Role of Soluble Fas/Fas Ligand Pathway and Osteoprotegerin in Diabetic Foot Ulceration

Diyabetik Ayak Ülserlerinde Solubl Fas/Fas Ligand ve Osteoprotegerin Yolağının Rolü

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Özet

Amaç: Apopitoz ateroskleroza katkıda bulunur. Fas/Fas ligand apopitoz gelişimini teşvik ederken osteoprotegerin ters yönde etki gösterir. Çalışmamızda diyabetik ayak patogenezinde bu apopitoz belirteçlerinin rolünü incelemeyi amaçladık.

Yöntem ve Gereçler: Diyabetik ayağı bulunan 38 ve bulunmayan 25 tip 2 diyabet hastası, diyabetik olmayan 25 kontrol hastasıyla birlikte çalışmaya alındı. Diyabetik ayak lezyonları Wagner sınıflandırmasına göre değerlendirildi. Serum örneklerinde solubl Fas, solubl Fas ligand ve osteoprotegerin düzeyleri ELISA yöntemiyle değerlendirildi.

Bulgular: Osteoprotegerin, solubl Fas ve solubl Fas ligand düzeyleri, diyabetik ayak grubunda diyabetik ayağı bulunmayan diğer tip 2 diyabetli hastalar ve kontrol grubuna göre belirgin olarak daha yüksekti (sırasıyla; $p<0.05$, $p<0.001$, $p<0.001$). İleri düzeyde diyabetik ayak lezyonları bulunan hastalarda (Wagner 4-5), solubl Fas ve solubl Fas ligand düzeyleri daha yüksekti (sırasıyla; $p<0.01$ ve $p<0.01$).

Sonuç: Apopitotik yolağın, Fas/Fas ligand ve osteoprotegerin aracılığıyla diyabetik ayak ülserleri gelişiminde rol aldığını düşünüyoruz. Gangrenöz ayak lezyonlarında sFas and FasL düzeyi belirgin olarak daha yüksek olduğu için sFas/FasL sistemi enflamasyon ve eşlik eden apopitoz sürecinin şiddetini gösterebilir.

Anahtar Sözcükler: Diyabetik ayak, Fas, osteoprotegerin

Abstract

Aim: Apoptosis contributes to atherosclerosis. Fas/Fas ligand promotes apoptosis while osteoprotegerin does the reverse. We aimed to investigate the role of these apoptosis markers in the pathogenesis of diabetic foot.

Material and Methods: Thirty-eight type 2 diabetic patients with diabetic foot, 25 type 2 diabetic patients without diabetic foot and 25 control subjects were enrolled in the study. Diabetic foot lesions were graded according to Wagner classification. ELISA method was used to measure soluble Fas, soluble Fas ligand and osteoprotegerin levels in serum samples.

Results: Osteoprotegerin, soluble Fas and soluble Fas ligand levels were significantly higher in the diabetic foot group than those without diabetic foot and the control subjects ($p<0.05$, $p<0.001$, and $p<0.001$ respectively). Patients with advanced diabetic foot lesions (Wagner grade 4-5) had higher soluble Fas and soluble Fas ligand levels ($p<0.01$ and $p<0.01$ respectively).

Conclusions: We conclude that apoptotic pathway plays a role in the development of diabetic foot ulcers via Fas/Fas ligand and osteoprotegerin. Since sFas and FasL were significantly higher in gangrenous foot lesions, sFas/FasL system may indicate the severity of inflammation and ongoing apoptosis.

Keywords: diabetic foot, Fas, osteoprotegerin

Introduction

Atherosclerotic vascular disease is the leading cause of death in diabetes mellitus (1). Endothelial dysfunction precedes overt atherosclerosis (2). Increased apoptosis and dysfunction of endothelial cells lead to procoagulant activity, loss of vasomotor tonus, and apoptosis of vascular smooth muscle cells (3). Normal adult arterial tissue has low apoptotic and mitotic indices (4). Various atherosclerotic risk factors increase apoptotic activity in endothelial cells (4). Deposition of immune cells such as macrophages and T cells in atherosclerotic regions cause secretion of proinflammatory cytokines4. These cytokines cause increased apoptosis in smooth muscle cells (4).

Tumor necrosis factor receptor (TNFR) family is the most important member of the membrane receptors involved in apoptosis. One of the most important members of this family is Fas (CD95) receptor. Fas ligand (FasL) either in membrane bound or soluble (sFasL) form, is synthesized by immune system cells and after binding to Fas receptor it forms a signal complex that triggers cell death. The secreted isoform of Fas (soluble Fas; sFas) lacking the receptor transmembrane domain, competes with membrane-associated Fas for binding to ligand (FasL) and suppresses Fas/FasL mediated apoptosis (5).

Osteoprotegerin (OPG) protein, another member of TNFR family, is produced by endothelium lining arterial wall and vascular smooth muscle cells (6). Certain regions of OPG are related to apoptotic signal transduction and OPG can inhibit apoptosis through binding to TNF related apoptosis inducing ligand (TRAIL). Therefore it is proposed that OPG serves as an autocrine survival factor for endothelial cells (7). Experimental models and human studies have showed that OPG plays a role in vascular calcification (8-10).

Lifetime risk of foot ulceration, which is a common and much feared complication of both types of diabetes, may be as high as 25% (11). It results from microvascular (neuropathy) and macrovascular complications (peripheral artery disease) via endothelial dysfunction and atherosclerosis. sFas/sFasL and OPG may be involved in macro- and microvascular complications of type 2 diabetes mellitus (12-14).

In this study we aimed to study changes in OPG and sFas/sFasL system and their contribution to the presence and severity of diabetic foot ulcers.

Material And Methods

Sixty three type 2 diabetic patients and 25 control subjects were enrolled to the study. The control subjects had normal thyroid, liver and renal function tests. They had no known cancer. They were non-diabetic (hemoglobin A1c less than %6.5 and fasting glucose less than 126 mg/dl) and not using corticosteroid and immunsuppressive medication. Four of them had hypertension and 3 had coronary artery disease. Diabetic patients were composed of two groups: those with diabetic foot (group 1, n=38) and those without this complication (group 2, n=25). Based on history, laboratory tests and physical examination patients with liver disease, chronic renal failure, rheumatologic diseases, malignancy, and thyroid dysfunction were excluded. Usage of corticosteroid and immunsuppressive medication was another exclusion criteria. Age, gender, smoking status, diabetes duration, blood pressure measurements, and their medication were recorded. The study was approved by the ethics commitee of our faculty and patients' written informed consent were obtained. The study was performed according to Helsinki declaration.

Diabetic foot lesions were classified according to Wagner classification (grade 0: no open skin lesion, grade 1: superficial ulcer and not infected, grade 2: deep ulcer, frequently infected but no osteomyelitis, grade 3: deep ulcer, abscess or osteomyelitis present, grade 4: localized gangrene of foot finger tips or forefoot, grade 5: gangrene entire foot) (15). In patients with diabetic foot, lower extremity peripheral artery disease was studied with Doppler sonography. Presence of osteomyelitis was determined by labeled leukocyte bone scintigraphy, leukocyte count, and CRP. Haemoglobin A1c levels and serum lipids were studied. LDL was calculated by Friedewald formula (16). Following 8 hours of fasting, blood samples were drawn for sFas, sFasL, and OPG levels measurement. After centrifugation at 3000 g for 10 minutes within 30 minutes of sampling, the supernatant part was collected in polyproline tubes and preserved at -80ºC. Serum sFas (Bender MedSystems GmbH, Vienne, Austria, Europe), FasL (Bender MedSystems GmbH, Vienne, Austria, Europe), and OPG (Biovendor Research And Diagnostic Products, Czech Republic) were measured by ELISA method.

Statistical analysis

SPSS version 17.0 was used for the statistical analysis.

The results were expressed as mean±SD. The data of the three main groups were compared by ANOVA. Student's t-test and Mann-Whitney-U test were done to compare data of subgroups of diabetic foot. In the diabetic foot group the relation of OPG, sFas, and FasL levels to clinical features and laboratory values were evaluated by using Pearson and Spearman correlation tests. A value of p less than 0.05 was accepted as statistically significant.

Results

The groups did not differ in terms of age, gender, diabetes duration, lipid levels, hemoglobin A1c level, and smoking habitus. There was no difference in medication between diabetic patients (insulin therapy, oral antidiabetics, antihypertensives, antihyperlipidemic medication, antiaggregant drugs) (data not shown). Haemoglobin A1c levels of diabetic patients with and without diabetic foot were similar and higher than control subjects. HDL cholesterol was lowest in the diabetic foot group. There was no difference in the other lipid parameters.

OPG, sFas, and sFas ligand levels were significantly higher in diabetic foot group than in those without foot ulceration ($p<0.05$, $p<0.001$, and $p<0.001$, respectively) and control subjects ($p<0.05$, $p<0.001$, and $p<0.001$, respectively) (Table 1). Although OPG, sFas, and sFas ligand levels were slightly higher in diabetics without ulceration than control subjects, they did not reach statistical significance.

CRP and leukocyte counts were also higher in diabetic foot group than in those without foot ulceration ($p<0.05$, $p<0.001$, and $p<0.001$, respectively) and control subjects ($p<0.05$, $p<0.001$, and $p<0.001$, respectively) (Table 1). Although CRP and leukocyte counts were slightly higher in diabetics without ulceration than control subjects, they did not reach statistical significance.

In the diabetic foot group OPG correlated positively with

Table 1. Characteristics of diabetic patients and control subjects.

	Diabetics with foot ulcers (Group 1) n= 38	Diabetics without foot ulcers (Group 2) n= 25	Control group (Group 3) n = 25	P
Gender (M/F)	29/9	18/7	20/5	>0.05
Age (years)	62.8±10.0	63.7±9.8	67.7±9.6	>0.05
DM duration (years)	11.1±7.6	10.3±9.1	-	>0.05
SBP (mmHg)	139±7	135±7	130±5	>0.05
DBP (mmHg)	78±5	77±4	77±3	>0.05
Current smoker (n)	4	3	4	>0.05
Total cholesterol (mg/dL)	165±48	189±47	190±55	>0.05
Triglyceride (mg/dL)	185±139	150±95	196±145	>0.05
HDL (mg/dL)	30.6±17.4	42.3±13.6	38.4±6.4	*
LDL (mg/dL)	99±35	123±47	104±35	>0.05
HbA1c (%)	10.5±2.1	10.2±2.0	4.6±0.8	&
OPG (pmol/L)	14.6±5.7	11.2±5.1	10.9±4.4	ß
sFasL (ng/mL)	3.29±1.49	1.27±0.28	1.25±0.99	#
sFas (pg/mL)	350.4±270.4	91.0±106.8	90.6±63.8	°
CRP (mg/dL)	13.0±11.4	1.6±2.5	1.1±0.8	a
Leukocyte (/mm^3)	14812±7060	8149±2459	7950±5303	b

***** Group 1- group 2 $p<0.05$; group 1-group 3 $p<0.05$; group 2-group 3 $p>0.05$
&Group 1- group 2 $p>0.05$; group 1-group 3 $p<0.05$; group 2-group 3 $p<0.05$
ß Group 1- group 2 $p<0.05$; group 1-group 3 $p<0.05$; group 2-group 3 $p>0.05$
Group 1- group 2 $p<0.001$; group 1-group 3 $p<0.001$; group 2-group 3 $p>0.05$
° Group 1- group 2 $p<0.001$; group 1-group 3 $p<0.001$; group 2-group 3 $p>0.05$
a Group 1- group 2 $p<0.001$; group 1-group 3 $p<0.001$; group 2-group 3 $p>0.05$
b Group 1- group 2 $p<0.001$; group 1-group 3 $p<0.001$; group 2-group 3 $p>0.05$
CRP: C reactive protein, **DBP:** diastolic blood pressure, **DM:** type 2 diabetes, **HbA1c:** haemoglobin A1c, **OPG:** osteoprotegerin, **sFas:** soluble Fas, **sFasL:** soluble Fas ligand, **SBP:** systolic blood pressure

sFas ligand (p<0.01, r=0.52), sFas (p<0.05, r=0.42), CRP levels (p<0.05, r=0.41) and leukocyte count (p<0.05, r=0.33). Fas levels also showed correlation with CRP levels (p< 0.01, r = 0.46) and leukocyte count (p<0.01, r=0.46). However there was no statistically significant correlation between sFas and sFasL in this group (Table 2).

Since the patients in each Wagner class was few in number, we reclassified diabetic foot group: gangrene group (Wagner grade 4 and 5; n=18) and non-gangrene group (Wagner grade 1, 2 and 3; n=20). sFasL and sFas levels were significantly higher in the gangrene group than non-gangrene group (p<0.01 and p<0.01, respectively). Although OPG levels were also higher in this group, the difference did not reach any statistical significance (Table 3).

Table 2. Relation of OPG and sFas levels to other parameters in diabetic foot group

	OPG (pmol/L)	sFas (pg/mL)
sFas (pg/mL)	p<0.05, r=0.42	-
sFasL (ng/mL)	p<0.01, r=0.52	p>0.05
CRP (mg/dL)	p<0.05, r=0.41	p<0.01, r=0.46
Leukocyte count (/mm^3)	p<0.05, r=0.33	p<0.01, r=0.46

CRP: C reactive protein, **OPG:** osteoprotegerin, s**Fas:** soluble Fas, **sFasL:** soluble Fas ligand

Table 3. OPG, sFasL/Fas levels according to Wagner classification (non-gangrene group: Wagner grade 1-2-3 and gangrene group: Wagner grade 4-5).

	Non-gangrene group n= 18	Gangrene group n= 20	p
OPG (pmol/L)	13.2±5.6	16.0±5.4	>0.05
sFasL (ng/mL)	2.5±0.6	3.9±1.71	<0.01
sFas (pg/mL)	205.6±137.5	472.2±296.9	<0.01

OPG: osteoprotegerin, **sFas:** soluble Fas, **sFasL:** soluble Fas ligand

Fifteen patients with diabetic foot were diagnosed with peripheral vascular disease using doppler USG. OPG (16.6±6.4 pmol/L, 13.5±5.1 pmol/L, p>0.05) sFasL (3.8±1.8 ng/mL, 2.9±1.1 ng/mL, p>0.05), and sFas (369±180 pg/mL, 336±326 pg/mL, p>0.05) levels did not show significant difference between patients with and without peripheral vascular disease. The patients diagnosed with osteomyelitis by leukocyte stained bone scintigraphy (n=20) were not different from those without osteomyelitis (n=18) in terms of OPG (14.5±5.7 pmol/L, 14.8±6.0 pmol/L, p>0.05), sFasL (3.2±1.7 ng/mL, 3.4±1.2 ng/mL, p> 0.05) and sFas (366±333 pg/mL, 328±162 pg/mL, p>0.05). sFas, sFasL and OPG levels were not higher in the diabetic foot group after adjustment for age, gender, smoking habitus, lipid levels, and A1c level.

Discussion

Despite being expressed in atherosclerotic plaques, the biologic roles of Fas and FasL are unclear (5). It was proposed that FasL attenuated the growth of atherosclerotic lesion and decreased T cell infiltration (17). However there are data showing that FasL is not athero-protective, but proliferative and proinflammatory instead. Increased FasL expression may contribute to atherosclerotic progression (18,19).

The studies evaluating the relation between microvascular complications and apoptosis indicated that Fas/FasL took part in development of diabetic retinopathy, nephropathy and neuropathy (19-21). García-Unzueta et al found higher sFas levels in diabetics with peripheral artery disease and proposed that sFas was an independent risk factor for peripheral artery disease (22). In our study the patients with peripheral artery disease (n=15) documented by Doppler ultrasonography did not have higher sFas and sFasL levels. Conflicting results may arise from different diagnostic criteria used for peripheral artery disease.

It has been reported that sFasL is chemotactic for human neutrophils (23). Fas and FasL is also expressed on lymphocytes and other immune cells in HIV infection and malignancies (5). We found that CRP and leukocyte count was significantly higher in diabetic foot group as expected due to infection and inflammation. sFas was positively correlated with CRP level and leukocyte count. There was no correlation between FasL and the inflammatory markers (CRP and leukocyte count). Antiapoptotic sFas could be increased compensatorily to suppress ongoing inflammation. sFas and FasL were also significantly higher in gangrenous foot lesions (Wagner 4-5) than non-gangrenous lesions (Wagner 1-2-3). We suggest that sFas may indicate the severity of inflammation and ongoing apoptosis and Fas/FasL system may play a role in progression as well as development of diabetic foot ulcers.

In addition to lipid transport HDL has antiinflammatory properties demonstrated by both in vitro and in vivo studies (24). It also inhibit endothelial cell apoptosis. HDL inhibits cytokine-induced expression of adhesion molecules in endothelial cells in concentration dependent manner and reduces binding of monocytes to endothelial surface. We found significantly lower HDL values in gangrenous foot group. It may due to extensive inflammation in gangrene. HDL level can be low in diabetic gangrene (25,26). Beyond low levels in peripheral artery disease, HDL may increase with improving diabetic foot gangrene (26).

Medial linear calcification is a strong risk factor for cardiovascular disease. The cause of calcification is not clear. Increased OPG concentration may induce osteogenic transformation of vasculature (27). OPG is also related to systemic inflammation and vascular stress. On the other hand OPG is a component of apoptosis system and inhibits apoptosis through binding to TRAIL (28). In our study OPG level was significantly higher in the diabetic foot group, and it positively correlated with sFas, sFasL, CRP and leukocyte count. OPG like sFas might have increased to compensate apoptosis. The increase may be due to intense inflammation as indicated by positive correlation with CRP and leukocyte count. OPG level was not different between gangrenous and non-gangrenous diabetic foot groups, therefore we suggest osteomyelitis cannot be the sole mechanism responsible for the increase.

In conclusion, we suggest that sFas/FasL system and OPG play a role in the pathogenesis of diabetic foot lesions. sFas/FasL system may indicate the severity of foot lesions. The observational cross-sectional design is a limitation of our study. Data were collected at a single time-point. Although it was not the aim of the study, changes over time and response to therapy should be assessed. A definite causal relationship between sFas/FasL system and OPG in diabetic foot lesions can be settled in a longitudinal study.

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