Exploring the role of VEGF in Indian Age related macular degeneration

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KEY WORDS

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ABSTRACT

Background: Age related macular degeneration (AMD) is major devastating neurodegenerative disorder characterized by progressive irreversible vision loss in the elderly persons. In spite of several genetic and environmental factors, the role of VEGF and CFH predispose the pathological phenomenon in the AMD patients.
Purpose: The aim of the study was to estimate the VEGF levels in the serum of AMD patients and its correlation with co-morbidity of the participants.
Methods: The study recruited the 98 AMD patients and 59 controls with proper consent of the participants as per the exclusion-inclusion criteria. The co-morbidity and socio-economic details were obtained by introducing the standard questionnaire amongst the participants. Serum levels of vascular endothelial growth factor (VEGF) was estimated by ELISA and compared with the control population of the study. The levels of VEGF in the serum of AMD patients and controls were compared with Mann-Whitney *U*-test. Kruskal Wallis one-way analysis of variance (ANOVA) was employed to analyze more than two variables in the study.
Results: Elevated level of VEGF was found in AMD patients as compared to controls. Surprisingly, we did not find significant changes among wet AMD subtypes i.e. minimal, predominant and classic wet AMD. However, we have demonstrated the intravitreal anti-VEGF treatment (avastin) in AMD patients could reduce the systemic VEGF levels although it was not significant. Moreover, the heart ailment in the AMD patients could also influence the VEGF levels.
Conclusion: Our study is consistent with previous studies describing the imperative significance of VEGF in AMD pathology. However, our study did not reveal the role of VEGF in wet AMD progression but it is well established causative agent for the same. The increased levels of VEGF in heart ailment among AMD patients are significant.

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Introduction

Neovascularization (new blood vessel formation) is a characteristic feature of several disease pathologies including cancer and age related macular degeneration (AMD). These newly formed blood vessels have tendency to spread the disease condition in the tissue due to their leaky nature. Neovascularization underlying choroid can percolate the Bruch's membrane and disrupt the integrity of retinal pigment epithelium (RPE) layer. Neovascularization process is guided by several angiogenic factors including Platelet-derived growth factor (PDGF)[1,2], matrix metalloproteinases (MMPs),[3,4] Pigment epithelium-derived factor (PEDF)[5], TGF-β and its receptors.[6,7] Among all, vascular endothelial growth factor (VEGF) is more potent to lead angiogenic process. Although, it has been found that TGF-β could induce the VEGF expression in RPE cells, with the involvement of MAP kinases, signifies the role of TGF beta as the pro-angiogenic protein in enabling angiogenic process.[8] Apart from these angiogenic factors, several pro-angiogenic factors (TIMPs family and FGF)[3,4] are also involved in the process. Hence, neovascularization is a complex process having role of both angiogenic and pro-angiogenic factors. Uncontrolled phenomenon of angiogenesis leads to the pathological condition like in cancer and AMD.

AMD is the leading cause of irreversible blindness in the elderly people and is characterized by age related changes in the retina like formation of drusen and new vessels in between the retinal pigment epithelial (RPE) layer and Bruch's membrane.[9] The components of drusen can also promote the angiogenic factors to perform their action and consequently result in wet AMD.[10] It has already been investigated that AMD is complex disorder and, both environmental and genetic factors equally distribute in the disease pathology. The various studies on AMD suggested the role of VEGF in disease pathology.[5,11–12] VEGF ligands regulate its functions through binding with tyrosine kinase receptors VEGFR-1 and VEGFR-2.[13] Receptor VEGFR-2, plays important role in angiogenesis of endothelial cells amongst other receptors.[14,15]

The significant associations have been shown in several genetic studies by SNP analysis of VEGF promoter and 5' UTR sequences with the AMD pathology. It has been demonstrated that the 'CC' genotype could influence the VEGF expression in serum of diabetic retinopathy patients in Japanese population.[13] It has also been found that the AMD pathology was associated with the +405C allele in AMD cohort.[13] Moreover, there are several studies which have demonstrated the conflicting results with CG genotype of VEGF gene and its association with diabetic retinopathy but the levels of VEGF was found to be higher in individuals carrying +405GG genotype in various populations.[16,17] Haines et al have shown the correlation of VEGF, low density lipoprotein receptor- related protein 6 (LRP 6), and very low density lipoprotein receptor (VLDLR) with AMD pathology

in Caucasian population suggesting the role of metabolizing genes and angiogenic genes in AMD pathophysiology.[13] Meanwhile Churchill et al have also screened the 14 SNPs of VEGF promoter. The genotype +674 CC was found to be significantly associated with disease pathology. Moreover, the sequences 'CTCCT' and 'TCACC' of SNPs +674, +4618, +5092, +9162 and +9512 of VEGF gene by haplotype analysis have shown significant correlation with AMD in 45 patients as compared to age matched controls.[18]

Recently, increased mRNA expression of VEGF-A121 isoform was found in excised choroidal neovascular membrane tissue from AMD patients as compared to control. Moreover, the study has also suggested the other isoform of VEGF i.e. VEGF-A165 in AMD cases.[19] The expression of VEGF-A could progressively affect the function of RPE cells in the mice. Mice with increased VEGF-A were found with abnormal morphology of RPE cells, reduced levels of retinal rhodopsins and impaired transport of retinoic acid between RPE and photoreceptors. The alfa and beta waves analyzed were also found to be distorted as compared to control mice.[20] Similarly, Marneros et al have also demonstrated the altered morphology of RPE cell layer and distorted visual function in VEGF rpe-/- mice and demonstrated that VEGF function is independent on hypoxia inducing factor-1α (HIF-1 α).[21] Several genetic polymorphism studies have also shown the association with AMD pathology. Several anti-VEGF treatments are available currently which includes aflibercept, bevacizumab, pegaptanib, ranibizumab etc. targeting different sites of VEGF protein to inhibit its functions.

Previously, we had shown the increased expression of VEGF receptor2 (VEGFR2) alongwith single nucleotide polymorphism (SNP) which were also found to be associated with AMD pathology.[22] In this study we have estimated the levels of VEGF in AMD patients.

Methodology

Participants

We have recruited 98 AMD and 59 control participants for this study from outdoor patient care facility of Advanced Eye Centre, PGIMER, Chandigarh, INDIA. The participants were recruited after getting their approval and sign of consent form. The ethical approval of the study has been taken from Institute Ethical Committee (IEC) vide letter No PGI/IEC/2015/881; dated 29.01.2015.

AMD diagnosis

The AMD participants were included after their proper AMD diagnosis, which included fluorescein fundus angiography (FFA) and optical coherence tomography (OCT).[23–26] The retinal specialist examined all the ophthalmic parameters like visual acquity, dilated fundus examination and slit lamp biomicroscopy of anterior chamber of eye.

Demographic information

Demographic parameters of subjects were collected by administrating the standard questionnaire. The questionnaire includes demographic as well as general life style details of the participants.[27–28] The participants were characterized on the basis of their smoking habit and associated co morbidity like cardiovascular problems, hypertension *etc* and summarized in table 1.

Table 1: Demographic characteristics of Controls and AMD patients

Variables	AMD	Controls
Total	98	59
Male	61	38
Female	37	21
Duration of disease¥	25.6 M	
Dry	29	
Wet	69	
Avastin treated	40	
Not treated	29	
Minimal Classic	7	
Predom Classic	14	
Occult	29	
One Eye Affected	29	
Both Eyes Affected	69	
Alcoholic	31	15
Non Alcoholic	67	38
Smokers	41	10
Non Smokers	57	43
Vegetarian	51	29
Non Vegetarian	47	24
Age	65.31 ± 6	60 ± 13

Clinical and demographic details of subjects. AMD, age related macular degeneration; M, Months; Age, Age of onset; Values are mean ± SD or (percentage), ¥ Duration of disease is the interval between appearance of first symptom of AMD and collection of sample. AMD subjects were asked to provide all clinical and demographic details at the age of disease-onset.

Inclusion and exclusion criteria

The criteria were based on the number and size of drusen, and age of the participants. The subjects with age of 50 years or more were included in the study. The AMD participants who were having choroidal vascularization and/or geographic atrophy were included in the study after FFA examination. In case of dry AMD, the participants who had >5 drusen in at least one eye were recruited in AMD group. The control subject included those with age of 50 or more and with <5 drusen and lacking other diagnostic parameters of AMD pathology.

The degenerative changes in their photoreceptors and retinal layers due to other ocular pathological condition like myopia, uveitis, retinal dystrophies, vein occlusion, diabetic retinopathy were excluded from the study. Moreover, those below 50 years of age were also excluded from study.

Serum separation

The serum samples were obtained from the 4 ml blood collected in serum separator tubes (BD bioscience, USA) and allowed the tube to coagulate for 30 minutes. Further, the tubes were centrifuged at 1800rpm for 30 minutes at room temperature. The separated serum samples were collected. The samples were labeled and stored at –80^{0}C to perform ELISA.

Total protein estimation

Total protein estimation was done with the standard procedure of Bradford's method as per the manufacturer's instructions. The serum samples were diluted with distilled water upto 1500 times. The standard curve was made using Bovine serum albumin (BSA). The coomassie brilliant blue G-250 dye (Bradford reagent) was used in 1:4 ratio. The absorbance was taken at 595 nm using the ELISA reader (680XR model of Microplate reader, Biorad, Hercules, CA, USA). Quadratic fit or linear method was used to obtain standard curve. Total protein was used to normalize the VEGF levels.

VEGF ELISA

VEGF level in serum was analyzed by ELISA as per the manufacturer's instructions (R&D, USA & Ray Bio, USA). The procedure was performed in duplicates. OD was taken at 450 nm in ELISA reader (Bio Rad, USA).

Statistics

Normality of the data was tested with the help of Q-Q plot and data was found not normally distributed. Two groups were compared by Mann-Whitney *U*-test and Kruskal Wallis one-way analysis of variance (ANOVA) followed by post-hoc was applied for more than two comparisons. Goodness of standard curve fit for ELISA and total protein were measure by R^2 (Coefficient of determination). All statistical analysis were performed with SPSS 20.0 software.

Results

All the demographic characteristics of studied population are in Table 1. AMD patients were both segregated as dry and wet AMD. The wet AMD patients were further subdivided into minimal classic, predominant classic and occult. Several other demographic factors were taken into consideration which included food habits (vegetarian vs non-vegetarian), smoking, co-morbidity (history of heart disease) *etc*. (Table 2). Serum VEGF expression ($p = 0.034$; z value = 2.11) was found to be higher in AMD cases as compared to controls (Figure 1). There was no significant difference in VEGF expression among dry and wet AMD patients ($p = 0.187$). Again the difference was not significant between minimal, predominant and classic wet AMD patients ($p = 0.079$). Moreover the history of heart disease was found to be associated with VEGF levels in AMD patients. VEGF level was found to be elevated in AMD patients as compared to controls ($p = 0.031$; Figure 2). The other factors like smoking history ($p = 0.974$), alcohol ($p = 0.912$), Food habits ($p = 0.076$) and use of anti-inflammatory drugs ($p = 0.912$) did not show any association with VEGF level in AMD patients. Not surprisingly, the anti-VEGF treatment (Avastine treatment) was found to have reduced VEGF levels at the systemic levels but it was not significant ($p = 0.058$).

Table 2: Serum VEGF levels comparison in AMD and controls

Subjects VEGF	No.	Mean Rank	Z Value	P value
AMD	98	84.96		
Control	59	69.10	2.11	0.034
Dry	29	43.66		
Wet	69	51.96	1.36	0.187
Male	61	49.95		
Female	37	48.76	0.202	0.840
Avastin treated	40	38.90		
Not treated	29	29.62	1.89	0.058
Minimal Classic	7	7.57		
Predom Classic	14	12.71	1.791	0.079
Occult	29	19.86	1.579	0.121
One Eye Affected	29	53.59		
Both Eyes Affected	69	47.78	0.922	0.356
Alcoholic	31	49.03		
Non Alcoholic	67	49.72	0.111	0.912
Smokers	41	49.42		
Non Smokers	57	49.61	0.032	0.974
Vegetarian	51	54.39		
Non Vegetarian	47	44.19	1.774	0.076
Anti inflammatory use	63	47.78		
No Anti inflammatory	32	48.44	0.110	0.912
Heart Disease	11	40.09		
No heart Disease	48	27.69	2.16	0.031

Discussion

The expression of VEGF in serum has been found to be elevated in AMD patients as compared to controls but no significant difference was observed between wet and dry AMD patients which suggests that VEGF is involved in both pathologies i.e. wet and dry form of AMD or possibly in progression of dry form of these patients into wet AMD. Functional studies of VEGF gene have revealed that the AMD progression could be successfully altered through targeting of this gene in mouse model of wet AMD.[28,29] These studies guide AMD research such that VEGF mediated pathology of wet AMD could be understood with additional evidence from various populations across the world. Consequently, most of AMD treatment strategies are only confined to targeting of VEGF molecules in order to suppress the disease phenotypes. The induction of human RPE and choroidal cells by chemokine cocktail (IFN-γ+ TNF-α+IL-1β) has been known to increase the production of VEGF-A and VEGF-C by over 10 times with the activation of proteins involves in JAK-STAT and NF-β pathways. Such studies suggest that the chemokines have important role in the regulation of angiogenic pathways and their molecules to influence pathological changes in the RPE cells and choroidal tissue.[9,30] We have

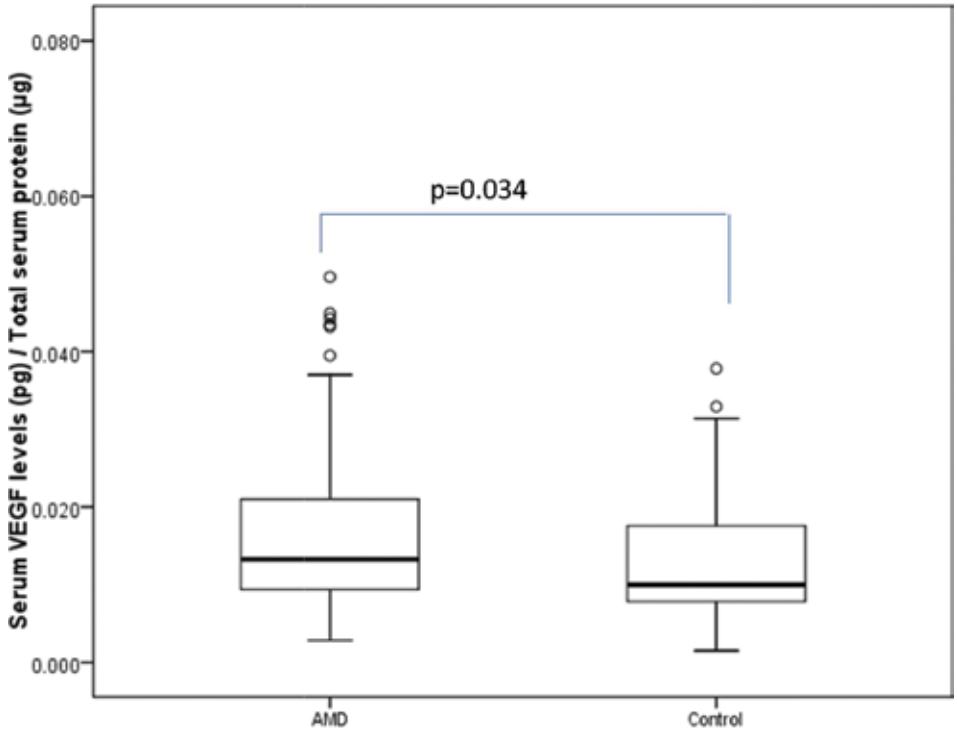

Fig. 1: Expression levels of VEGF in serum of AMD patients with comparison of controls. Representative boxes include the values from first quartile (25%) to third quartile (75%). The outliers in the experiments are shown with circles. The values of VEGF levels normalized with protein of the serum. AMD: age related macular degeneration; pg: picogram; µg: microgram; VEGF: vascular endothelial growth factor.

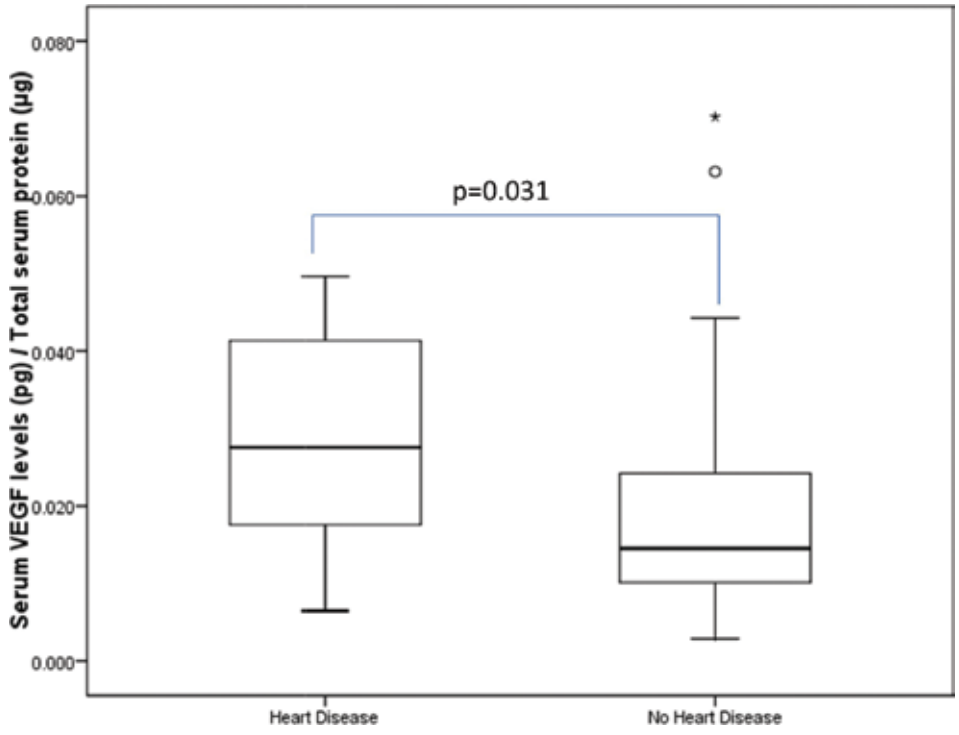

Fig. 2: VEGF Serum levels in heart ailment of AMD patients. The plotted values in the boxes includes from first quartile (25%) to third quartile (75%). The outliers and extreme values are designated with circles and asterisk respectively. The expression levels of VEGF (pg) standardized with total protein (µg). AMD: age related macular degeneration; pg: picogram; µg: microgram; VEGF: vascular endothelial growth factor.

investigated the monocyte chemoattractant protein-1 (MCP-1 or CCL2) and its receptor (CCR2) and reported its association with Indian AMD. We demonstrated the CCR2 (rs1799865) and CCL2 (rs4586) polymorphism was significantly associated with AMD pathogenesis. Moreover, the expression of both genes was also found to altered in AMD patients as compared to controls.[31] Additionally, we have also examined the other chemokines like CCR-3 (G-protein coupled receptor) and eotaxin-2 in Indian AMD patients and reported these to be associated with disease phenotype.[32,33] Both chemokines are involved in recruitment of eosinophils and other inflammatory cells which reveals that their actions are exerted through inflammatory cells recruitment and activation which might further result in increased VEGF expression.

AMD and cardiovascular diseases (like coronary heart disease) share common pathological features including deposition of lipoproteins and impair angiogenesis.[34] Smoking, age, hypertension, high blood cholesterol levels *etc* are known widespread causative agents for both diseases.[35] In this study we have shown that VEGF level was less in AMD patients without heart disease as compared to AMD patients with history of heart disease. Similarly, the homeostasis between hemorheologic factors (e.g. von Willebrand factor, fibrinogen, blood viscosity *etc*), VEGF levels and endothelial dysfunction were shown to be impaired in both AMD and hypertensive patients sharing common pathological changes including angiogenic (VEGF) and plasma factors.[36,37]

Some studies with Avastin treatment in AMD patients have reported decreased VEGF levels in vitreous as well as in systemic circulation.[32,33] In this study, AMD patients with anti-VEGF (Avastin) treated group did not show any significant reduction of systemic VEGF levels but it was found that there was a trend of its decrease in treated group. Our data did not show that VEGF was overexpressed in wet AMD as compared to dry AMD even though the role of VEGF is well defined in wet AMD progression.[32,33] Together these studies indicate a need to undertake Mendedial Randomization analysis of SNP-Biomarker involvement so that this complex disease can be successfully predicted and personalized medicine can be instituted appropriately. Levels of VEGF in this study is supporting and consistent with previous discoveries in the field that showing the increased VEGF levels in the serum, human choroids and RPE cells.[5,37,40]

Authorship contribution

Akshay Anand: Study PI and executed study plan, Manuscript editing; **Ramandeep Singh:** Provided samples for study, **Kaushal Sharma:** Manuscript writing, **Neel K Sharma:** Conducted experiment, Statistics analysis, Manuscript editing.

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