The Relationship Between Fibronectine Levels and Oxidation - Glycosylation End Products in Type 1 Diabetic Patients

Tip 1 Diyabeliklerde Fibronektin Düzeylerinin Oksidasyon Glikolizasyon Son Ürünleriyle İlişkisi

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Abstract
Purpose: Fibronectin might play an important role in pathogenesis and progression of chronic diabetic complications. The aim of this study is to estimate the plasma concentrations of fibronectin, AGEs (Advanced Glycosylation End Products), AOPPs (Advanced Oxidation Protein Products) T1DM (Type 1 Diabetes Mellitus) patients as well as the relationship between each other and diabetic complications.
Material and Methods: Fourty T1DM patients attending to the Diabetes and Endocrinology outpatient clinic in Haseki Hospital and 17 healthy volunteers as control group were included in this study. AGEs and AOPPs were measured spectrophotometrically and fibronectin was measured with nephelometric method.
Results: Fibronectin levels of diabetic patients were significantly higher than control group ($p<0.05$). There was not any significant difference in fibronectin levels between patients with and without diabetic complications. In addition, no statistically significant difference was observed in AGE and AOPP levels among diabetic patients and control group. No correlation was determined between plasma fibronectin and AGE and AOPP levels ($r=0.4$) either.
Conclusion: No significant correlation was determined between fibronectin levels and AGE and AOPP which play an important role in progression of diabetic complications in T1DM. The lack of significant difference between healthy controls and patients with T1DM suggests that treatment counteracts the increase in free radical production.

Key Words: **Type 1 Diabetes Mellitus; Fibronectins; Advanced Glycosylation End Products.**

Özet
Amaç: Fibronektin birçok hücre yüzeyinde, extrasellüler matrixte ve plasmada bulunan bir glikoproteindir. Bu çalışmanın amacı komplikasyonlu ve komplikasyonsuz Tip 1 *Diabetes Mellitus* hastalarında plasma fibronektin seviyelerini ve onun komplikasyonlarla bağlantılı olduğu bilinen ileri glikozillenmiş son ürün (AGE) ve ileri oksidasyon protein ürünü (AOPP) düzeyleri ile ilişkisini incelemekti.
Gereç ve Yöntem: Tip I Diyabet'li 40 hasta ve kontrol grubu olarak 17 sağlıklı bireyle çalışıldı. İleri Oksidasyon Protein Ürünü (AOPP) ve ileri Glikozillenmiş Son Ürün (AGE), spektroflorometrik fibronektin düzeyleri ise nefelometrik yöntemle çalışıldı.
Bulgular: Diyabetli hastaların plasma fibronektin ortalamaları, kontrol grubuna göre yüksek bulundu ($P<0,05$). Diyabetli hastalar kendi içinde komplikasyonlu ve komplikasyonsuz olarak karşılaştırıldığında aralarında anlamlı fark olmadığı görüldü ($P>0,05$). AOPP ve AGE düzeyleri açısından hasta grubu ile kontrol grubu arasında da fark bulunamadı ($P>0,05$). Hasta grubunda plasma fibronektin seviyesi ile AOPP ve AGE düzeyleri arasında korelasyon olmadığı görüldü ($r = 0,4$).
Sonuç: Tip1 diyabetli hastalarda AGE, AOPP gibi diyabetik komplikasyonların gelişimi açısından önem taşıyan oksidasyon ve glikozilasyon son ürünleri ile fibronektin düzeyleri arasında anlamlı farklılık bulamadık. Tip 1 diyabetli hastalarda serbest radikallerin artışının tedavi ile önlendiği düşünülebilir.

Anahtar Sözcükler: **Fibronektin; İleri Glikolizasyon Son Ürünleri; Tip 1 Şekerli Diyabet.**

Introduction

Diabetes mellitus is a disorder characterized by hyperglycemia which is associated with non-enzymatic glycation, oxidative stress and carbonyl stress (1). Through these mechanisms hyperglycemia induces increased production of advanced glycosylation end products (AGE) and advanced oxidation protein products (AOPP) which cause severe damage on biologically important compounds (2). Hyperglycemia increases oxidative stress, supports leucocyte endothelium interaction and helps glycation of every protein in the body included lipoproteins, apolipoproteins and coagulation factors. In the course of time, following complex dehydration and oxidation reactions, glycation end products occur. AGE may cause accumulation of LDL particles by stimulating cross-binding of collagens and especially extracellular matrix proteins which are present in blood vessels (3). Deposition of AGE's in tissues has an extremely toxic effect. By changing the structure of proteins AGE may damage extracellular matrix structure and metabolism directly. They occasionally implement these effects through receptors (RAGE- receptor for advanced glycation end–products). The interaction of AGE-RAGE enhances activation of nuclear factor-κB (NF-κB) and stimulates gen transcription of cytokins, growth factor and adhesive molecules. This mechanism stimulates the migration of macrophages and produces highly deleterious effects (4). Besides, it contributes to the progression of diabetic complications such as nephropathy, retinopathy, neuropathy and macroangiopathy. Furthermore it modificates LDL particles and accelerates the progression of atherosclerosis (5).

Advanced oxidation protein product (AOPP) was first described in 1996 by Witko-Sarsat et al. They occur during oxidative stress by the action of hypochlorous acid and chloramines which are produced by myeloperoxidase. They act like AGEs in induction of proinflammatory cytokins and adhesive molecules (6). Reactive oxygen species (ROS) effects the proteins directly which leads to formation of oxydated amino acids. It oxydates thyrosine amino acid directly and generates dithyrosine structure causing agreggation and fragmantation in protein structure. The product produced by this configuration is called AOPP (7). Chromatographic and electrophoretic techniques demonstrate that AOPP consists of heavy chain disulfide bonds or/and aggreagates ofalbuminwith thyrosine cross binding (7).

Endothelial dysfunction is considered to be the first step in the development of arterial disease. It is possible to assess endothelial dysfunction by vascular function tests which use invasive or noninvasive techniques or by determining plasma concentrations of specific endothelial proteins. Fibronectin is also another marker of endothelial activation (8).

Fibronectin is a large glycoprotein present in extracellular matrix and on cell surfaces which improves cell-cell and cell-matrix interactions and thus plays an important role in tissue construction and reconstruction (9). It has two major forms of which one is predominantly soluble in body fluids like plasma, CSF, sinovial, amniotic and seminal fluid. The other form is found on cell surfaces and in extracellular matrix in an insoluble multimeric form. A fibronectin molecule has a length of 600 A, width of 25 A and its molecular weight is 550 KD. It consists of two subunits. The disulfide bonds which bind these subunits with each other stand near the carboxy terminal ends of each one (10).

Fibronectin plays an important role in embryogenesis, oncogenic transformation, cell adhesion, wound healing, tissue reparation, platelet functions and cell migration Furthermore it may have a role in inflammation as an opsonin and chemotactic agent. While the majority amount of circulating fibronectin is synthesized by hepatocytes it is also synthesized and secreted by several types of cells like macrophages, thrombocytes, fibroblasts, amniotic cells, endothelial cells, melanocytes, mast cells, schwann cells, sinovial cells and chondrocytes. Plasma fibronectin has a half life of 24-72 hours. Average concentration of plasma fibronectin in normal people is between 250-400 pg/ml. It is reported that the plasma levels do not show any difference considering age and gender (10).

Elevated levels of plasma fibronectin are reported in rheumatoid arthritis, pre-eclamptic pregnancies, collagenous vascular diseases, acute trauma, septic syndrome and thrombotic thrombocytopenic purpura (11-15). These results indicate that intravascular accumulation of fibronectin damages blood vessels.

Characteristic endothelial extracellular matrix alterations and damages in blood vessels are also present in diabetic patients. In diabetic patients the accumulation of proteins in subendothelial matrix is seen as PAS positive in light microscobe. The increased plasma levels of fibronectin reflect the vessel wall injury and matrix modifications in diabetic patients (16).

Although oxidative stres is increased in both types of DM we could not find satisfactory number of studies concerning the patients with T1DM. With this study, we aimed to assess fibronectin, AOPP and AGE levels in T1DM diabetic patients as well as the interaction of these parameters with each other and diabetic complications.

Materials and Methods

We studied 40 T1DM patients presenting to the outpatient clinic of Diabetes, Endocrinology and Obesity in Haseki Training and Research Hospital and 17 healthy individuals as control group.(Table I). The study was approved by the local ethics committee and written consent was obtained from each participant.

Fifty of diabetic patients had diabetic complications. 11 had retinopathy, 7 had nephropathy and 9 had neuropathy. Biochemical parameters like glucose, creatinine, cholesterol, triglyceride, HDL and HbA1c levels were determined by Olympus AU 2700 (Japan) with original reactive and methods. Fibronectin levels were determined through nephelometric method with Dade Behring ProsPec analyser (Australia).

The results were expressed as mean ±SD (Standard deviation of mean). Data were evaluated by SPSS 2.0 for Windows (SPSS Inc., Chicago, II, USA). Statistical analysis was performed using Mann Whitney-U and Sperman correlation tests. A P value less than 0.05 was considered as statistically significant. *Levene F test* was used for assessing the compliance of the data to normal distribution. Nonparametric test was used because variance was not homogenous and the distribution was not normal.

AGEs assay. The sera were diluted 1:50 with phosphate buffered saline (PBS; pH 7.4). Fluorescence intensity was recorded at the emission maximum 350 nm (spectrofluorimeter Biorad). Maximum emission was recorded as 440 nm. Fluorescence intensity was expressed in arbitrary units (AU) and in AU/g protein (6).

AOPP assay. Chloramine–T stock solution (10 mmol), 1.16mol/L of potassium iodure (KI), glaciel acetic acide (% 100 v/v) and PBS (20 mmol/L) were used as reactives. Chloramine–T stock solution was diluted 100 times with PBS and main standart solution of 100µmol/L concentration was prepared. Chloramine–T standards for PBS calibration curve were prepared by diluting this solution with PBS to concentrations of 12.5, 25, 50, 75, 100 µmol/L. The stability of Chloramine–T stock solution is 3 to 4 months at 5° C, whereas stability of the diluted solution is 3 to 4 days at 5° C. The samples were processed at room temperature with a programme adapted to Olympus AU 2700 analyser. 160 µL of PBS reactive was added on 40µL plasma and incubated for 25 seconds. The absorbance of the mixture was measured at 340 nm. Subsequently 20µL of acetic acide was added and incubated for 25 seconds. the absorbance was re-measured. Finally 10µL of KI solution was added and absorbance was re-measured after another 25 sec. of incubation. Concentration of AOPP was determined in chloramines units (ìmol/L) and values adjusted for albumin levels were calculated (µmol/L/g albumin). All of the steps of the process were performed in a single measure cell at 37°C. Depending on the programme characteristics of the analyser used, time intervals might be arranged at 25 seconds or longer for each step (17).

Results

There was a significant difference between diabetic patients and healthy control group regarding fibronectin levels (0.264±0.026 g/L; 0.175±0.012 g/L, respectively. $p<0.05$). AOPP (8.4±3.5 µmol/g Albumin; 7.94±3.38µmol/g Albumin) and AGE (18.19±5.85–AU/g T. Protein; 15.7±6.36 AU/g T. Protein) levels were similar in diabetic and control group. There was not any significant difference in cholesterol, triglyceride, HDL, creatinine levels between diabetic and control group either (Table II). No significant difference was determined between the groups with and without complication regarding all parameters. (Table II). Correlation test between fibronectin, AGE and AOPP levels did not reveal any relationship between each other ($r = 0.4$).

Table I. The demography of patients and control group

	Type 1 DM	Control group
Number of patients	40	17
Age (year, mean±SD)	30.11±9.6	32.6±8.7
Female	29	10
Male	11	7
Smoker	14	
Complications	15	
Duration of DM (year, mean±SD)	7.6±7.1	

Table II. Data of patients and control group (mean ± SD).

	Type1 DM	Control group	P Value
Glucose (mg/dl)	216 ± 109.07	102.5 ± 34.2	>0.05
Cholesterol (mg/dl)	167 ± 38.1	176.4 ± 40	>0.05
Triglyceride (mg/dl)	98.2 ± 60.9	100.9 ± 45	>0.05
Creatinine (mg/dl)	0.9 ± 0.9	0.82 ± 0.15	>0.05
Total Protein (g/L)	7.15 ± 0.65	7.27 ± 0.48	>0.05
Albumin (g/L)	4.38 ± 0.38	4.5 ± 0.24	>0.05
Fibronectin (g/L)	0.264 ± 0.026	0.175 ±0.012	<0.05. r = 0.4
HDL (mg/dl)	52.8 ± 13.9	47 ± 11	>0.05
HbA1c (%)	8.17 ± 1.5	5.01 ± 1.17	<0.05
AOPP/Alb (3mol/g Albumin)	8.4 ± 3.5	7.94 ± 3.38	>0.05. r = 0.4
AGE /T. Prot (AU/g T.Protein)	18.19 ± 5.85	15.7 ± 6.36	>0.05. r = 0.4

No significant difference was determined between the groups with and without complication regarding all parameters. Correlation test between fibronectin, AGE and AOPP levels did not reveal any relationship between each other.

Discussion

Plasma fibronectin levels are elevated in diabetic patients. There are many indicators of microangiopathy in this jgroup of patients. Some of them are increased urinary albumin excretion, high concentrations of vWF, high concentrations of vascular cell adhesion molecules and impaired endothelial vasodilatation (18-22). Fibronectin is one of these indicators. These findings are nonspecific for diabetic vasculopathies and they may be present in atherosclerosis without diagnosis of diabetes. High levels of triglyceride, smoking, increased urinary albumin excretion are not affected by high levels of fibronectin in diabetic patients. High levels of fibronectin may be evaluated as a form/kind of endothelial cell activation. Advanced studies will demonstrate that fibronectin concentration may be used as a marker of endothelial dysfunction in diabetic patients (11-15).

Some matrix proteins are found in high concentrations in diabetes mellitus. Big vessels also involve laminin and fibronectin molecules in big amounts like small ones. Unfortunately polimerisation in intracellular matrix causes fibronectin to be released in the circulation in an insoluble form. The polarised secretion modifications induces an increase in fibronectin concentrations in diabetic patients (8).

Simo and co-workers did not demonstrate any significant difference between fibronectin concentrations of diabetic patients with and without complications (23). We could not demonstrate any significant difference either. Zozulinska et al found fibronectin levels higher in type 1 diabetic patients than healthy people but when patients with and without complications were compared there was not any difference (24). The results of this study support our data. These studies point out that elevation of plasma concentration in diabetic patients is independent of microangiopathy. A study by Testa and co-workers also revealed elevated levels of fibronectin in diabetic patients and their findings supported our study (25). Lee and co-workers demonstrated that fibronectin levels were higher in diabetic patients compared with healthy subjects (26).

We compared AOPP and AGE levels in diabetic and healthy group and could not find any significant difference between two groups. Furthermore these two parameters were correlated with fibronectin, cholesterol, triglyceride, HDL and creatinine levels of the patients and no correlation was found (r=0.4). Wagner and co-workers found a positive correlation between AGE levels and creatinine levels of the diabetic patients with renal insufficiency. They did not define an increase in AGE levels of diabetic patients with normal renal functions (27). In our study, renal functions of our patients were within normal ranges. Kalousova and co-workers described a positive correlation in AGE and triglyceride levels in diabetic patients. They believe that these results are because of the fact that diabetes mellitus is a complex metabolic disorder (6).

Witko and Sarsat found AOPP levels elevated in patients undergoing hemodialysis regardless of being diabetic or not (4). However Kalousova and co-workers did not find AOPP levels elevated in diabetic patients with normal renal functions (6). We also could not find AOPP levels

significantly different in patient and control group. Both group of investigators found significant correlation between AOPP and triglyceride levels. Early on, the association between lipids and AOPP was not so clear but now it is known that AOPP is effective on oxidative modification of LDL and this effect causes atherosclerosis. Wagner and co-workers could not find an association between AGE levels and diabetic complications (27). Stitt and co-workers could not demonstrate detrimental effects and the accumulation AGE in damaged tissues (28). We also could not demonstrate a significant association in AGE and AOPP levels of patients with or without complication ($p>0.05$). Kalousova and co-workers found AGE and AOPP levels slightly elevated in patients with micro and macrovascular complications but it was not statistically significant (10) while Gleisner and co-workers assessed AOPP levels in type 1 diabetic patients and failed to show any significant difference from the control group (29). Kalousova and co-workersl found these levels markedly increased in type 2 diabetic patients. Probably oxidative stress has a more important role in production of AGE in type 2 diabetic patients. It is supposed to be the compensation mechanism of the illness (6). With another point of view, it may be suggested that antidiabetic treatment prevents the formation of free radicals caused by oxidative stress.

Xie XM and co-workers demonstrated by a study in 2006 that expression of fibronectin mRNA is induced by AGE when compared to control group. They suggested that elevation of fibronectin levels induced by AGEs contributed to progression of diabetic complications (30). We conclude that fibronectin levels were higher in diabetic group than control group yet this elevation was not related with complications. We could not demonstrate the effects of formation of AOPP and AGE on diabetic complications and fibronectin levels.

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