Increased Serum N-CML, VEGF and ICAM-1 is Associated with Photoreceptor Inner Segment Ellipsoid Disruption in Diabetic Retinopathy

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ABSTRACT

Consecutive cases of type 2 diabetes mellitus in the 40–65 age group were included to study the association of serum N-CML, VEGF and ICAM-1 with photoreceptor inner segment ellipsoid disruption in diabetic retinopathy. Study subjects included patients with type 2 DM [diabetes mellitus with no retinopathy (No DR; n=40); non-proliferative diabetic retinopathy (NPDR; n=39); proliferative diabetic retinopathy (PDR; n=39)] and healthy controls (n=39) between the ages of 40 and 65 years. Disruption of photoreceptor inner segment ellipsoid (ISel) was graded by spectral domain optical coherence tomography. The serum levels of N-CML, VEGF and ICAM-1 were analyzed using the standard protocol. Data was statistically analyzed. A significant difference was found between the serum levels of N-CML, VEGF and ICAM-1 and the various study groups (p<0.001). A positive correlation was found between the photoreceptor ISel disruption and the levels of N-CML (r=0.68, p<0.0001), VEGF (r=0.36, p=0.001) and ICAM-1 (r=0.36, p=0.001). A significant positive correlation was found between log MAR visual acuity and grade of disruption (r=0.9, p<0.0001). Present study highlighted the positive correlation of N-CML, VEGF and ICAM-1 with photoreceptor structural integrity and visual acuity in diabetic retinopathy providing further insight into the pathogenesis of the disease. Thus serum N-CML, VEGF and ICAM-1 levels can be used as prognosticator of photoreceptor disruption in diabetic retinopathy.

ABBREVIATIONS

DM: Diabetes Mellitus; DR: Diabetic Retinopathy; VEGF: Vascular Endothelium Growth Factor; ICAM-1: Intracellular Cell Adhesion Molecule -1; N-CML: N-Carboxy Methyl Lysine; ISel: Inner Segment ellipsoid; SD-OCT: Spectral Domain- Optical Coherence Tomography; DME: Diabetic Macular Edema

INTRODUCTION

Diabetic retinopathy, a micro vascular complication with several underlying pathogenic mechanisms, is the leading cause for blindness in the world [1].

Strong relationship between chronic hyper glycemia and the development and progression of diabetic retinopathy has been well established but the underlying mechanism that leads to the development of micro vascular damage remains unclear [2, 3]. A number of interconnecting biochemical pathways have been proposed as potential links between hyper glycemia and diabetic retinopathy. These include increased polyol pathway flux, activation of diacylglycerol- protein kinase C pathway, increased expression of growth factors such as vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1), haemodynamic changes, accelerated formation of advanced glycation end products (AGEs), oxidative stress, activation of the rennin – angiotensin - aldosterone system, and subclinical inflammation and leukostasis [4-8].

Advanced glycation end products, product of non-enzymatic reaction of sugars with proteins, are known to accumulate in retinal endothelial pericytes in diabetic retinopathy and their levels are found to be associated with severity of the disease [9]. N-carboxy methyl lysine (N-CML) is one of the most common and widely studied AGEs. AGEs act on pericytes to stimulate VEGF expression [10]. VEGF, a vascular permeability factor, is considered as a crux factor in the pathogenesis of diabetic retinopathy [11]. VEGF levels have been found to correlate with the breakdown of the blood- retinal barrier [12,13]. AGEs have also been found to be involved in the process of vascular

inflammation as AGEs were found to increase leukocyte adhesion to cultured retinal micro vascular endothelial cells by inducing intracellular cell adhesion molecule-1 (ICAM-1) expression [14]. Retinal VEGF induces ICAM-1 expression leading to leukostasis and breakdown of blood–retinal barrier [15].

The importance of photoreceptor integrity in various retinal diseases and association of its disruption with poor visual acuity has already been emphasized in several studies [16-18]. Photoreceptor dysfunction may be a significant predictor of visual acuity in patients with diabetic retinopathy [19,20]. Recently Spaide et al and Curcio et al highlighted that the outer highly reflective band next to retinal pigment epithelium was located at the ellipsoid in the inner segments which correlated between the retinal microstructure on the SD-OCT images and the histologic findings [21,22]. Thus it is more appropriate to consider the previously termed inner segment–outer segment (IS-OS) junction to be photoreceptor inner segment ellipsoid band (ISel). Photoreceptor ISel has been found to be visible on optical coherence tomography (OCT) due to its biological activity rather than the structure in an animal model [23]. Our recent study highlighted that an increase in serum VEGF and ICAM-1 levels is associated with an increase in the severity of diabetic retinopathy and grades of subfoveal external limiting membrane (ELM) and ISel disruption [24].

In the present study we evaluated the association of serum N-CML, VEGF and ICAM-1 levels with photoreceptor inner segment ellipsoid disruption in diabetic retinopathy, using SD-OCT.

**MATERIALS AND METHODS**

The authors confirm adherence to the tenets of the Declaration of Helsinki. An institutional review board clearance was obtained. A written informed voluntary consent was received from all the study subjects. The study was a tertiary-care-centre–based cross-sectional study of cases of type 2 diabetes mellitus and healthy controls. Consecutive cases of diabetes mellitus in the 40–65 age groups were included. Subjects with any of the following conditions were not enrolled in the study: other ocular or systemic diseases affecting the retinal vascular pathology, previous intravitreal injection(s), ophthalmic surgical or laser interventions, vitreous hemorrhage and tractional retinal detachment, media haze at any level giving signal strength of less than 5 on OCT, systemic diseases that may affect ICAM-1 and VEGF levels such as malignancies, inflammatory disorders (e.g., asthma and rheumatoid arthritis), ischemic heart disease, or current or planned dialysis. Patients having conditions in which serum level of N-CML is raised like Alzheimer’s disease, pulmonary fibrosis, atherosclerosis, end stage renal disease, tobacco smoking were also excluded. The best-corrected visual acuity was recorded on the log MAR scale. Information regarding the patient’s age, gender, and disease duration was also recorded. All the study subjects underwent detailed fundus evaluation using stereoscopic slit-lamp bio microscopy and indirect ophthalmoscopy. Digital fundus photography and fluorescein angiography were done using a Zeiss fundus camera FF 450 Plus with a pixel width of 0.0054 and an image size of 2588×1958. Cases were divided into three groups: diabetes patients without retinopathy (No DR) (n = 40), with non-proliferative diabetic retinopathy with diabetic macular edema (NPDR) (n = 39), and with proliferative diabetic retinopathy (PDR) (n = 39) with DME according to the early treatment of diabetic retinopathy study (ETDRS) classification [25]. Healthy controls (n = 39) were also studied. Subsequently, photoreceptor ISel disruption was studied using three-dimensional SD-OCT (Cirrus High Definition OCT from Carl Zeiss Meditec Inc. CA) with scans passing through the fovea (Figure 1) Every patient underwent macular thickness analysis using the macular cube 512 × 128 feature.

Blood samples of 7 ml were collected from the study subjects. Blood was transferred to glass tubes for separation of serum. The tubes containing blood were set on a stand and left for 30 min to allow the blood to clot. Soon after, the samples were centrifuged at 1000 × g for 10 min, and the serum was carefully poured into other tubes. All samples were stored at −80 °C till assay of sICAM-1, VEGF and N-CML.

**N-CML assay**

Assay of n-CML in serum was carried out using the Human N-CML ELISA kit procured from Uscn, Life Science Inc., USA. The reagents were prepared following the standard protocol provided with the kit. Briefly, n-CML standard provided with the kit was reconstituted with standard diluents buffer. Serial dilutions of the N-CML standard (0, 61.7, 185.2, 555.6, 1666.7, 5000 ng/ml) was done following the instructions and run in parallel. Standard or sample (50µl) and detection reagent A (50µl) was added to the appropriate micro titer wells plate pre coated with monoclonal antibody, specific for the N-CML protein. Following this, the plate was incubated at 37°C temperature for one hour. The contents of the plate were removed using multichannel pipettes after the incubation was over. The plate was washed with the wash buffer times to remove unbound antigens (proteins), if any. Detection reagent B (100µl) was added to each well, the plate was incubated at 37°C temperature for 30 minute. After the incubation, the plate was washed with the wash buffer four times.
as previous step. Substrate Solution (90µl) was added to each well and the well of solution began to turn blue. Following this, the plate was incubated at room temperature for 2 h. The contents of the plate were removed using multichannel pipettes after the incubation was over. The plate was washed with the washing buffer four times to remove any unbound antigens (proteins). Biotinylated Hu VEGF (Biotin Conjugate, 100 µl) was added to each well and the well solution began to turn blue. Following this, the plate was incubated at room temperature for 1 h. When TMB Substrate Solution (100 µl) was added to each well, the well solution began to turn blue. Following this, the plate was washed with the washing buffer three times to remove any unbound antigens (proteins). The VEGF standard provided with the kit was reconstituted with the standard diluent buffer. Serial dilutions of the VEGF standard (0, 0.625, 1.25, 2.5, 5, 10 ng/ml) were done following the instructions and run in parallel. The standard amount (100 µl) was added to the appropriate microtiter wells. A sample of 100 µl diluted to 1:100 with the diluent buffer of the controls and the cases was added to these wells, and diluted HRP-Conjugate (50 µl) was added to each well. Following this, the plate was incubated at room temperature for 2 h. The contents of the plate were removed using multichannel pipettes after the incubation was over. The plate was washed with the washing buffer four times to remove any unbound antigens (proteins). The blue colour that developed earlier then turned yellow. The intensity of the colour was read with an ELISA plate reader (Synergy HT, Biotech) at 450 nm. The calibration curve of the standard VEGF was plotted against the VEGF with absorbance on the x-axis and concentration on the y-axis. The concentration of VEGF in the serum sample was calculated based on the standard curve. The values were expressed as pg/ml.

**ICAM-1 assay**

Assay of sICAM-1 in the serum was performed using the Human sICAM-1 ELISA kit procured from Invitrogen. The reagents were prepared following the standard protocol provided with the kit. Briefly, an incubation buffer (50 µl) was added to the multi well plate pre coated with a monoclonal antibody specific for the sICAM-1 protein. The VEGF standard provided with the kit was reconstituted with the standard diluent buffer. Serial dilutions of the sICAM-1 standard (0, 0.625, 1.25, 2.5, 5, 10 ng/ml) were done following the instructions and run in parallel. The standard amount (100 µl) was added to the appropriate microtiter wells. A sample of 100 µl diluted to 1:100 with the diluent buffer of the controls and the cases was added to these wells, and diluted HRP-Conjugate (50 µl) was added to each well. Following this, the plate was incubated at room temperature for 2 h. The contents of the plate were removed using multichannel pipettes after the incubation was over. The plate was washed with the washing buffer three times to remove any unbound antigens (proteins). The blue colour that developed earlier then turned yellow. The intensity of the colour was read with an ELISA plate reader (Synergy HT, Biotech) at 450 nm. The calibration curve of the standard sICAM-1 was plotted against the sICAM-1 with absorbance on the x-axis and concentration on the y-axis. The concentration of sICAM-1 in the serum sample was calculated based on the standard curve. The values were expressed as ng/ml.

**Statistical analyses**

The VEGF and ICAM-1 levels in the study groups were compared by single-factor analysis of variance (ANOVA). For pair wise comparison between the groups Turkey’s test for multiple comparisons was used. Spearman’s and Pearson’s correlation analysis was used to assess the association between the variables. The association of VEGF, ICAM-1 and N-CML with severity of

| Table 1: Mean value of different study variables and distribution of sex in the study groups. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Variable                        | Control (n=39)  | No DR (n=40)    | NPDR (n=39)     | PDR (n=39)      |
| Age (years) (mean ± SD)         | 50.5 ± 10.6     | 56.25 ± 7.7     | 53.5 ± 6.7      | 51.1 ± 9.2      |
| Sex                                  |                 |                 |                 |                 |
| Male                                | 25              | 19              | 23              | 26              |
| Female                              | 14              | 20              | 16              | 14              |
| Duration of diabetes (years) (mean ± SD) | Not applicable | 6.1±4.9        | 10.4±6.1       | 10.3±5.2        |
| Glycosylated haemoglobin (%) (mean ± SD) | 6.181±0.61     | 7.197±1.13     | 8.064±1.64     | 8.248±1.48     |
| Serum N-CML (ng/ml) (mean ± SD)    | 31.3±21.23      | 73.88±35.01    | 91.21±66.65    | 132.08±84.07   |
| V-VEGF ± SD (pg/ml)                | 138.7±64.38     | 210.7±120.2    | 307.0±125.9    | 404.7±192.5    |
| ICAM-1 ± SD (ng/ml)                | 484.1±784.1     | 592.6±119.3    | 643.7±108.0    | 742.8±175.8    |

No DR: no diabetic retinopathy; NPDR: non proliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy
retinopathy and photoreceptor disruption was obtained using Pearson’s correlation analyses.

RESULTS AND DISCUSSION

Table 1 shows the distribution of age, sex and duration of diabetes in the study groups. On comparing the mean age of the four groups, ANOVA revealed no significant difference (p>0.05). Similar sex proportion was found among all the four groups (p=0.068). On comparing the duration of diabetes a significant difference among the study groups was present (p<0.0001). The level of diabetic retinopathy increased as the duration of the disease increased.

Visual acuity decreased as the level of retinopathy increased. Mean log MAR visual acuity was 0.05 for control, 0.27 for No DR, 0.65 for NPDR and 1.16 for PDR. A significant positive correlation was found between log MAR visual acuity and grade of disruption (r=0.9, p<0.0001). Visual acuity decreased as the grade of ISel photoreceptor disruption increased. ANOVA revealed that the grade of disruption increased significantly as the level of retinopathy increased (p<0.0001). The grade of disruption correlated significantly with the level of retinopathy (r=0.73; p<0.001).

The mean levels of N-CML, VEGF and ICAM-1 in each group are shown in Table 1. ANOVA revealed that N-CML, VEGF and ICAM-1 were significantly different between the study groups (p<0.0001). Turkey’s multiple comparisons showed that VEGF and ICAM-1 were significantly different between controls and NPDR, between controls and PDR, and between No DR and PDR (p<0.0001). On multiple comparison N-CML showed significant difference between control and NPDR (p = 0.008), control and PDR (p < 0.0001), and No DR and PDR (p = 0.01).

The association of N-CML, VEGF and ICAM-1 with disruption of ISel photoreceptor was evaluated independently. Strong association using Pearson’s correlation analysis was noted between their increased serum levels and disruption of ISel photoreceptor. [N-CML: r = 0.68, p < 0.0001; VEGF: r = 0.36, p=0.001; ICAM-1: r = 0.36, p = 0.001] (Figure 2-4).

Hyperglycaemia has been advocated to be one of the leading causes for the micro vascular complications in diabetic retinopathy [26]. Several biochemical pathways and mediators are induced by it. Formation of advanced glycation end products along with mediators like VEGF, ICAM-1 has gained importance not only for better understanding of the pathogenesis of micro vascular complications but are also important for establishing better therapeutic regimens [27-29].

AGE receptor complex has been recently reported in retinal pigment epithelial cells by McFarlane et al. They highlighted that exposure of bovine RPE cells and the human D407 RPE cell line to AGE modified albumin led to formation of AGE receptor complex within the cells [30]. The accumulation of lipofuscin and reduction of lysosomal degradative capacity in RPE cells may result from AGE formation which is responsible for impaired degradation of engulfed photoreceptor remnants [31,32].

Increased levels of photoreceptor ISel disruption has been demonstrated to be associated with increased VEGF levels in our earlier study [24]. In a rat model, Foulds et al found that in the hypoxic retina, angiogenic and vascular permeability factors such as VEGF, nitric oxide synthases, and insulin-like growth factor-1 are up regulated in retinal astrocytes and Müller cells but are
also present in large amount in the photoreceptors [33]. Muller cells play a crucial role in the neurotropic factors mediated photoreceptor cell response to injury [34,35]. Activation of intracellular pathways triggering PI3K/Jack-stat pathway in the muller cells results in changes in the levels of endogenous neurotropic factors [36,37]. Activation of the PI3K/mTOR/translational pathway has been reported to be important for IFNγ-mediated VEGF expression in RPE cells [38]. Thus, up regulation of VEGF might be a response to ISEI photoreceptor disruption, implying diabetic retinopathy to be a secondary effect of this pathway. ICAM-1 is the key mediator of the effect of VEGFs on retinal leukostasis [39]. Thus all the three biochemical parameters of our study intricately effect the photoreceptors in diabetic retinopathy.

Maheshwary et al demonstrated percentage disruption of photoreceptor ISEI as an important predictor of visual acuity among diabetic macular edema patients [40]. The transverse length of the disrupted or absent ISEI has been related to visual impairment [41]. Yoshanan et al in a study on association of retinal sensitivity to integrity of photoreceptor ISEI in patients with diabetic macular edema found that disruption of the ISEI is correlated with a significant decrease in point sensitivity in eyes with diabetic macular edema [42]. Lee et al found that foveal ischemia in diabetic macular edema resulted in photoreceptor outer segment shortening and ISEI disruption resulting in outer retinal layer atrophic changes and subsequent visual loss [43]. Photoreceptor ISEI integrity serves an important indicator for visual acuity in diabetic retinopathy.

In present study, we correlated the levels of N-CML, VEGF and ICAM-1 with disruption of ISEI. The accurate delineation of the photoreceptor ISEI on SD-OCT images encouraged us to evaluate its structural integrity in vivo. Increased serum levels of N-CML, VEGF and ICAM-1 were significantly related with increased severity of retinopathy. Also, all the three biochemical parameters were found to be strongly associated with photoreceptor ISEI disruption. Decreased visual acuity was found to be positively associated with increased severity of retinopathy, photoreceptor ISEI disruption and increased serum levels of N-CML, VEGF and ICAM-1.

CONCLUSION

Present study highlighted the positive correlation of N-CML, VEGF and ICAM-1 with photoreceptor structural integrity and visual acuity in diabetic retinopathy providing further insight into the pathogenesis of the disease. Thus serum N-CML, VEGF and ICAM-1 levels can be used as prognosticator of photoreceptor disruption in diabetic retinopathy.

REFERENCES


