

RESEARCH ARTICLE

A decrease in VEGF and inflammatory markers is associated with diabetic proliferative retinopathy

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ABSTRACT. Diabetic retinopathy is the most severe ocular complication of diabetes mellitus (DM), is associated with micro-vascular damage. The more advanced stage, proliferative diabetic retinopathy, has been linked to an increased risk of cardiovascular morbidity and mortality. Our hypothesis was that inflammatory and angiogenic markers will detect the different stages of type 2 diabetes, and may predict development of micro-vascular damage. **Methods.** Seventy three type II diabetic patients were randomly assigned to three groups (A - 25 patients [12 males], no diabetic retinopathy; B - 25 patients [19 males], non-proliferative retinopathy; and C - 23 patients [13 males], proliferative retinopathy), when they came for a routine follow-up visit in the ophthalmologic outpatient clinic. Twenty-three healthy subjects (14 males) served as controls. High-sensitivity C reactive protein (hs-CRP), soluble vascular cell adhesion molecule 1 (sVCAM-1) and vascular endothelial growth factor (VEGF) were studied. **Results.** The duration of type II diabetes differed between group A (9 ± 6 years) and B (17 ± 9 years) patients ($p = 0.001$). No such difference was revealed between groups B and C (19 ± 6 years) ($p = 0.30$). A difference in hemoglobin A1C (HbA1C) levels was detected between groups A ($7.1 \pm 2.7\%$) and B ($8.5 \pm 1.5\%$) ($p = 0.02$), but none was found between groups B and C ($8.5 \pm 1.6\%$) ($p = 0.98$). Only six patients (out of 23) used insulin treatment in group A, compared with 16 in group B (out of 25) and 17 in group C (out of 25) ($p = 0.004$). All three groups of diabetic patients were older (62.8 ± 10.8 , 61.9 ± 9.4 , 59.2 ± 10.3 years, respectively) than the controls (44.3 ± 11.6 years) ($p \leq 0.001$). Hs-CRP levels were higher in diabetic patients ($4,391 \pm 4,175$, $4,109 \pm 4,533$, $3,005 \pm 3,842$ ng/mL, respectively) than in controls ($1,659 \pm 1,866$ ng/mL); however, only the levels in patients of groups A ($p = 0.01$) and B ($p = 0.03$) were significantly different from those of the controls, in contrast to group C, which did not differ ($p = 0.180$). Similar findings were observed for sVCAM-1 (706 ± 347 , 746 ± 328 , 638 ± 208 ng/mL, respectively, vs. controls [552 ± 143 ng/mL]); sVCAM-1 levels of groups A and B, but not C, differed from the controls ($p = 0.05$, $p = 0.01$ and $p = 0.125$, respectively). With the exception of group B ($p = 0.03$), soluble VEGF DM type II levels (493 ± 353 , 625 ± 342 , 368 ± 223 pg/mL, respectively) did not vary from those of the controls (392 ± 355 pg/mL, $p \geq 0.05$). However, as the disease progressed, there was a significant decrease in VEGF levels, accompanied by a significant difference between groups B and C ($p = 0.006$). **Conclusions.** Patients with diabetes type 2 with no retinopathy and with non-proliferative retinopathy had high levels of inflammatory and angiogenic markers, which decreased in patients with diabetic proliferative retinopathy. Biomarkers of inflammation and angiogenesis may detect the progression of diabetic vascular disease and may lead towards earlier interventions that would prevent systemic complications.

Keywords: diabetic retinopathy, VEGF, AVASTIN

The earliest clinical signs of diabetic retinopathy are microaneurysms [1], and after 20 years of disease they are present in nearly all patients with type I diabetes [2] and in 80% of the patients with type II diabetes [3]. Epidemiological studies have shown that proliferative retinopathy predicts cardiovascular mortality and morbidity in both diabetes type 1 [4-7] and type 2 [8-12] populations. Non-proliferative retinopathy could predict mortality (all

cause and cardiovascular) in women with type II diabetes [13], while another study (2103 type II diabetic patients, 7 years' follow up) showed that only diabetic retinopathy (especially in its more advanced stages) was associated with an increased cardiovascular disease incidence independent of other known cardiovascular risk factors [14]. The mechanism of the association between diabetic proliferative retinopathy and the increased incidence of

cardiovascular morbidity and mortality raises questions about the ability to predict vascular disease progression (micro- and macro-vascular) by non-invasive means or by using biomarkers that will alert doctors regarding deterioration of this complicated metabolic cardio-vascular disease.

Our aim was to find an association between stages of diabetic retinopathy (no-retinopathy, non-proliferating retinopathy and proliferating retinopathy) and biochemical markers, which could define each group and would assist in early detection of vascular complications that lead to blindness and cardiovascular events.

DONORS AND METHODS

We randomly selected 73 type II diabetes patients that came for a routine follow up visit in the ophthalmic outpatient clinic between January 2010 and February 2011 and separated them into three groups (25 patients {12 males}, age 62.8 ± 10.8 years, without diabetic retinopathy [group A]; 25 patients {19 males}, age 61.9 ± 9.4 years, with non-proliferative retinopathy [group B]; and 23 patients {13 males}, age 59.2 ± 10.3 years, with proliferative retinopathy [group C]). Twenty three healthy subjects (14 males; age 44.3 ± 11.6 years) served as controls.

Inclusion criteria: patients with type 2 diabetes in all ages, without any limitation to gender, age, weight, but without chronic diseases and that are capable of signing a consent form.

Exclusion criteria: patients with type 2 diabetes that have renal failure (even mild), documented coronary heart disease, heart failure, myocardial disease, any immunological or inflammatory disease, any oncological disease, or any other debilitating disease.

We planned to recruit within 100 patients within 12 months (we screened more than 125 patients but only 73 patients were found eligible to participate in the study). We also recruited 25 healthy volunteers that were employees of the hospital. We assumed that 100 patients will give a strong statistical power to answer our hypothesis, that inflammatory and angiogenic markers will separate between groups of patients with type 2 diabetes and retinopathy, and may also suggest future development of micro-vascular disease typical of type 2 diabetes, but the 73 patients that were eventually recruited had enough statistical power for data analysis.

On entry to the study an ophthalmologist diagnosed retinopathy by funduscopy after papillary dilatation using a clinical disease severity scale [15]. Retinopathy was classified as follows: group A – no retinopathy; group B – non-proliferative retinopathy; group C – proliferative retinopathy with intra-retinal hemorrhage and/or hard exudates. None of our patients was treated with AVASTIN. The study was approved by the internal review board of the hospital, and all participants signed a consent form before enrollment in the study. **Clinical characteristics** included age, gender, height, weight, hemoglobin A1C% (HgBA1C%), length of disease and the need for insulin treatment. All patients had hypertension and hypercholesterolemia. Fasting blood was withdrawn early in the morning (before 8:00 AM). The serum was separated and frozen at -80°C until processed as one batch in the end of the

study. Biochemical markers of inflammation included high-sensitivity C reactive protein (hs-CRP), soluble vascular cell adhesion molecule 1 (sVCAM-1) and vascular endothelial growth factor (VEGF), which were measured by enzyme-linked immunosorbent assay (ELISA) methods (Quantikine Human C-Reactive Protein, Cat. No. DCRP00; and Quantikine Human sVCAM-1, Cat. No. DVC00, R&D System, Inc. 614 McKinley Place NE Minneapolis MN 55413 USA, and for VEGF we used Quantikine ELISA Human VEGF Immunoassay catalog number DVE00, SVE00, PDVE00 from the same manufacturer with a sensitivity of detectable dose of VEGF less than 5.0 pg/mL. Using Calibrator Diluent RD6U the minimum detectable dose is typically less than 9.0 pg/mL. The specificity – this assay recognizes natural and recombinant VEGF. This assay also recognizes recombinant human VEGF_{165b}).

Statistical analysis

The student's T test and Chi square test were used for categorical variables, and one way ANOVA was used for continuous variables to compare differences between patients in different stages of DM. T-tests included two tailed distribution with paired and two sample unequal variance.

RESULTS

Seventy three type II diabetes patients were recruited to the study after signing a consent form. We grouped them according to the ophthalmologic examination (funduscopy) after papillary dilatation, using a clinical disease severity scale [15]. There were 25 patients in group A (12 males, age 62.8 ± 10.8 years), 25 patients in group B (19 males, age 61.9 ± 9.4 years) and 23 patients in group C (13 males, age 59.2 ± 10.3 years). In the control group, there were 23 healthy subjects (14 males, age 44.3 ± 11.6 years) ($p \leq 0.001$) (table 1). The patients had lower statures (165 ± 9 cm, 168 ± 7 cm, 165 ± 9 cm, respectively, to groups A, B and C) vs. controls (173 ± 8 cm) ($p \leq 0.05$), larger waist circumferences (110 ± 14 cm, 109 ± 13 cm, 109 ± 13 cm, respectively) than controls (93 ± 12 cm) ($p \leq 0.001$) and larger body mass index (BMI) (30 ± 6 , 29 ± 4 , 30 ± 5 , respectively) vs. controls (25 ± 4) ($p \leq 0.001$) (table 1). The duration of diabetes differed between groups A (9 ± 6 years) and B (17 ± 9 years) ($p = 0.001$). No such difference was found between groups B and C (19 ± 6 years) ($p = 0.30$) (table 1). A significant difference in HgA1C% was observed between groups A ($7.1 \pm 2.7\%$) and B ($8.5 \pm 1.5\%$) ($p = 0.02$), but no such difference was noted between groups B and C ($8.5 \pm 1.6\%$) ($p = 0.98$) (table 1). Only six patients in group A, 16 patients in group B and 17 patients in group C used insulin treatment ($p = 0.004$) (table 1).

Hs-CRP levels were high in all three groups of patients ($4,391 \pm 4,175$ ng/mL, $4,109 \pm 4,533$ ng/mL, $3,005 \pm 3,842$ ng/mL, respectively) vs. controls ($1,659 \pm 1,866$ ng/mL); however, only group A ($p = 0.01$) and B ($p = 0.03$) had significantly different hs-CRP levels than the controls, while patients in group C did not demonstrate such a difference ($p = 0.180$) (table 2). A similar phenomenon was observed for sVCAM-1 levels

Table 1
Clinical Characteristics

	Controls	Group A	Group B	Group C
No.	23	25	25	23
Men	14 (60%)	12 (48%)	19 (76%)	13 (56%)
Age (years)	44.3 ± 11.6	62.8 ± 10.8	61.9 ± 9.4	59.2 ± 10.3
P-value*		0.75		0.35
P-value**		≤0.001	≤0.001	≤0.001
Height (cm)	173 ± 8	165 ± 9	168 ± 7	165 ± 9
P-value*		0.15		0.34
P-value**	≤0.001	0.046	≤0.001	
BMI	25 ± 4	30 ± 6	29 ± 4	30 ± 5
P-value*		0.78		0.76
P-value**	0.001	≤0.001	≤0.001	
Waist circumference	93 ± 12	110 ± 14	109 ± 13	109 ± 13
P-value*		0.76		0.91
P-value**	≤0.001	≤0.001	≤0.001	
Insulin Rx	6	16	17	
P-value*		0.004		0.80
Duration of DM (y)	9 ± 6	17 ± 9	19 ± 6	
P-value*		0.001		0.30
P-value***	0.001			
HBgA1C%	7.1 ± 2.7	8.5 ± 1.5	8.5 ± 1.6	
P-value*		0.02		0.98

BMI – body mass index

DM – diabetes mellitus type II

HBgA1C% - hemoglobin A 1 C %

P-value* - comparison between the groups of patients

P-value** - comparison between each group and the control group

P-value*** - comparison between group 3 and group 1.

Table 2
Biochemical Markers

	Controls	Group A	Group B	Group C
hs-CRP	1,659 ± 1,866 4,391 ± 4,175	4,109 ± 4,533	3,005 ± 3,842 (ng/mL)	
P-value*		0.84		0.32
P-value**		0.01	0.03	0.18
sVCAM-1	552 ± 143	706 ± 347	747 ± 328	638 ± 208 (ng/mL)
P-value*		0.71		0.21
P-value**		0.05	0.01	0.12
VEGF	392 ± 355	493 ± 353	625 ± 342	368 ± 223 (pg/mL)
P-value*		0.22		0.006
P-value**		0.52	0.53	0.35

hs-CRP – high sensitivity C reactive protein

sVCAM-1 – soluble vascular cell adhesion molecule

VEGF – vascular endothelial growth factor

P-value* - comparison between the groups of patients

P-value** - comparison between each group and the control group

(706 ± 347 ng/mL, 746 ± 328 ng/mL, 638 ± 208 ng/mL, respectively) vs. controls (552 ± 143 ng/mL); group A (p=0.05) and B (p=0.01) had significantly different sVCAM-1 levels than the controls (*table 2*). Patients in group C did not demonstrate such a difference (p=0.125) as a result of their low sVCAM-1 levels (*table 2*).

VEGF levels in groups A and B, but not in group C, were higher than those of the controls (493 ± 353 pg/mL, 625 ± 342 pg/mL, 368 ± 223 pg/mL, respectively, vs. 392 ± 355 pg/mL); a significant differ-

ence was observed between group B (625 ± 342 pg/mL) and C (368 ± 223 pg/mL) (p=0.006) (*table 2*). The lowest VEGF levels were observed in group C.

DISCUSSION

We found that patients with type 2 diabetes with no-retinopathy and with non-proliferative retinopathy had high levels of VEGF and inflammation markers, which

decreased steeply in patients with diabetic proliferative retinopathy. Patients with proliferative diabetic retinopathy were revealed to have the lowest levels of inflammation markers (hs-CRP and sVCAM-1), in comparison to diabetic patients with non-proliferative retinopathy or to those that did not have retinopathy.

The traditional belief is that elevated levels of hs-CRP accompany diabetic retinopathy, providing a link between inflammation and the development of microvascular complications of diabetes [16]. Another clinical study with 18 patients suffering from diabetic proliferative retinopathy had significantly raised plasma VEGF levels when compared with controls ($P=0.001$). After pan-retinal photocoagulation there was a significant reduction in plasma VEGF level at 4 months' follow-up ($P<0.001$). Patients with complete resolution of neovascularization had a trend toward lower median VEGF levels (80 versus 150 pg/mL, $P=0.062$) [17]. Based on this paradigm the anti-VEGF treatment was developed, having a significant clinical success in about 50% of patients that were treated with AVASTIN injections.

However, a recent study demonstrated that high CRP levels in patients with type 2 diabetes were associated with a lower prevalence of diabetic retinopathy [18]. Diabetic patients with the highest CRP quartiles were less likely to have any diabetic retinopathy [18]. This study supports our findings, that higher CRP levels were observed in diabetic patients without retinopathy and in patients with non-proliferative retinopathy. Another study reported that in diabetic patients with retinopathy, hs-CRP levels and the frequency of patients with hs-CRP values above 1.0 mg/l were low [19].

Our results demonstrated that the same phenomenon was true also for another marker of inflammation (ICAM-1) in the most advanced state of diabetes. We also found a significant decrease in VEGF level in patients with proliferative retinopathy ($p=0.006$), and that patients with proliferative retinopathy had the lowest levels of VEGF (even lower than controls). Induction of adhesion molecules is an essential step in inflammation mediated by leukocyte adhesion. Previous reports have shown that VEGF did not affect the expression of ICAM-1 and VCAM-1 in human dermal micro-vascular endothelial cells [20], whereas VEGF did increase the expression of ICAM-1 in vivo in retinal endothelial cells [21]. VEGF elevated VCAM-1 and ICAM-1 protein levels and leukocyte adhesiveness in an NF-k-B dependent manner [22]. In addition, VEGF mRNA and protein were induced to high levels after corneal injury and were correlated with inflammation and neo-vascularization. Moreover, specific inhibition of VEGF by antibodies suppressed corneal neo-vascularization, and VEGF may be required for inflammatory neovascularization of the cornea and can be identified as a functional endogenous corneal angiogenic factor [22]. Endoglin(CD105) is a type I membrane glycoprotein located on cell surfaces and is part of the TGF-beta receptor complex. It has been found on endothelial cells, activated macrophages, fibroblasts and smooth muscle cells. CD105 has a role in the development of the cardiovascular system and in vascular remodeling. Plasma CD105 levels were significantly higher in diabetic patients than in non-diabetic patients and were elevated in diabetic

patients at all stages of retinopathy. Interestingly, the highest levels were found in patients that had no retinopathy, followed by non-proliferative retinopathy, and the lowest levels were found in advanced proliferative retinopathy [23]. These findings support ours, which indicated that angiogenic factors are inhibited in the advanced proliferative retinopathy stage.

Our findings are further supported by another trial where limbal insufficiency was created surgically, after which sub-conjunctival injections of 2.5 mg bevacizumab (Avastin) were started twice weekly for 1 month - immediately (early-treatment group), 1 week (mid-treatment group) and 1 month after injury (late-treatment group). Avastin injections inhibited corneal neo-vascularization in the early and mid-treatment groups, but not in the late-treatment group [23]. Since VEGF levels were not measured, there is no data on its level at each stage of vascular injury; however, the synthesis of our results with these data may lead to the hypothesis that anti-VEGF treatment is effective only in early stages of vascular complications, when VEGF levels are still high and can be manipulated. Low VEGF levels may explain the lack of response to Avastin treatment in the proliferative retinopathy stage (advanced micro-vascular disease) that was observed in this study [24].

Ischemia and occlusion of retinal (and elsewhere) small blood vessels trigger angiogenic factors (VEGF) and inflammatory mediators, which induce angiogenesis in order to overcome and bypass the occluded small blood vessels. However, at a certain point, inflammatory inhibition begins in order to stop the continuous uncontrolled "wild" growth of new blood vessels and vascular complications in the retina (and elsewhere) through inhibition of VEGF production; thus, less cell adhesion molecules and pro-inflammatory growth factors and cytokines are produced and secreted. Exhaustion of the inflammatory system after years of intense continuous inflammatory reaction could be another plausible explanation.

Study limitations

The relatively small group of patients, the significantly younger age of subjects in the control group, the significant difference between patients' BMI and controls, and the significant difference of waist circumference between patients and controls may limit our ability to form firm conclusions. Larger studies with more age-matched controls and with a control group of healthy volunteers that will have larger BMIs and will be more obese should be sought and pursued in the near future.

Summary

All patients with type II diabetes in our study had high levels of inflammatory and angiogenic markers in the serum but a decrease of these markers was observed in patients with progressive vascular disease (diabetic proliferative retinopathy). Biomarkers of inflammation and angiogenesis may detect the progression of diabetic micro-vascular disease at an earlier stage and may prevent blindness and systemic complications by timely interventions.

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