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SUPPLEMENT ARTICLE

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Induction of vascular endothelial growth factor-A_{165a} in human retinal and endothelial cells in response to glyoxal

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Abstract

Low-density lipoprotein (LDL) apheresis is effective and safe for patients with diabetes, proteinuria, and dyslipidemia. Diabetes mellitus is accompanied by ocular microvascular complications like retinal neovascularization or diabetic macular edema. These are leading causes of blindness and can be mediated by abnormal vessel growth and increased vascular permeability due to elevated levels of vascular endothelial growth factor (VEGF) in diabetic patients. In this study, we established methods to study the expression of different VEGF isoforms in human retinal and endothelial cells. The VEGF-A_{165a} isoform is much higher expressed in retinal cells, compared to endothelial cells. Stimulation with glyoxal as a model of oxidative stress under diabetic conditions lead to a pronounced induction of VEGF-A_{165a} in human retinal and endothelial cells. These data suggest that diabetes and oxidative stress induce VEGF-A isoforms which could be relevant in regulating the ingrowths of novel blood vessels into the retina in diabetic patients.

KEYWORDS

diabetes, endothelial cells, glyoxal, retinal cells, VEGF-A

1 | INTRODUCTION

Low-density lipoprotein (LDL) apheresis is effective and safe for patients with diabetes, proteinuria, and dyslipidemia [1]. Apheresis is even considered as potential therapeutic strategy in COVID-19 patients [2–4]. Diabetes mellitus is accompanied by retinal diseases like retinal neovascularization, proliferative vitreoretinopathy, or diabetic macular edema (DME) [5]. DME is one of the leading causes of blindness in the industrial world. DME could be caused by abnormal vessel growth and increased vascular permeability due to elevated levels of vascular endothelial growth factor (VEGF) in vitreous probes of diabetic patients. Enhanced expression of the VEGF-A might be pivotally involved in hyperpermeability of capillaries and breakdown of the blood-retinal barrier [6, 7]. Indeed, vascular endothelial growth factor (VEGF or VEGF-A), also known as vascular permeability factor, plays an important role in the regulation of physiological and pathophysiological blood vessel growth [8]. The

formation of new blood vessels by angiogenesis and the inhibition of this process involves stimulation and inhibition of VEGF [9].

For the treatment of one of the most common and severe microvascular complications of diabetes, DME, anti-VEGF-A therapy became the current standard of care [10]. Inhibitors of VEGF are approved drugs for the treatment of age-related macular degeneration (AMD; pegaptanib, ranibizumab, or bevacizumab) [11]. Pegaptanib (Macugen) is an adaptamer representing a selective anti-VEGF-A_{165a} treatment [12]. Furthermore, aflibercept (VEGF-Trap) is a potent modulator of vasoregenerative responses and protects the retina in degenerative ischemic neovascular retinopathy [13]. These new therapeutic strategies might also have a positive impact on diabetic retinopathy [14, 15]. On the other hand, systemic safety of intravitreal anti-VEGF therapy is a matter of debate [16, 17]. Therefore, further research is necessary to achieve a better understanding of the underlying molecular mechanisms that regulate VEGF-A expression and release.

Different VEGF isoforms generated by alternative splicing have been described [18]. In the eye, retinal and the endothelial cells grow in close vicinity [19]. They might express different VEGF-A isoforms. In particular microvascular aberrations in the inner retinal vascular plexus might contribute to the pathogenesis of DME [20]. Therefore, we developed techniques to measure the expression of different VEGF isoforms in human retinal and endothelial cells. In addition, we stimulated human retinal and endothelial cells with glyoxal as model of oxidative stress under diabetic conditions and analyzed the VEGF-A_{165a} expression.

2 | MATERIALS AND METHODS

2.1 | Cell culture of retinal and human endothelial cells

Arising retinal pigment epithelial cell line-19 (ARPE-19) (ATCC) are a human adult diploid RPE cell population derived from a 19-year-old donor [21]. ARPE-19 cells were grown in Dulbecco's MEM (DMEM) and Ham's F12 nutrient mixture (DMEM/F12; Life Technologies) supplemented with 10% fetal calf serum (Biochrom AG). Cells were analyzed between passages P25 to P28. Trypsinized cells were counted and plated at a density of 3000 cells/cm^2.

The human microvascular endothelial cell line-1 (HMEC-1) [22] was kindly provided by Dr. Francisco J. Candal, Centers for Disease Control and Prevention and cultured on gelatin-coated plates in medium M199 supplemented with 10% (v/v) serum.

2.2 | Exposure of ARPE-19 and endothelial cells to glyoxal

Optimal glyoxal concentrations and incubation times were determined in preliminary experiments [23]. As a result, glyoxal (Sigma-Aldrich) was added at a concentration of 0–5 mM to the culture medium for 5 h at 24 h after seeding. In all experiments, cells cultured in medium without glyoxal served as internal time-matched controls.

2.3 | Cell viability

Cell viability was determined by Cell Titer Blue® as previously described [24].

2.4 | RNA preparation and RT-PCR

Total RNA from endothelial cells was isolated from human retinal and endothelial cells using peqGOLD TriFast™ (peqlab Biotechnologie), reverse transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen), and quantified by real-time PCR using a BioRad iQ iCycler Detection System (BioRad Laboratories) with Maxima™ SYBR Green qPCR Master Mix (2X) (Thermo Fisher Scientific Inc.). Optical density of amplified PCR fragments was quantified using AIDA software (Raytest) and normalized to EF as previously described [25].

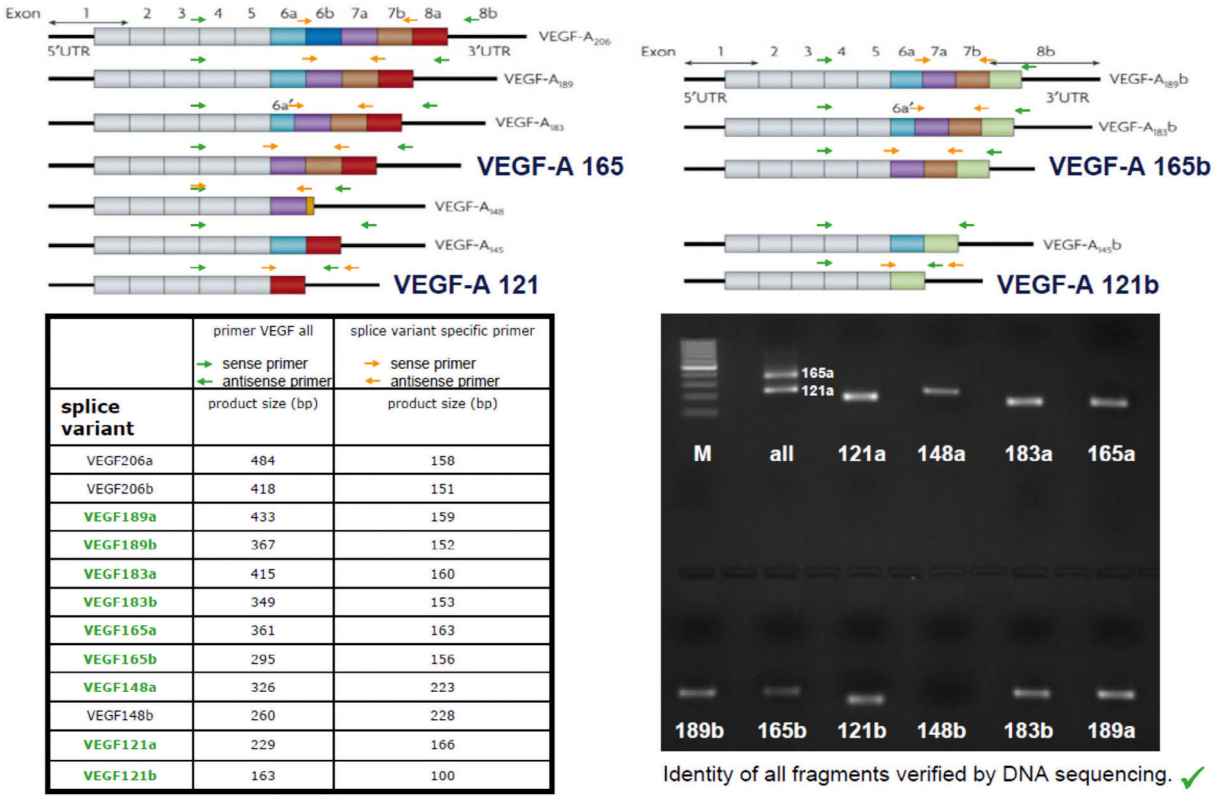
2.5 | Data analysis

Values are expressed as mean ± standard deviation (SD). The statistical analysis was performed using Graph Pad Prism 6 Software (GraphPad Software, Inc.). Differences in means with p values < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Development of methods to detect human VEGF-A splice variants

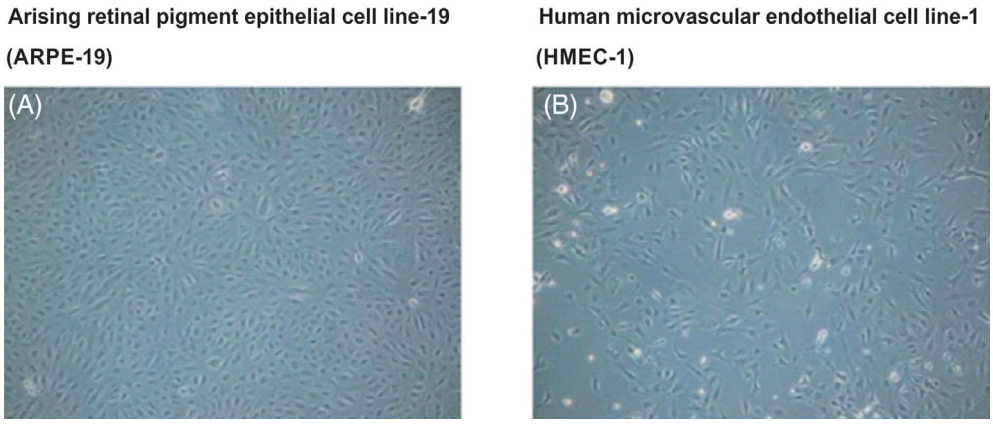
In this study, we established methods to study the expression of different VEGF isoforms in human retinal and endothelial cells. Isoform-specific primers (position indicated in orange and green in Figure 1) have been generated. They specifically detect the VEGF isoforms 121a, 121b, 148a, 165a, 165b, 183a, 183b, 189a, and 189b (Figure 1). Their molecular identity was confirmed by DNA sequencing. Primers for the isoforms VEGF 148b, 206a, and 206b did not amplify



	primer VEGF all sense primer antisense primer	splice variant specific primer sense primer antisense primer
splice variant	product size (bp)	product size (bp)
VEGF206a	484	158
VEGF206b	418	151
VEGF189a	433	159
VEGF189b	367	152
VEGF183a	415	160
VEGF183b	349	153
VEGF165a	361	163
VEGF165b	295	156
VEGF148a	326	223
VEGF148b	260	228
VEGF121a	229	166
VEGF121b	163	100

FIGURE 1 Development of methods to study the expression of different vascular endothelial growth factor isoforms. The upper part is modified after Harper & Bates [18]

FIGURE 2 Morphology of arising retinal pigment epithelial cell line-19 (ARPE-19) (A, left) and human microvascular endothelial cell line-1 (HMEC-1) (B, right)



specific fragments from RNA of human endothelial cells.

3.2 | Induction of VEGF-A_{165a} by glyoxal as model of oxidative stress in diabetes in human retinal and endothelial cells

Diabetic conditions can lead to increased glyoxal formation and the generation of oxidative stress. Therefore, we studied the impact of glyoxal on human retinal pigment epithelial cells (ARPE-19) and human microvascular endothelial cells (HMEC-1) (Figure 2).

We analyzed the impact of increasing dosages of glyoxal (0–5 mM) on the VEGF-A_{165a} expression in human retinal cells (ARPE-19) as a model of oxidative stress under diabetic conditions. Stimulation of ARPE-19 cells with glyoxal for 5 h increased VEGF-A_{165a} expression in human retinal cells reaching its maximum at 4.5 mM glyoxal (313 ± 54% of control, $p < 0.05$ vs. control, n 5) (Figure 3). The viability of the human retinal cells was not affected by glyoxal treatment (data not shown).

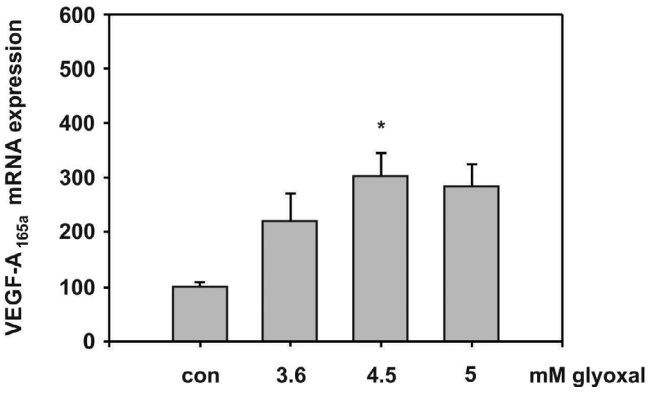


FIGURE 3 Dose-dependent impact of glyoxal on VEGF-A_{165a} expression in human retinal cells. Total RNA was isolated from human retinal cells exposed to glyoxal (0–5 mM) for 5 h, compared to internal time-matched control. Amplification of VEGF-A_{165a} variant was performed by real-time RT-PCR assay and normalized to EF2 ($n = 5$). *$p < 0.05$ vs. con

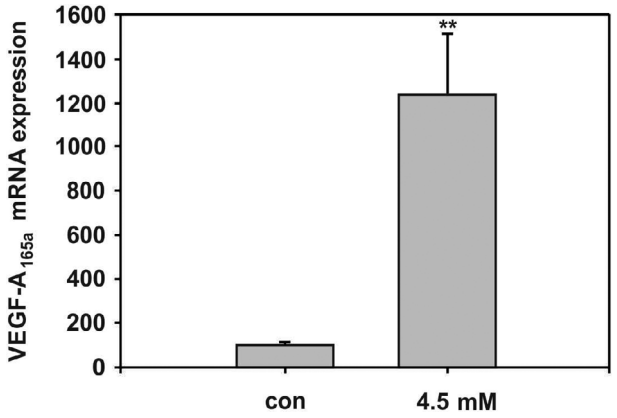


FIGURE 4 Impact of glyoxal on VEGF-A_{165a} expression in human endothelial cells. Total RNA was isolated from human endothelial cells exposed to glyoxal (4.5 mM) for 5 h, compared to internal time-matched control. Amplification of VEGF-A_{165a} variant was performed by real-time RT-PCR assay and normalized to EF2 ($n = 3$). **$p < 0.01$ vs. con

Next, we stimulated human endothelial cells with the same concentration of glyoxal for 5 h. We found an increased VEGF-A_{165a} expression in human endothelial cells reaching its maximum at 4.5 mM glyoxal (1236 ± 299% of control, $p < 0.01$ vs. control, n 3; Figure 4). Glyoxal treatment did not affect the viability of the endothelial cells (data not shown).

4 | DISCUSSION

The expression of pro- and antiangiogenic VEGF-A isoforms is differentially regulated by alternatively splicing and growth factors such as IGF1, TNFα, and TGFβ [26]. Whether glyoxal as a model of oxidative stress and diabetes is able to induce VEGF-A isoforms in different cell types is currently not well understood. In this study, we developed methods to detect VEGF-A isoforms in human retinal and endothelial cells. We could detect the proangiogenic isoforms VEGF-A 121a, 148a, 165a, 183a, and 189a and the antiangiogenic VEGF-A isoforms 121b, 165b, 183b, and 189b. Furthermore, we found a significant upregulation of the most important proangiogenic isoform VEGF-A_{165a} by glyoxal in retinal and endothelial cells. We could previously show that glyoxal induces advanced glycation end products and reactive oxygen species formation, VEGF-A protein release and apoptosis in retinal cells after 24 h [23]. Therefore, in this study we exposed human retinal and in addition microvascular endothelial cells to glyoxal for 5 h. The cell viability was not affected under these conditions in both cell types. The observed induction of the proangiogenic VEGF-A_{165a} in both human retinal and endothelial cells might contribute to enhanced vessel formation in patients with diabetic retinopathy [27, 28]. Furthermore, a cross-talk between retinal cells and the endothelial cells from microvessels could potentiate the deleterious effects of proangiogenic VEGF-A isoforms. Further studies are necessary to investigate a potential cross-talk between retinal and endothelial cells and the impact of oxidative stress. Selective inhibition of VEGF-A isoforms might provide a more specific therapeutic strategy in the prevention of enhanced vessel formation during diabetic retinopathy.

We observed in previous studies that lipoprotein apheresis reduced the expression of the proatherosclerotic oxLDL receptor LOX-1 and adhesion molecule VCAM-1 and increased the expression of vasoprotective and NO generating eNOS in human endothelial cells in response to serum of hypercholesterolemic patients [29]. This shows the potential of LDL apheresis to mediate positive effects on endothelial gene expression and function. Corresponding experiments will be the focus of future studies.

5 | CONCLUSION

In this study, we could detect pro- and antiangiogenic VEGF-A isoforms in human retinal and endothelial cells. The proangiogenic isoform VEGF-A_{165a} was induced by glyoxal in both cell types. These data suggest that diabetes and oxidative stress induce VEGF-A and the ingrowths of new blood vessels into the retina of diabetic patients. This might provide the basis for

novel and more selective therapeutic strategies in diabetic retinopathy.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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