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Serum VEGF and CFH in Exudative Age-Related Macular Degeneration

Paulina Haas^{1,2}, Kerstin Steindl¹, Tina Aggermann², Katharina Schmid-Kubista², Walter Krugluger³, Gregory S. Hageman⁴, and Susanne Binder^{1,2}

¹Ludwig Boltzmann Institute for Retinology and Biomicroscopic Lasersurgery, Rudolf Foundation Clinic, Vienna, Austria

²Department of Ophthalmology, Rudolf Foundation Clinic, Vienna, Austria

³Department of Clinical Chemistry, Rudolf Foundation Clinic, Vienna, Austria

⁴University of Utah, Center for Translational Medicine, Ophthalmology and Visual Science, Salt Lake City, Utah, USA

Abstract

Purpose—To determine serum vascular endothelial growth factor 165 (VEGF165) levels and the association of the complement factor H gene (CFH) Y402H polymorphism in patients with exudative age-related macular degeneration (AMD) in comparison to unaffected control subjects.

Methods—Sixty-six AMD patients and 66 healthy age- and gender-matched controls were included in this case-control study. The serum VEGF165 was assayed by ELISA (R&D). Genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism analysis. Chi-squared tests were used regarding the polymorphism, a *t*-test regarding the VEGF-levels.

Results—Levels of serum VEGF165 were similar in both groups (*p*-value = 0.2112). Genotype frequency differed significantly between patients with exudative AMD and the healthy control group (*p* = 0.003136). The serum VEGF165 levels were similar irrespective of the presence of the CFH Y402H polymorphism (*p* = 0.4113) and independent of the specific genotype (*p* = 0.9634).

Conclusion—In the present study exudative AMD is not associated to serum VEGF165 levels; furthermore, our data does not establish a statistical link between VEGF165 and the CFH Y402H polymorphism.

Keywords

Age-related macular degeneration; Complement; Genetics; Neovascular; Vascular endothelial growth factor

Introduction

Age-related macula degeneration (AMD) is a progressive degenerative disorder in the central region of the retina. It is the leading cause of legal blindness in people aged over 55 years in developed countries. Among those patients with severe visual impairment, nearly 90% are caused by exudative AMD, which is a subtype of late-stage AMD found in approximately 20% of all AMD cases.¹

Choroidal neovascularization (CNV) is the hallmark of exudative AMD.² Neovascular stimulus is initiated by increased secretion of vascular endothelial growth factor (VEGF) from retinal pigment epithelium (RPE) cells, preceded by different inflammatory stimulus including cytokines and other proinflammatory molecules, which in turn causes migration and proliferation of choroidal endothelial cells.³

Vascular endothelial growth factor 165 (VEGF165) appears to be the isoform of VEGF responsible for pathological ocular neovascularization.⁴⁻⁶ VEGF expression is upregulated by a number of growth factors, including epidermal growth factor, transforming growth factor- α and - β , keratinocyte growth factor, insulin-like growth factor-I, FGF, and platelet-derived growth factor. Thus, a variety of local interactions can modulate local VEGF concentrations.⁷

Complement components C3a and C5a also induce VEGF expression, which could induce choroidal neovascularization.⁸ Complement factor H normally inhibits C3 deposition and C5a release after complement activation,⁹ dysfunctional CFH induces increased production of C3a and C5a.

This study was conducted to determine the difference in levels of serum VEGF165 between patients with exudative AMD and healthy controls. Additionally, we determined the frequency of the CFH Y402H polymorphism in patients with age-related macular degeneration in the Austrian population of Caucasian descent.

Methods

Study Design: Case-Control Study

One hundred thirty-two consecutive patients seen in our department of ophthalmology were included in this study: these included 66 unrelated patients with exudative AMD and 66 control subjects, mostly patients registering for cataract surgery. All patients were aged 55 or older, were of Caucasian origin, and lived in the same geographical area of Austria.

Written informed consent was obtained prior to enrollment. The study was performed in accordance with the Austrian Gene Technology Act, the tenets of the Declaration of Helsinki, and the guidelines of the local ethics committee.

Exudative AMD was diagnosed by ophthalmoscopic fundus examination, optical coherence tomography, and fluorescein/indocyanine angiography. Patients with hereditary diseases, polypoidal choroidal vasculopathy or secondary CNV due to pathologic myopia (>-2 diopters, spherical equivalent), angioid streaks, inflammatory or infectious chorioretinal

disease, trauma, or diabetic retinopathy were excluded from the study. AMD patients with previous performed anti-VEGF therapy have been excluded.

All AMD patients enrolled in the present study were classified according to the Age-Related Eye Disease Study system; subgroups were noted (predominantly classic choroidal neovascularization, minimal classic choroidal neovascularization, occult choroidal neovascularization, and retinal angiomatous proliferation) according to the angiographic findings.

Exclusion criteria for the age- and sex-matched controls were evidence of age-related maculopathy (drusen as well as pigmentary changes), macular hemorrhages of any cause. Media opacities resulting in impaired visualization of the macula (cataract grade 3 and 4) was an exclusion criteria for both groups.

Serum VEGF165 Levels

Peripheral venous blood samples were collected according to standard hospital procedure. Red topped tubes were centrifuged at 3000 r/min for 10 min at 4°C and the serum separated and stored at -70°C.

The serum VEGF165 level was determined using a quantitative sandwich enzyme immunoassay technique according to the manufacturer's guidelines (R&D Systems, Inc., Minneapolis, Minnesota, USA).

A quantitative sandwich enzyme immunoassay technique was used for the quantitative determination of human vascular endothelial growth factor (VEGF165) concentrations according to manufacturer's guidelines. A monoclonal antibody specific for VEGF165 was precoated onto a microplate. Standards and samples were pipetted into the wells and any VEGF165 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF165 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of VEGF165 bound in the initial step. The color development was stopped and the intensity of the color measured. The recombinant human VEGF standards were measured to form a standard curve. Results were expressed in ng/L.

Genotyping

Genotyping was carried out in a random order by an experienced technician who was masked to the disease status of the samples. An aliquot of 5 mL venous blood from each subject was withdrawn and collected in an EDTA-containing tube. Genomic DNA was isolated from whole blood using a commercial kit (QIA-AMP DNA blood mini kit, Qiagen, Vienna, Austria). The single-nucleotide polymorphisms (Y402H; rs 1061170) located in exon 9 of CFH was genotyped by PCR-directed sequencing using the following primer sequences: 5'-CTT TAG TTC GTC TTC AGT TAT AC-3' (forward) and 5'-GTC ATC TAT GTT ACT TAG AAA GT-3' (reverse). PCR products were then purified and sequenced by Single-Read Sequencing reaction (VBC-Biotech, Vienna, Austria).

Statistics

A sample size calculation was performed using an alpha error of 0.05 (5%), a beta error of 0.20 (20%), and a clinical significant difference of 0.18 (18%) to determine the power calculation.

To find differences in groups depending on the polymorphism, Chi-squared tests were observed. If the premises were not fulfilled, Fisher's exact tests were performed.

To find out the height of a VEGF-level depending on AMD or healthy controls, a *t*-test was computed. Negative values of the titer were replaced by zero, because these are not possible and may occur by incorrect measurements of the instruments used.

Analysis was performed using R 2.4.0 and SAS 9.1. All *p*-values smaller than 0.01667 were considered to be statistically significant. The critical boundary of 0.0125 results from correction for multiplicity according to Bonferroni due to the number of tests (three tests were performed, $0.05/3 = 0.01667$).

Results

In the exudative AMD group, 66 patients were enrolled in this study. Subgroups were noted and classified according to angiographic findings consisting of 16 patients with predominantly classic CNV, 11 patients with minimal classic CNV, 31 patients with occult CNV, and 8 patients with retinal angiomatous proliferation. Sixty-six healthy controls were age- and gender-matched. The AMD group consisted of 35 (53.0%) females and 31 (47.0%) males, and the mean age was 78.0 ± 7.1 years (range: 56–92 years). The control group consisted of 34 (51.6%) females and 32 (48.4%) males, and the mean age was 76.5 ± 6.5 years (range: 62–94 years).

Regarding the VEGF165 levels, there was no statistically significant difference between the exudative AMD group and the control group ($p = 0.2112$) (Figure 1). Serum VEGF levels according to the angiographic type of CNV are shown in Table 1.

Genotype frequency differed significantly between the patients with exudative AMD and the control group ($p = 0.003136$), (1277TT 21.2%, 1277TC 53.0%, and 1277CC 25.8% in the AMD group; 1277TT 54.5%, 1277TC 43.9%, and 1277CC 15.2% in the control group) (Figure 2).

Genotype distribution according to the angiographic type of CNV is shown in Table 2.

The serum VEGF165 levels were similarly irrespective of the presence of the CFH Y402H polymorphism ($p = 0.4113$) and independent of the specific genotype ($p = 0.9634$); (Tables 3 and 4).

Discussion

This study was conducted to determine whether differences in levels of serum VEGF165 exist between patients with exudative age-related macular degeneration and healthy controls.

Second, the prevalence of the CFH Y402 polymorphism in patients with age-related macular degeneration as compared to a healthy control group in the Austrian population was investigated.

VEGF is a member of the platelet derived growth factor (PDGF) family and plays a central role in the angiogenesis and embryonic vasculogenesis. This growth factor increases microvascular permeability of post capillary venules and capillaries; it is able to stimulate all major functions of endothelial cells needed in angiogenesis—including increased permeability, migration, proliferation and tubeformation.^{10,11} VEGF messenger RNA has been demonstrated in almost all tissues of the normal eye, most notably in the ciliary body, conjunctiva, RPE/choroids, and the lens.

VEGF selectively stimulates endothelial cells by binding to two receptors, VEGFR-1 and VEGFR-2, which respond in typical fashion to ligand binding by activation of signal transduction cascades. VEGFR-2 is believed to be the principal receptor for VEGF signalling in angiogenesis.⁷

Alternative splicing of the VEGF gene results in at least four major biologically active human isoforms, containing 121, 165, 189, and 208 amino acids. The isoforms appear to have identical biological activity but differ in molecular weight and affinity to heparin. VEGF165, the predominant isoform in the human eye, is a secreted, heparin-binding, homodimeric, 45-kDa glycoprotein.^{10–13}

VEGF165 is the isoform responsible for pathological ocular neovascularization, whereas VEGF121 appears to be essential for normal retinal vascular function (20–22). Elevated VEGF levels have been found in aqueous humor and vitreous of most patients with proliferative diabetic retinopathy and ischemic central retinal vein occlusion, but only rarely in patients without ocular neovascularization.^{14–16} Immunohistochemical studies of autopsy eyes found VEGF to be elevated in the RPE and choroidal blood vessels of maculas with AMD.¹⁷ In subfoveal fibrovascular membranes, the expression of VEGF mRNA was higher in areas with marked inflammatory signs.^{18,19}

In our study serum VEGF165 levels showed no statistically significant difference between the exudative AMD patients and healthy controls (p -value = 0.2112).

In contrast, one previous study showed that AMD patients with active CNV had a significantly higher plasma level of VEGF165 than normal controls.²⁰ The study of Lip et al. showed only a weak association between plasma VEGF165 level and CNV in AMD patients.²¹

Our study revealed higher levels of serum VEGF165 in patients with predominantly classic CNV, as compared to levels in patients with minimal classic CNV, occult CNV, and RAP. This might be explained by the pathogenesis of the predominantly classic CNV and correlated to the progressive dye-leakage as seen in the early phase of the fluorescein angiogram.

VEGF expression is upregulated by a number of growth factors, including epidermal growth factor, transforming growth factor- α and - β , keratinocyte growth factor, insulin-like growth factor-I, FGF, and platelet-derived growth factor, so that a variety of local interactions can modulate local VEGF concentrations.⁷

The route of new vessel growth has been suggested to be in areas of lesser resistance, for example defects in Bruch membrane and RPE irregularities.³

C3a and C5a induce VEGF expression and, as such, can stimulate choroidal neovascularization. The complement factor H inhibits C3 deposition and C5a release after complement activation. The impairment of this regulation in the alternative pathway of the complement activation leads to excessive liberation of different cleavage fragments, such as C3a and C5a, and to the formation of the C5b9 complex, which in turn are able to induce endothelial cell activation and release of growth factors, such VEGF.^{8,9}

Several studies have confirmed an association of the complement factor H (CFH) Y402 gene polymorphism to the risk of AMD.^{12, 22–29} Our data confirms the higher frequency of the Y402 polymorphism of CFH in exudative AMD patients, as compared to healthy controls (p -value = 0.003136). Regarding the different subgroups of AMD, homozygosity and heterozygosity was found nearly equally in patients with occult CNV. Interestingly, homozygosity was more prevalent in patients with occult CNV in comparison to those with RAP, predominantly classic CNV, and minimal classic CNV.

Serum VEGF165 levels showed similar results independent of the presence of the CFH polymorphism and independent of the specific genotype distribution in patients with exudative AMD.

In conclusion, our data shows that exudative AMD is not associated to seric VEGF165. These findings underline that AMD is a localized disease with no affect on the circulating serum VEGF165 levels.

Our data confirms that the CFH Y402H polymorphism is a risk factor for exudative age-related macular degeneration with a higher frequency of the Y402 polymorphism in AMD patients.

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Figure 1.

Mean (with standard deviation) VEGF levels (y-axis) in exudative AMD patients compared to the control group (x-axis).

Figure 2. Genotype distribution (y-axis) of the CFH Y402H polymorphism in the exudative AMD patients compared to the control group (x-axis) in percentages.

Table 1

Serum VEGF165 level according to AMD subtypes.

Exudative AMD subtypes	n	Serum VEGF165 level (ng/L)		
		Mean (ng/L)	Min	Max
Predominantly classic CNV	16	531.89575	155.182	999.618
Minimally classic CNV	11	324.537273	73.828	674.201
Occult CNV	31	375.936767	71.768	1123.194
RAP	8	289.307875	98.543	613.443
				177.1

CNV = choroidal neovascularisation; RAP = retinal angiomatous proliferation; SD = standard deviation.

Table 2

CFH genotype distribution of the complement factor H polymorphism Y402H in different subtypes of exudative AMD.

Exudative AMD	CFH-polymorphism		
	Normal <i>n</i> (%)	Hetero <i>n</i> (%)	Homo <i>n</i> (%)
Predominately classic CNV	2 (12.5% YY)	10 (62.5% YH)	4 (25.0% HH)
Minimally classic CNV	3 (27.3% YY)	7 (63.3% YH)	1 (9.0% HH)
Occult CNV	6 (19.4% YY)	13 (41.9% YH)	12 (38.7% HH)
RAP	3 (37.5% YY)	5 (62.5% YH)	0 (0% HH)

CNV = choroidal neovascularisation; RAP = retinal angiomatous proliferation.

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Table 3
VEGF165 level according to positive or negative complement factor H polymorphism Y402H.

Polymorphism	n = 66	VEGF165 Level (ng/L)		
		Mean (ng/L)	Min	Max SD
Yes	52	387.401904	71.768	1123.194 242.1
No	14	414.320571	88.245	825.582 239.3

SD = Standard Deviation.

Table 4

VEGF165 level according to genotype distribution of the CFH polymorphism Y402 in patients with exudative AMD.

Genotype	n = 66	VEGF165 level (ng/L)			
		Mean (ng/L)	Min	Max	SD
YY (Normal)	14	414.320571	88.245	825.582	239.3
YH (Hetero)	35	362.407229	73.828	999.618	228.3
HH (Homo)	17	438.861529	71.768	1123.194	267.9

SD = Standard Deviation.