ORIGINAL ARTICLE

Imbalance between the Serum Levels of VEGF, MMP-2 and α1-AT in Patients with Diabetic Retinopathy

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Abstract:

Background: Diabetic Retinopathy (DR), a microvascular complication of Type 2 Diabetes Mellitus (T2DM) is due to retinal neovascularization mediated by Vascular Endothelial Growth Factor (VEGF). Neovascularization involves proteolytic degradation of Extracellular Matrix (ECM) by Matrix Metalloproteases (MMPs). Alpha 1-antitrypsin (α1-AT), an antiprotease is known to inhibit MMP-2. *Aim and Objectives:* To evaluate the serum levels of VEGF, MMP-2, and α1-AT in DR patients. *Material and Methods*: A three-group comparative study was carried out by considering patients with DR (n = 32), T2DM (n = 32), and healthy controls (n =32). Serum levels of VEGF, MMP-2, and α1-AT were determined by the ELISA technique. *Results:* Serum levels of VEGF and MMP-2 were significantly higher in DR than in T2DM and controls (p <0.001), while the serum levels of α1-AT were significantly lower in DR when compared to T2DM and controls (p < 0.001). *Conclusion:* An imbalance between the serum levels of VEGF, MMP-2 and α1-AT may be involved in the pathogenesis of DR.

Keywords*:* Diabetic Retinopathy, Vascular Endothelial Growth Factor, Matrix Metalloproteases -2, Alpha 1-antitrypsin, Angiogenesis, Extracellular Matrix Degradation

Introduction:

Diabetic Retinopathy (DR) is one of the predominant microvascular complications of Type 2 Diabetes Mellitus (T2DM) that involves the development of neovascular structures in the retina [1, 2]. Approximately 6.28% of the world's population is affected by T2DM [3] among which 6.7% of individuals develop DR in the late-onset [4]. Being one of the predominant complications of T2DM it leads to vision impairment and ultimately results in blindness [3].

The progression of DR is a complex process involving molecular, cellular as well as physiological changes in the retinal tissue [5]. Prolonged hyperglycemia induces damage to the blood-retinal barrier and causes hypoxia in the cells of retinal tissue [6]. Hypoxia, in turn, upregulates the secretion of Vascular Endothelial Growth Factor (VEGF) from neighbouring Müller cells [7]. The cognate binding of VEGF to its receptor on endothelial cells elicits angiogenesis, a classic hallmark feature of progressive DR [8]. Proteolysis of Extracellular Matrix (ECM) facilitates VEGF to promote angiogenesis. VEGF-activated endothelial cells induce secretion of Matrix Metalloproteinases (MMPs), a zinc-dependent endopeptidase, which is responsible for ECM remodelling and degradation [9-11]. Key MMPs involved in neoangiogenesis of DR are MMP-2 and MMP-9 [12]. MMP-2 is known to degrade type IV collagen which is a major structural component of ECM [13].

Regulation of the ECM degradation to maintain the microvascular structures of the retina is important to avoid the progression of the disease [14]. Physiologically the proteolytic activity of MMP-2 is regulated by a group of endogenous inhibitors such as Tissue Inhibitors of Metallo-proteinase (TIMPs), α2-Macroglobulin, and Alpha 1-Antitrypsin (α1-AT). α1-AT is a major serine protease inhibitor produced by the liver [15-16]. Several clinical and experimental studies have supported the potential protective role of α1-AT in DR as a result of its multiple activities. The process of neovascularization requires remodelling of the ECM; thereby inhibition of several MMPs through α1-AT may partly decrease the action of VEGF [15]. An adequate level of α1-AT inhibitor is critical for the prevention against proteolytic degradation of ECM and subsequent angiogenesis. Thus, the objective of this study was to assess the serum levels of VEGF, MMP-2 and α1-AT in DR patients to evaluate their role in the pathogenesis of retinopathy.

Material and Methods:

Study Design and Patient Selection:

This was a comparative study carried out from November 2019 to March 2021. The study participants were randomly selected that fulfilled the criteria for the patient selection. Study participants were recruited from the Department of Ophthalmology and Department of General Medicine of R.L Jalappa Hospital and Research Centre, teaching hospital of Sri Devaraj Urs Medical College, Tamaka, Kolar, Karnataka. The study was approved by the Institutional Ethics Committee. Informed consent was obtained from all the participants prior to their recruitment for the study. The exclusion criteria for patient selection were: T2DM and DR patients with other co-morbidities and with a history of smoking and alcohol consumption.

The study included 96 subjects in the age group of 30-70 years who were divided into 3 groups.

Group I (n=32): included DR patients confirmed by fundoscopy and were further categorized into Non-Proliferative Diabetic Retinopathy (NPDR, n=15) and Proliferative Diabetic Retinopathy (PDR, n=17). Group II (n=32): included confirmed T2DM patients with Fasting Blood Sugar (FBS) (>100mg/dl), Postprandial Blood Sugar (PPBS) (>140 mg/dl) and Glycated Haemoglobin levels (HbA1c) >5%. Group III (n=32): Volunteers without a known history of chronic infections, smoking, alcohol consumption, and with FBS (<100mg/dl) and HbA1c levels (<5%) were included under the Control Group.

Sample Collection:

Venous blood (6 ml) from the study subjects was collected in different vacutainers like EDTA (for HbA1c analysis), sodium fluoride (for FBS and PPBS), and no-anticoagulant for liver enzymes Aminotransferase (AST), Alkaline Phosphatase (ALP), C-reactive Protein (CRP), Gamma Glutamyl Transferase (GGT) and Enzyme-Linked Immunosorbent Assays (ELISA). Basic biochemical parameters were analysed immediately by standard methods using Vitros 5.1 FS autoanalyzer. For ELISA estimation, serum was separated within 2 hours of the sample collection by centrifugation. The serum was then aliquoted and stored at -80˚C until further analysis. Before the analysis, the samples were thawed at room temperature, vortexed, and centrifuged.

Estimation of VEGF, MMP-2, and α1-AT:

Serum levels of VEGF, MMP-2, and α1-AT were estimated using commercially available ELISA kits (Cloud Clone Corp, USA, #SEA143Hu, #SEA100Hu and #SEB697Hu, respectively).

Statistical Analysis:

The results were analysed statistically using SPSS V20 (International Business Machine Corporation, Armonk, New York) software and Prism Graphpad 9.1. Shapiro Wilk test was performed with Q-Q plots and normality plots to check for the normal distribution of the data. The Shapiro wilk test showed p value > 0.05 and the data were found to be normally distributed. Results are expressed as mean and standard deviation. One-way ANOVA was used to compare means between the groups. *P*-value <0.05 was considered statistically significant and p <0.001 as highly significant.

Results:

Demographic and biochemical characteristics of study groups are represented in Table 1 and Table 2 which are reflective of the clinical features of the study groups. The VEGF levels were 474.8 ± 27.3 pg/ml in the DR group, 363.4 ± 118 pg/ml in the T2DM group, and 233.4 ± 55.8 pg/ml in the control group, indicating significantly high levels in the DR group when compared to T2DM and controls (p<0.001) (Fig. 1a). When the serum levels of MMP-2 were compared, mean MMP-2 levels were significantly high in the DR group (530.1 ± 136.2 ng/ml) when compared to the T2DM group (413.5 ± 121.1 ng/ml) and control group (238.8 ± 81.8 ng/ml) (p<0.001) (Fig. 1b). The results for the levels of α1-AT showed significantly decreased levels in DR group (10.63 ± 2.86 mg/dl) as compared to T2DM (48.72 ± 6.68 mg/dl) and controls (76.50 ± 3.41 mg/dl) (p<0.001) (Fig. 1c). The subgroup analysis showed no significant difference in serum levels of VEGF and MMP-2 between the NPDR and PDR groups (Figs. 2a and 2b respectively). However, α1-AT levels were significantly decreased in PDR subjects as compared to NPDR (Fig. 2c). The ratio of the means of MMP-2 and α1-AT was 49.86 in the DR group, 8.48 in the T2DM group, and 3.1 in the control group (Fig. 3). This indicates that the ratio is substantially higher in DR than in T2DM and control groups.

Table 1: Demographic Characteristics of the Study Groups

Parameters	Groups			Comparison between Groups (p-value)		
	DR (n=32)	T2DM (n=32)	Controls (n=32)	DR vs Controls	T2DM vs Controls	DR vs T2DM
Age	53.93 ± 8.12	55.93 ± 8.12	56.34 ± 7.37	0.21	0.83	1
Duration	9.24 ± 3.01	8.96 ± 2.82	-	-	-	0.71
Gender(M/F)	21/11	20/12	18/14	-	-	-

DR- Diabetic Retinopathy, T2DM- Type 2 Diabetes Mellitus

Table 2: Biochemical Parameters Characteristics of the Study Groups

Parameters	Groups			Comparison between Groups (p-value)		
	DR (n=32)	T2DM (n=32)	Controls (n=32)	DR vs Controls	T2DM vs Controls	DR vs T2DM
FBS	167.22 ± 77.22	189.91 ± 53.95	86.71 ± 7.75	<0.001*	<0.001*	0.04*
PPBS	298.13 ± 61.16	260.09 ± 88.15	106.71 ± 10.78	<0.001*	<0.001*	0.04*
HbA1c	9.65 ± 2.73	8.71 ± 2.08	4.88 ± 0.54	<0.001*	<0.001*	0.1
AST	29.21 ± 5.46	27.84 ± 5.55	27.09 ± 6.90	0.1	0.6	0.9
ALKP	70.56 ± 21.70	79.16 ± 18.23	73.28 ± 18.96	0.5	0.2	0.3
GGT	31.3 ± 13.54	31.59 ± 13.288	27.81 ± 11.18	0.2	0.2	0.8
Urea	26.15 ± 12.60	24.15 ± 9.66	22.15 ± 6.63	0.1	0.3	0.1
Creatinine	0.81 ± 0.23	0.69 ± 0.27	0.68 ± 0.17	0.1	0.3	0.3
CRP	5.29 ± 0.48	5.27 ± 0.41	5.13 ± 0.53	0.2	0.2	0.3

*ANOVA test was used. Results represented as mean ± SD.*p<0.001 were considered statistically significant, FBS-Fasting blood sugar, PPBS-Postprandial blood sugar, HbA1c-Haemoglobin A1c, AST-Aminotransferase, ALP-Alkaline phosphatase, GGT- Gamma glutamyl transferase CRP- C-reactive protein, DR- Diabetic Retinopathy, T2DM- Type 2 Diabetes Mellitus*

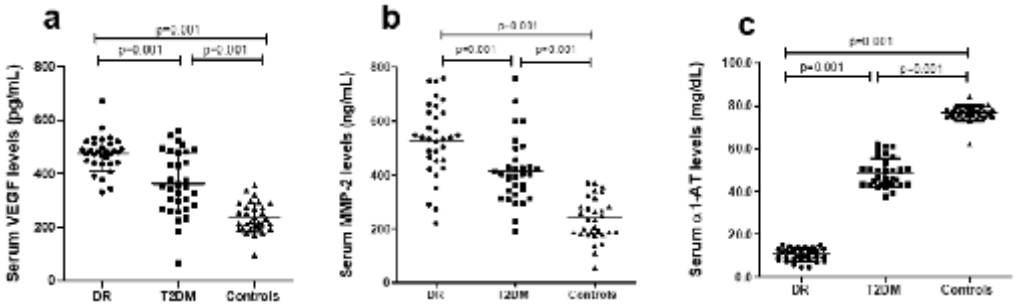

Fig. 1: Serum Levels of VEGF, MMP-2 and α 1-AT in Study Groups. (a) VEGF Levels in the Study Groups. (b) MMP-2 Levels in the Study Groups. (c) α 1-AT Levels in the Study Groups.

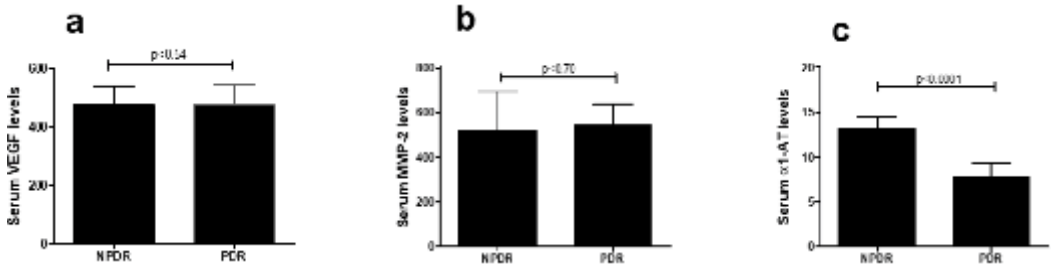

Fig. 2: Serum Levels of VEGF, MMP-2 and α 1-AT in NPDR and PDR Groups. (a) VEGF Levels in the NPDR and PDR Groups. (b) MMP-2 Levels in the NPDR and PDR Groups. (c) α 1-AT Levels in the NPDR and PDR Groups.

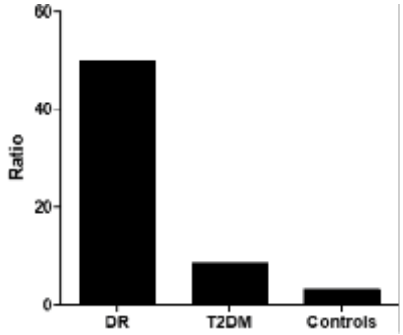

Fig. 3: Ratio between MMP-2 and α 1-AT in Study Groups

Discussion:

In PDR, the hypoxic condition of patients leads to the overexpression of VEGF which ultimately results in the formation of new blood vessels. In the case of NPDR, leakage in the retinal capillary leads to the release of VEGF and results in vascular leakage [17]. VEGF has been identified as a primary initiator of PDR, and as a potential mediator of NPDR. VEGF was demonstrated to cause vascular permeability and the production of new blood vessels in DR [18]. VEGF expression was found to be elevated in the retina of diabetic mice whereas, suppression of VEGF expression inhibited angiogenesis in a time-dependent manner [19, 20]. Elevated serum levels of VEGF in DR patients have been observed in several studies and the abnormality is confirmed at the level of meta-analysis suggesting VEGF as a reliable biomarker for DR. Our results recapitulate the previous reports on the serum levels of VEGF observed in DR [21-25]. Ahuja *et al.* observed an incremental trend in the VEGF levels with the increasing severity of DR [25]. However, in the current study, no significant difference was observed between PDR and NPDR patients.

Neoangiogenesis in DR is initiated by the migration of the endothelial cells [26]. Under normal conditions, the endothelial cells are prevented from migrating out of the endothelial lining due to the phenomenon of contact inhibition. During neoangiogenesis, endothelial cells secrete

MMP-2 for the degradation of the ECM. Degradation of the ECM eliminates contact inhibition and thereby facilitates the migration of endothelial cells [27, 28]. In our study, MMP-2 levels were found to be elevated in DR and stand on par with the previously reported studies. Elevated levels of MMP-2 have been reported in the vitreous fluid and the blood of DR [29-31]. In a study carried out by Rodrigues *et al*. It was observed that the VEGF induces MMP-2 enzymatic activity in endothelial cells [30]. Studies have demonstrated that MMP-2 promotes an angiogenic phenotype while its suppression exhibited reduced angiogenesis [31, 32]. These observations demonstrate the potential role of MMPs in ECM degradation and hence are implicated in the development of DR.

The most significant observation of this study was significantly reduced levels of α1-AT in retinopathy patients than in T2DM and control subjects. The possible explanation for this observation could be attributed to increased proteolytic activity of MMP-2. An imbalance between MMP-2 and α1-AT levels could lead to uncontrolled ECM proteolysis contributing to DR progression, suggesting α1-AT as a possible therapeutic option in DR [15].

Downregulation of MMP-2 by α1-AT has been demonstrated by Geraghty *et al.,* both in *in-vitro* and *in-vivo* [16]. In the current study, we have observed significantly lower levels of α1-AT in PDR patients as compared to NPDR patients indicating α1-AT as a potential marker for grading the severity of DR. However, further research with larger sample size is needed to elucidate this association. The main novelty of this study lies in the combined measurement of VEGF, MMP-2, and α1-AT. This approach emphasizes on assessing the relative balance between the factors particularly, MMP-2 and α1-AT.

Conclusion:

Significantly increased levels of VEGF and MMP-2 and decreased levels of α1-AT in DR suggest their role in the pathogenesis of DR. A protease-antiprotease axis may be developed as a therapeutic target to ameliorate the progression of T2DM into DR. Due to the increased proteolysis of ECM in DR, ECM can be placed as an emerging target for antiangiogenic therapies.

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