87.5 ± 91.5 pg/mL) ($P < 0.001$; Mann-Whitney test).

PGE_2 was detected in all vitreous samples from patients with PDR and nondiabetic control patients (Table 1). The detected PGE_2 levels in PDR patients (28.7±10.6 pg/mL) were significantly lower than that in nondiabetic control patients (58.0 ± 18.8 pg/mL) ($P < 0.001$; Mann-Whitney test). eNOS was not detected in vitreous samples from patients with PDR and nondiabetic control patients.

3.2. Relationship between Angiogenic and Vasculogenic Factors and Activity of PDR. Comparison of mean levels of angiogenic and vasculogenic factors among active PDR patients, inactive PDR patients, and nondiabetic control patients was conducted using the Kruskal-Wallis test, and the results are shown in Table 2. Mean levels differed significantly between the 3 groups for VEGF ($P < 0.001$), sVEGFR-2 ($P = 0.007$), SCF ($P = 0.001$), s-kit ($P < 0.001$), and PGE_2 ($P < 0.001$). Post-ANOVA pairwise comparisons of means indicated that mean VEGF level was significantly higher in patients with active PDR than in patients with inactive PDR ($Z = 3.67$) and nondiabetic control patients ($Z = 5.25$). For sVEGFR-2, mean level for patients with active PDR was significantly higher than that in nondiabetic control patients ($Z = 3.11$). For SCF, the mean levels for patients with active PDR and patients with inactive PDR were significantly higher than that in nondiabetic control patients ($Z = 3.42$; $Z = 3.02$, resp.). For s-kit, the mean level for patients with active PDR was significantly higher than that in nondiabetic control patients ($Z = 4.0$). For PGE_2, the mean levels for patients with active PDR and patients with inactive PDR were significantly lower than that in nondiabetic control patients ($Z = 3.73$; $Z = 2.73$, resp.).

3.3. Relationship between Angiogenic and Vasculogenic Factors and Vitreous Hemorrhage. When patients with PDR were divided into those with or without hemorrhage, the mean levels of angiogenic and vasculogenic factors differed significantly between PDR patients with hemorrhage, PDR patients without hemorrhage, and nondiabetic control patients for VEGF ($P < 0.001$), sVEGFR-2 ($P = 0.003$), SCF ($P = 0.001$), s-kit ($P = 0.001$), and PGE_2 ($P < 0.001$) (Table 3). Post-ANOVA pairwise comparisons of means highlighted that for VEGF, the mean levels for PDR patients with or without hemorrhage were significantly higher than those for nondiabetic control patients ($Z = 4.75$; $Z = 2.52$, resp.),

Table 2: Comparisons of mean angiogenic and vasculogenic factor levels in proliferative diabetic retinopathy (PDR) patients with or without active neovascularization.

Disease group	VEGF (pg/mL)	sVEGFR-2 (pg/mL)	SCF (pg/mL)	s-kit (pg/mL)	PGE_2 (pg/mL)
Active PDR	1099.9 ± 1535.6	1692.5 ± 1873.9	233.7 ± 626.8	697.4 ± 1528.1	28.2 ± 11.1
Inactive PDR	150.7 ± 237.7	1050.1 ± 403.2	88.6 ± 66.2	205.3 ± 106.4	30.0 ± 10.1
Controls	31.6 ± 37.9	678.0 ± 471.5	21.6 ± 38.5	87.5 ± 91.5	58.0 ± 18.8
ANOVA P value	<0.001*	0.007*	0.001*	<0.001*	<0.001*

*Statistically significant at 5% level.
VEGF: vascular endothelial growth factor; sVEGFR-2: soluble vascular endothelial growth factor receptor-2; SCF: stem cell factor; s-kit: soluble c-kit; PGE_2: prostaglandin E_2.

Table 3: Comparisons of mean angiogenic and vasculogenic factor levels in proliferative diabetic retinopathy (PDR) patients with or without hemorrhage.

Disease group	VEGF (pg/mL)	sVEGFR-2 (pg/mL)	SCF (pg/mL)	s-kit (pg/mL)	PGF_2 (pg/mL)
PDR with hemorrhage	994.1 ± 1357.4	1567.9 ± 2118.8	253.1 ± 660.2	682.7 ± 1643.0	24.6 ± 9.3
PDR without hemorrhage	453.6 ± 1156.7	1404.7 ± 425.8	84.7 ± 56.4	291.4 ± 149.3	34.1 ± 10.3
Controls	31.6 ± 37.9	678.0 ± 471.5	21.6 ± 38.5	87.5 ± 91.5	58.0 ± 18.8
ANOVA P value	<0.001*	0.003*	0.001*	0.001*	<0.001*

*Statistically significant at 5% level.
VEGF: vascular endothelial growth factor; sVEGFR-2: soluble vascular endothelial growth factor receptor-2; SCF: stem cell factor; s-kit: soluble c-kit; PGE_2: prostaglandin E_2.

and the mean level for PDR patients with hemorrhage was significantly higher than that for PDR patients without hemorrhage ($Z = 2.40$). For sVEGFR-2, the mean levels for PDR patients with or without hemorrhage were significantly higher than those for nondiabetic control patients ($Z = 3.37$; $Z = 2.45$). For SCF, the mean levels for PDR patients with or without hemorrhage were significantly higher than those for nondiabetic control patients ($Z = 3.27$; $Z = 3.21$, resp.). For s-kit, the mean levels for PDR patients with or without hemorrhage were significantly higher than those for nondiabetic control patients ($Z = 3.5$; $Z = 2.88$, resp.). For PGE_2, the mean level was significantly lower for PDR patients with hemorrhage than that for nondiabetic control patients ($Z = 4.23$).

There were no statistically significant relationships between vitreous levels of angiogenic and vasculogenic factors and systemic disease variables (Table 4).

3.4. Correlations. When all patients with diabetes were considered, there were significant correlations between vitreous fluid levels of sVEGFR-2 versus SCF ($r = 0.950$, $P < 0.001$), sVEGFR-2 versus s-kit ($r = 0.941$, $P < 0.001$), and SCF versus s-kit ($r = 0.970$, $P < 0.001$) (Table 5).

4. Discussion

The present study is to our knowledge the first to assess the levels of sVEGFR-2, SCF, and s-kit in the vitreous fluid from patients with PDR. Because activation of VEGFR-2 plays an important role in tumor angiogenesis, clinical interest in monitoring plasma sVEGFR-2 levels in cancer patients has focused on its potential use as a surrogate biomarker for disease progression as well as assessing efficacy/activity of antiangiogenic drugs particularly those that target VEGF or VEGFR-2 [9, 27]. In vitro studies showed that VEGFR-2 downregulation from the cell surface leads to reduced sVEGFR-2 levels in the conditioned media from endothelial cells, and that sVEGFR-2 is derived mainly from shedding from endothelial cell surface. These findings imply that expression levels of VEGFR-2 and its soluble form are linked [9]. Therefore, increased levels of sVEGFR-2 in the vitreous from patients with PDR, particularly in patients with active PDR, may reflect increased cellular VEGFR-2. A VEGF-dependent increase in the shedding of sVEGFR-2 by endothelial cells was demonstrated [28, 29]. In the present study, we detected higher levels of VEGF in patients with active PDR compared with patients with inactive PDR and nondiabetic patients. Collectively, these findings are consistent with our previous immunohistochemical studies in which we demonstrated the presence of $VEGFR\text{-}2^+$ $CD34^+$ cells and $VEGF^+$ cells in the vascular endothelium of blood vessels and in the stroma in PDR fibrovascular epiretinal membranes. Furthermore, the numbers of blood vessels and stromal cells expressing VEGFR-2 and VEGF in membranes from patients with active PDR were significantly higher than that in membranes from patients with quiescent PDR [3, 30].

In a previous immunohistochemical study, we showed that bone marrow-derived $c\text{-}kit^+$ cells coexpressing the chemokine stromal cell-derived factor-1 receptor CXCR4 and eNOS contribute to new vessel formation in PDR fibrovascular epiretinal membranes. In addition, SCF was expressed by vascular endothelial cells and stromal cells. Furthermore, the expression of SCF and c-kit in membranes from patients with active neovascularization was significantly higher than that in membranes from patients with quiescent PDR [4]. We have extended those observations by showing that both SCF and s-kit levels were significantly elevated in vitreous from patients with PDR and were further increased in patients with

Table 4: Relationship between angiogenic and vasculogenic factors and systemic disease variables.

Variable	VEGF (pg/mL)	sVEGFR-2 (pg/mL)	SCF (pg/mL)	s-kit (pg/mL)	PGE_2 (pg/mL)
Type of diabetes					
Insulin-dependent	1125.4 ± 1819.4	1594.0 ± 1950.7	209.9 ± 614.4	601.3 ± 1502.7	29.6 ± 10.6
Noninsulin-dependent	472.9 ± 507.9	1338.2 ± 569.3	116.5 ± 75.5	320.3 ± 182.6	28.8 ± 11.1
P value	0.611	0.698	0.118	0.578	0.972
Fasting blood glucose					
Controlled	1296.1 ± 1115.8	2133.0 ± 2601.7	313.4 ± 809.2	867.0 ± 2014.4	31.0 ± 11.0
Uncontrolled	912.9 ± 1815.2	1158.1 ± 504.8	906 ± 74.2	297.9 ± 177.0	28.4 ± 10.5
P value	0.081	0.375	0.846	0.649	0.733
Hypertension					
Yes	1117.3 ± 1757.7	1639.4 ± 1873.0	209.4 ± 573.0	548.0 ± 1358.0	29.4 ± 10.6
No	672.6 ± 1063.8	1008.8 ± 535.1	59.7 ± 28.0	330.6 ± 196.2	29.2 ± 12.5
P-value	0.374	0.409	0.422	0.716	0.804

VEGF: vascular endothelial growth factor; sVEGFR-2: soluble vascular endothelial growth factor receptor-2; SCF: stem cell factor; s-kit: soluble c-kit; PGE_2: prostaglandin E_2.

Table 5: Pearson correlation coefficients.

	PGE_2	VEGF	sVEGFR-2	SCF
VEGF				
r =	0.054			
P =	0.849			
sVEGFR-2				
r =	−0.060	−0.151		
P =	0.785	0.590		
SCF				
r =	−0.085	−0.002	0.950	
P =	0.699	0.991	<0.001*	
s-kit				
r =	0.001	−0.021	0.941	0.970
P =	0.998	0.926	<0.001*	<0.001*

*Statistically significant at 5% level.
Where the row and column meet, is the correlation coefficient and the *P* value for the two variables.
VEGF: vascular endothelial growth factor; sVEGFR-2: soluble vascular endothelial growth factor receptor-2; SCF: stem cell factor; s-kit: soluble c-kit; PGE_2: prostaglandin E_2.

active PDR. Taken together, these findings suggest a role for the SCF/c-kit signaling in the progression of PDR.

Several reports demonstrated that SCF/c-kit signaling axis has been implicated in the regulation of angiogenesis. SCF/c-kit signaling promoted the survival, migration, differentiation, and capillary tube formation of endothelial cells [16, 17] and induced a potent angiogenic response in vivo [17]. In addition, SCF/c-kit signaling played an important role in ischemia-induced neovascularization [10, 13, 15, 18]. SCF has also been implicated in the mobilization of bone marrow-derived endothelial progenitor cells required for neovascularization [12, 15]. Interestingly, several studies showed that SCF/c-kit signaling upregulates the transcription factor hypoxia-inducible factor-1α (HIF-1α). SCF-induced HIF-1α was transcriptionally active and transcribed HIF-1α target genes, such as VEGF [31, 32]. On the other hand, Han et al. [33] demonstrated that hypoxia upregulates SCF gene expression through HIF-1α. These findings suggest a reciprocal effect between SCF and HIF-1α, thus forming a positive feedback in several cell lines coexpressing SCF and c-kit. In a previous report we demonstrated the presence of HIF-1α immunoreactivity in PDR epiretinal membranes [34]. Treatment of small cell lung cancer cell line with imatinib, a specific inhibitor of the protein tyrosine kinases c-kit, and platelet-derived growth factor receptor resulted in inhibition of c-kit-induced HIF-1α and VEGF expression [31]. Imatinib has been shown to have clinical activity as an anticancer agent [35]. These findings suggest that inhibition of SCF/c-kit signaling could have clinically relevant antiangiogenic effects in PDR.

Several studies demonstrated that circulating levels of s-kit correlate with the clinical course of tumors and that the concentration of s-kit may be a useful clinical biomarker of disease severity in patients with tumors [36, 37]. The serum levels of s-kit increase when the population of cells that release c-kit is pathologically expanded such as in acute myelogenous leukemia and mastocytosis [38, 39]. Because s-kit is thought to be generated by the proteolytic cleavage of the membrane-bound receptor [19], it is possible that the increased levels in the vitreous from patients with PDR in part reflect increased c-kit$^+$ cell numbers in PDR fibrovascular epiretinal membranes [4]. In addition, Nakamura et al. [40] demonstrated that exogenous s-kit induces mobilization of hematopoietic stem cells from bone marrow into peripheral blood. This finding is consistent with the observations of other investigators who demonstrated that the levels of s-kit in the serum showed a positive correlation with the numbers of peripheral blood stem cells [41]. The present study showed positive correlations between vitreous levels of SCF, s-kit, and sVEGFR-2. Similarly, c-kit and VEGFR-2 amplifications were strongly associated in glioblastoma multiforme, suggesting coamplification [42]. On the other hand, Turner et al. [43] demonstrated that s-kit retains high-affinity SCF binding activity suggesting that s-kit may bind SCF and function as a receptor antagonist.

eNOS is required for neovascularization in ischemic tissue [22, 23]. In a mice model of hind-limb ischemia, impaired

neovascularization in mice lacking eNOS is related to a defect in progenitor cell mobilization [22]. eNOS is involved in migration of EPCs [21] and is crucial and specific factor for firm c-kit$^+$ cell adhesion in the vascular endothelium [20]. In cell culture models, eNOS plays an essential role in endothelial cell proliferation and is a central mediator of several endothelium growth factors, such as VEGF and PGE_2 [44]. In a previous immunohistochemical study, we showed immunoreactivity for eNOS in vascular endothelial cells and stromal cells in PDR fibrovascular epiretinal membranes [4]. In the present study, however, eNOS levels were below the detection limit. This discrepancy might be explained by the primary localization of eNOS on the Golgi apparatus and plasma membrane caveolae in endothelial cells [45] and therefore might not release into the vitreous fluid. The crucial role of PGE_2 in EPCs homeostasis following tissue ischemia has been demonstrated. In vivo blockade of PGE_2 production by selective cyclooxygenase-2 inhibition virtually abrogated ischemia-induced EPCs mobilization. In addition, EPCs are a rich source of PGE_2, and PGE_2 stimulates the number and function of EPCs [24]. Furthermore, PGE_2 directly stimulates angiogenesis, and this stimulatory effect is not dependent on VEGF signaling. PGE_2 stimulation of endothelial cells enhances VEGF expression. In turn, VEGF stimulates PGE_2 expression in endothelial cells [25]. However, in the present study, PGE_2 levels in PDR patients were significantly lower than that in nondiabetic patients. These findings are consistent with the observations of other investigators [46].

The vitreous fluid, collected from patients with PDR during pars plana vitrectomy, is an ideal material for analysis of local, intraocular concentrations of selected proteins which take part of this pathology. However, when measuring these factors in the vitreous, some considerations should be kept in mind. Vitreous hemorrhage, associated with active neovascularization or traction on the retina induced by involuted fibrovascular proliferation during posterior vitreous detachment, can provide an influx of serum proteins into vitreous fluid. In a previous study, we demonstrated that there was no correlation between hemoglobin levels, as a measure of the amount of erupted blood, and total protein levels in vitreous fluid from patients with PDR [47]. In addition, we showed that chemokine levels in vitreous samples were significantly higher than that in serum samples and that there was no correlation between vitreous fluid and serum chemokine levels in patients with PDR [48]. Our previous immunohistochemical studies demonstrated the expression of c-kit, SCF, VEGF, and VEGFR-2 by vascular endothelial cells and stromal cells in PDR fibrovascular epiretinal membranes [3, 4, 30, 34]. In our laboratory, we also recently demonstrated upregulated expression of VEGF, VEGFR-2, and SCF in the retinas from diabetic mice (Mohammed et al., unpublished data). In addition, our subgroup analysis demonstrated that the levels of VEGF, sVEGFR-2, SCF, and s-kit were also increased in vitreous fluid from PDR patients without hemorrhage. These findings suggest that local cellular production is the relevant source of these factors within the ocular microenvironment and that systemic inflow mechanism is rather improbable.

In conclusion, our findings suggest that the upregulation of VEGF, sVEGFR-2, SCF, and s-kit in the vitreous fluid from patients with PDR reflects angiogenesis and vasculogenesis in PDR. These results have implications for understanding the pathogenetic mechanisms that underlie the neovascularization that develops as a complication of this disorder.

Conflict of Interests

The authors declare that they have no conflict of interests.

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