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Relationship Between Vascular Endothelial Growth Factor and Interleukin-6 in Diabetic Retinopathy

[1]Amina A. Baiomy, [1]Lamiaa F. Arafa and [2]Asaad A. Ghanem
[1]Department of Medical Biochemistry, [2]Department of Ophthalmology,
Faculty of Medicine, Mansoura University, Egypt

Abstract: The present study has to investigated the relationship between diabetic retinopathy and the levels of vascular endothelial growth factor and IL-6 in aqueous humor and plasma and also determination of VEGF gene expression. The current study was carried out on undiluted aqueous humor samples and the corresponding plasma samples of 24 diabetic patients (24 eyes) and 12 non diabetic patients (12 eyes) who underwent cataract surgery. VEGF and IL-6 were measured in aqueous humor and plasma samples by Enzyme Linked Immunosorbent Assay (ELISA) kits and total RNA content was measured and VEGF gene expression was analysed by RT-PCR. VEGF and IL-6 levels revealed a high significant rise in aqueous humor of diabetic patients and non-significant rise in plasma of the same patients. A high +ve correlation was found between VEGF and IL-6 in aqueous humor samples of diabetic patients. In addition, present results revealed increased expression of three molecular forms of VEGF ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$) in diabetic patients. VEGF and IL-6 are produced together in the intraocular tissues and are involved in the pathogenesis of DR. VEGF gene expression is increased early in DR. So, anti-VEGF drugs may have future application for treatment of diabetic retinopathy.

Key words: VEGF, diabetic retinopathy, aqueous humor, plasma

INTRODUCTION

Diabetic eye disease develops as a complication of long-term diabetes and is the most common cause of blindness. It includes retinopathy and less frequently, rubiosis iridis, neovascular glaucoma and cataract[1]. Diabetic retinopathy begins with a nonproliferative phase involving increased vascular permeability, thickening of the basement membrane and loss of pericytes in the retinal capillaries. It progresses to the proliferative phase, in which neovascularization causes visual impairment. The severity increases as diabetes progresses and nearly all diabetic patients develop retinopathy within 20 years of diagnosis, half of which have proliferative retinopathy[2].

Many cytokines and growth factors including TNF-α, IL-1 β, IL-8, IL-6 and Vascular Endothelial Growth Factor (VEGF) are involved in the pathogenesis of diabetic retinopathy[3].

Vascular Endothelial Growth Factor (VEGF), a major mediator of vascular permeability and angiogenesis, may play a pivotal role in mediating the development and progression of diabetic retinopathy[4]. VEGF, a 45 kDa homodimeric glycoprotein, has drawn much attention as important mediator of retinal ischemia-associated intraocular neovascularization[5].

VEGF is produced from many cell types within the eye: retinal pigment epithelial cells, pericytes, endothelial cells, Muller cells and astrocyte[6]. The observation of increased retinal VEGF expression early in diabetic retinopathy and the finding in non diabetic animals that exogenous intraocular VEGF administration can elicit retinal abnormalities resembling diabetic retinopathy suggest that VEGF may also play a role in the development of the earliest stages of retinopathy[4].

Interleukin-6 (IL-6) is one of several proinflammatory cytokines that have been associated with insulin resistance and type II diabetes[7]. IL-6 is a multifunctional cytokine and it is synthesized by a variety of cells including fibroblasts, macrophages, epidermal cells, vascular endothelium and within the eye, the sources of IL-6 include, the retinal pigment epithelial cells, corneal epithelial cells, keratocytes, iris and ciliary body[6]. IL-6 was reported to be related to hyperglycemia and diabetic nephropathy[8]. Also, IL-6 is considered to be an inducer of angiogenesis that exerts its activity through the induction of VEGF[9].

Ray *et al.*[10] postulated that human VEGF gene is an independent risk factor for the development of diabetic retinopathy in patients with long-standing diabetics. They proposed that in the future individuals at increased risk of development DR may be identified genetically and offered

Corresponding Author: Dr. Amina A. Baiomy, Department of Medical Biochemistry, Faculty of Medicine, Mansoura University, Egypt E-mail: amina_baiomy@hotmail.com

enhanced screening or possibly novel interventions targeting VEGF action.

The above mentioned finding suggested that IL-6 might play a role in the pathogenesis of diabetic retinopathy in co-operation with VEGF. So, the aim of this study was to measure the concentrations of IL-6 and VEGF in the aqueous humor and plasma samples of diabetic patients and make a correlative study between them. Also, we studied the expression of VEGF gene to assess its possible role in the pathogenesis of diabetic retinopathy.

MATERIALS AND METHODS

Subjects: Present study was carried out on undiluted aqueous humor samples and corresponding plasma samples from 24 diabetic patients (24 eyes) (12 men and 12 women) and 12 non diabetic patients (6 men and 6 women) who underwent cataract surgery at Mansoura Ophthalmic Center.

The mean age of the patients with diabetes mellitus was 57.5 years (range 40-75 years) and that of non diabetic patients was 65.7 years (range 52.5-79 years). Exclusion criteria included prior ocular surgery, a history of intraocular inflammation and history of intraocular ischemia due to causes other than diabetic retinopathy.

Sample collection: Five milliliter fasting venous blood samples were withdrawn from the patients and control groups. Each blood sample was collected in EDTA containing tube and divided into two parts:

a. One milliliter was used immediately for RNA extraction.
b. The rest of the sample was centrifuged at 3000 rpm for 5 min at 4°C and the separated plasma was rapidly frozen at -80°C for storage until the time of assay.

Analysis of VEGF and IL-6: The concentration of VEGF and IL-6 were measured using the Enzyme Linked-Immunosorbent Assay (ELISA) kits according to Hyodo *et al.*[11] and Brailly *et al.*[12], respectively. The VEGF kit was able to detect two of the four known VEGF isoforms ($VEGF_{121}$ and $VEGF_{165}$), probably because these two shorter isoforms are secreted, while the two longer isoforms appear to be cell associated[13]. The levels of both factors in the aqueous humor and plasma were generally within the detection range of the assays, since the minimum detectable concentration was 15.6 pg/mL for VEGF and 0.156 pg/mL for IL-6.

Determination of VEGF gene expression

RNA extraction

- Total RNA was extracted from blood samples using purescript (USA) total RNA isolation kit, according to the method of Ausubel *et al.*[14] which is dependent on the lysis of red blood cells to facilitate their separation from the white blood cells.
- In brief, 300 μL whole blood with 900 μL RBC lysis solution were centrifuged at 16.000 rpm for 20 sec. The supernatant was removed and 300 μL cell lysis solution were added again to lyse the residual cells. One hundred microliter of protein-DNA precipitation solution were added to the cell lysate then centrifugation at 16.000 rpm for 3 min and the supernatant was transferred to an OaK ridge centrifuge tube containing 300 μL of 100% isopropanol then centrifugation at 16.000 rpm for 3 min. The RNA appeared as a small, translucent pellet which was washed by adding 300 μL 70% Ethanol then centrifugation at 16.000 rpm for 1 min. Fifty microliter of RNA hydration solution was added to RNA on ice for 30 min at least.

RNA was quantified spectrophotometrically at 260 nm and the purity was estimated from the relative absorbance at 260 and 280 nm. Pure RNA will exhibit an A_{260}/A_{280} ratio 1.7-2.1 RNA samples were stored at -70°C until time of use within the same week.

RT-PCR and analysis of the products

- RT-PCR kit supplied by Amersham Biosciences was used according to method of Berchtold[15].
- RT-PCR was carried out on total RNA isolated from different groups studied.
- All primers were synthesized at the Biosource Europe Laboratories. The sequences of the primers used, as designed by Klauspodar *et al.*[16] are shown as follow;
- Sense primer: 5^{-}-TCGGGCCTCCGAAACCATGA-3^{-}.
- Antisenseprimer: 5^{-}-CCTGGTGAGAGATCTGGTTC-3^{-}.
- One-step protocol for RT-PCR in which a total reaction volume 50 μL, containing 3 μL primer a,b each 1.5 mM $MgCl_2$, 34 μL DEPC treated water, 5 μL template RNA. The mixture was overlayed with 50 uL of mineral oil and then RT-PCR was performed in programmable thermal minicycler.

- The cycle conditions were as follow: 10 min at 95°C and 30 min at 42°C followed by 45 cycles of 5 min at 95°C for (denaturation). One min at 55°C (annealing) and lastly 2 min at 72°C (extension). Then one cycle 5 min at 72°C for final extension. Then the samples were then rapidly cooled to 40°C.
- The solution containing the PCR product (9 μL) was mixed with 1 μL of loading dye (0.1 Bromophenol blue and 30% glycerol in water) and loaded into agarose gel containing 0.5 μg mL^{-1} ethidium bromide in 1XTBE buffer. The samples were run in 1XTBE buffer for 30 min at 140 V in a mini-gel apparatus. A DNA marker (DNA/Hae III) was run in parallel as size marker[17].

The product of PCR amplification were subjected to agarose gel electrophoresis using 1.8% agarose gel, containing 10 μg ethidium bromide (purchased from sigma-Aldrich, st. Louis. Mo), for one hour at 100 volt.

Statistical analysis: Results were expressed as mean±SE for (n) experiment. Statistical significance between groups were determined using unpaired student-t-test. Correlation coefficients (r) between the different variables were calculated with spearmann rank test. P values less than 0.05 were considered significant. These tests were done on an IBM compatible personal computer using the Statistical Package for Social Scientists (SPSS vesion 10).

RESULTS

Aqueous humor concentration of VEGF was highly significantly increased when compared with that of non-diabetic patients (control) [255.20±24.235 pg/mL vs. 46.2666±9.83 pg/mL, p = 0.0001] as shown in Table 1.

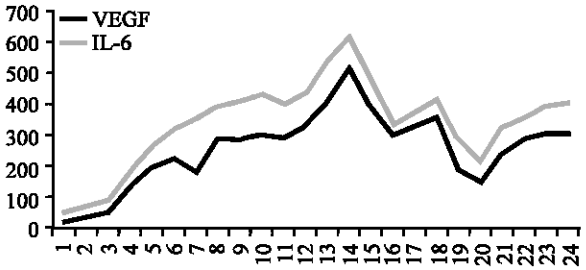

Fig. 1: The correlation between the aqueous levels of VEGF and IL-6

Also, aqueous humor concentration of IL-6 was highly significantly increased when compared with that of non-diabetic patients [83.56±7.95 pg/mL vs. 13.60±2.34 pg/mL, p = 0.0001] (Table 1).

On the other hand, VEGF concentration in plasma of diabetic patients was non significantly increased when compared with that of non-diabetic patients (control) [44.281±6.68 pg/mL vs. 36.733±6.84 pg/mL, p = 0.943] (Table 1). Also, IL-6 concentration in plasma of diabetic patients was non significantly increased when compared with that of non-diabetic patients [2.163±0.713 pg/mL vs. 1.834±0.704 pg/mL, p = 0.665] (Table 1).

VEGF and IL-6 concentration in aqueous humor of diabetic patients were significantly higher when compared with the corresponding concentrations in plasma of the same patients (P = 0.0001 in both) (Table 1).

Aqueous concentrations of VEGF in diabetic patients were significantly correlated with those of IL-6 [r = 0.305, p = 0.03] (Table 2 and Fig. 1).

No significant correlation between aqueous and plasma levels of VEGF and IL-6 (Table 2).

No cDNA was amplified from control group (Lane 3). VEGF specific amplifications of cDNA yielded a PCR

Table 1: Vascular Endothelial Growth Factor (VEGF) and Interleukin-6 (IL-6) concentrations in aqueous humor and plasma of diabetic patients and non-diabetic patients (control)

	VEGF in aqueous humor (pg/mL)		VEGF in plasma (pg/mL)		IL-6 in aqueous humor (pg/mL)		IL-6 in plasma (pg/mL)	
	Control (n = 12)	Diabetic patients (n = 24)	Control (n = 12)	Diabetic patients (n = 24)	Control (n = 12)	Diabetic patients (n = 24)	Control (n = 12)	Diabetic patients (n = 24)
Range	8.80-106.80	23.5-515	12.5-81.00	18.3-181.60	0.92-30.30	3.81-171.20	0.15-9.22	0.15-18.1
Mean	46.266	255.204	36.733	44.281	13.602	83.56	1.834	2.163
SE of Mean	±9.783	±24.235	±6.845	±6.68	±2.344	±7.95	±0.704	±0.713
T	6.219		0.074		5.815		0.444	
P	<0.0001		<0.943		<0.0001		<0.665	
	t		8.419		t		10.000	
	p		<0.0001		P		<0.0001	

Table 2: Kendall's correlation between VEGF and IL-6 in aqueous humor and plasma of diabetic patients and nondiabetic patients (control)

Correlation between studied variables	r	p	Level of significance
VEGF (aqueous) versus VEGF (plasma)	0.284	0.189	Non significant
IL-6 (aqueous) versus IL-6 (plasma)	0.150	0.308	Non significant
VEGF (aqueous) versus IL-6 (aqueous)	0.305	0.030	High significant
VEGF (plasma) versus IL-6 (plasma)	0.383	0.071	Non significant

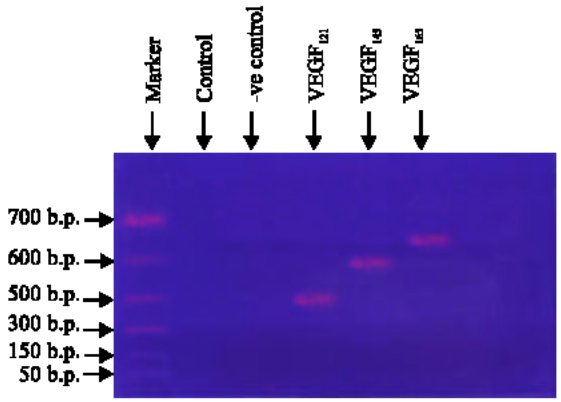

Fig. 2: cDNA of VEGF gene isoforms

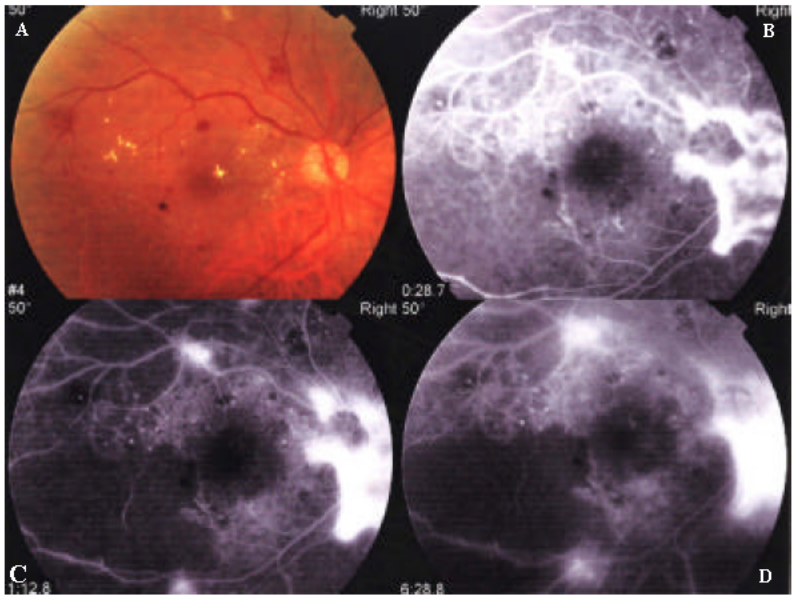

Fig. 3: Female aged 55 years showing red free fundus photography (A) and fluorescein angiography (B, C, D) with proliferative diabetic retinopathy

product of 516 bp of $VEGF_{121}$ (lane 4) and 588 bp of $VEGF_{145}$ (lane 5) and 648 bp $VEGF_{165}$ (Lane 6). Marker (Lane 1) and negative control in (Lane 2) as shown in Fig. 2.

A case showing fundus photography and fluorescein angiography with proliferative diabetic retinopathy (Fig. 3).

DISCUSSION

Diabetes leads to specific microvascular complications of retinopathy, nephropathy and neuropathy, as well as increased risk of atherosclerosis, which may reflect underlying endothelial dysfunction. The risk of developing these complications increases with poor glycemic control[18].

Diabetic Retinopathy (DR) is a major cause of new-onset blindness among diabetic adults and is characterized by increased vascular permeability, tissue ischemia and neovascularization. Neovascularization of retina carries a high risk of blindness as a result of vitreous hemorrhage and fibrosis[10]. The exact mechanism by which diabetes causes retinopathy remains unclear. Certain cytokines and growth factors appear to play a causative role in the development and progression of diabetic retinopathy such as Vascular Endothelial Growth Factor (VEGF) and interleukin-6 (IL-6)[19].

In the current study we simultaneously measured the aqueous humor and plasma levels of VEGF which induces an increase of vascular permeability and angiogenesis and those of IL-6, a proinflammatory factor, in patients with diabetic retinopathy.

The present study showed high significant increase of VEGF levels in aqueous humor of diabetic patients when compared with the control group ($p = 0.0001$). VEGF increase in aqueous humor of Diabetic Retinopathy (DR) patients, was also reported by Funatsu *et al.*[6], Caldwell *et al.*[20], Ray *et al.*[10] and Song *et al.*[21].

Funatsu *et al.*[6] stated that diabetic patients with an apparently normal fundus show significant breakdown of the blood-retinal barrier (BRB) in the early stage of retinal involvement in diabetes. VEGF increases vascular permeability and is a major factor promoting BRB breakdown in simple DR. VEGF expression in the retina and the optic nerve proceeds retinal neovascularization in patients with diabetes. VEGF concentrations are elevated in both the vitreous and aqueous humor of patients with active proliferative DR[6].

VEGF is increased in the retina of streptozotocin-induced diabetic rats at 5 months and in the retina of patients with non-proliferative and proliferative DR[20].

Ray *et al.*[10] postulated that VEGF can stimulate angiogenesis, enhance collateral vessel formation and increase the permeability of the microvasculature. So, VEGF plays a role in the neovascularization of proliferative retinopathy and in the breakdown of the blood retinal barrier that is characterized by hyperpermeability of retinal vessels. So, VEGF have been found to be markedly elevated in the vitreous and aqueous fluids in the eyes of patients with DR[22].

VEGF production is known to be stimulated by high glucose levels, advanced glycosylation end products, IGF-1, angiotensin II and hypoxia, all of which are present in the retinal microvascular bed[22].

These reports and the present study suggested that VEGF might be implicated in the pathogenesis of both proliferative and non proliferative diabetic retinopathy.

The development of DR cannot be explained by the actions of VEGF alone, suggesting that various cytokines may form a network that influences the formation and exacerbation of DR along with VEGF[23]. The present study shows that, Aqueous humor concentration of IL-6 was significantly higher in the diabetic group when compared with that of the control group ($p = 0.0001$).

IL-6 are reported to be higher in patients with active proliferative DR than in patients with inactive proliferative DR[6].

The IL-6 level in aqueous humor is elevated in patients with uveitis and endotoxin-induced uveitis[24]. Endotoxin-induced uveitis is characterized by breakdown of the blood-ocular barrier and by infiltration of the anterior chamber with polymorphonuclear cells and macrophages which are the predominant cells of IL-6 production[24].

The IL-6 concentration in vitreous fluid is also elevated in proliferative vitreoretinopathy which is characterized by breakdown of the blood-ocular barrier[25]. The neuroprotective effect of IL-6 was evaluated by giving intravitreal injections of IL-6 to eyes immediately after retinal ischemia reperfusion injury in rats[26].

Nakamura *et al.*[27] suggested that increased formation of advanced glycation end products in the vitreous may be involved in the development of diabetic retinopathy by inducing the production of IL-6 from retinal Muller cells. Also, IL-6 derived from blood during the breakdown of the blood-retinal barrier and produced by retinal pigment epithelial cells and hematogenous soluble IL-6 receptors (S IL-6R) cause retinal pigment epithelial cells proliferation[28].

It has been shown that a wide range of ocular tissues can produce IL-6 *in vitro* and *in vivo* such as cultures of cornea epithelial, stromal and endothelial cells, iris and ciliary body explants; cytokine-stimulated human pigment epithelial cells, ischemic retina and hypoxia-induced or cytokine-stimulated vascular endothelial cells and vascular smooth muscle cells. Inflammatory cells, especially mast cells are known to be able to stimulate IL-6 secretion from leukocytes and human vascular endothelial cells in ischemic and inflammatory conditions. Therefore, there is a strong possibility that IL-6 in aqueous humor of eyes comes from intraocular sources, such as ocular and inflammatory cells[29].

Balasubramanyam *et al.*[30] postulated that preretinal proliferative membrane formation which is regulated by various cytokines, is a very important step in the pathogenesis of diabetic retinopathy. In addition to VEGF, transforming growth factor-2, IL-6,IL-8 and TNF-α have been shown to play a key role in preventing membrane formation. Contraction of neovascular and proliferative membranes is closely associated with cytokine expression by retinal cells, particularly Muller cells. These reports taken together with the current study suggested that IL-6 might be implicated in the pathogenesis of DR.

In the current study, the VEGF level in aqueous humor was significantly correlated with that of IL-6 ($p = 0.03$). This can be illustrated by the fact that IL-6 is known to indirectly induce angiogenesis via the induction of VEGF expression and IL-6 and VEGF are involved in paracrine tumor stromal cell interactions in multiple myeloma[31]. VEGF can promote the activation and migration of monocytes and IL-6 mediated mechanisms

are involved in the production of VEGF through an interaction between monocytes and vascular smooth muscle cells[32]. These reports and the present study results suggest that VEGF is directly involved in the pathogenesis of proliferative and non-proliferative DR and conversely, IL-6 may have a direct role in non proliferative DR and an indirect role in the proliferative stage via VEGF. So, the correlation between VEGF and IL-6 may also be important in the pathogenesis of DR.

In the present study, the levels of both VEGF and IL-6 in the aqueous humor were higher than the corresponding plasma levels, while there was no correlation between the aqueous and plasma levels of VEGF or IL-6. These results suggested that the elevation of VEGF and IL-6 levels in aqueous humor were not related to breakdown of the BRB and/or ocular blood.

There was no significant correlation between aqueous and plasma levels of VEGF and/or IL-6 and VEGF and IL-6 levels were much higher in the aqueous than in the plasma. These results are in agreement with those of Funatsu *et al.*[6], Funatsu *et al.*[31] and Tsukamoto *et al.*[33].

Funatsu *et al.*[31] stated that vitreous levels of VEGF are not influenced by its serum concentration in DR. Up-regulation of VEGF is induced by local hypoxia in DR, which is caused by occlusion of retinal vessels. It is unknown whether increased aqueous levels of IL-6 are due to enhanced production in other organs and/or in the eye itself but production in the eye may be the cause.

The increased expression of VEGF has become a focal point of current research on the pathogenesis of DR, as well as other retinal and choroidal vascular diseases[34]. Normaly, VEGF expression decreases substantially after birth, but some cells constitutively secrete picomolar amounts, cells in the neural retina and cells in the combined choroid and retinal pigment epithelium[35].

VEGF expression is enhanced by hypoxia which is a major stimulus for retinal neovascularization[36]. Reduced retinal blood flow and accompanying hypoxia may be present even before the early signs of retinopathy and are likely to be accompanied by an increase in the synthesis and secretion of VEGF[37].

The current study shows an increased expression of three molecular forms of VEGF gene ($VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$) in diabetic patients while no cDNA was amplified from the control group. Song *et al.*[21] stated that the expression of VEGF was increased in DR and VEGF began to cooperate with the increased Basic Fibroblast Growth Factor (b FGF) which probably due to the inducement of anoxia. The expression of VEGF and bFGF are the signals of neovascularization which is the main pathological change in DR.

The current study suggests that both VEGF and IL-6 are produced together in the intraocular tissues and are involved in the pathogenesis of diabetic retinopathy. The association of VEGF gene expression with DR is an indicative to the very important role for VEGF in pathogenesis of DR.

The most effective over all strategy for DR is to prevent it as much as possible, so, anti-VEGF drugs may have future application for treatment of diabetic retinopathy.

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