Vasculopathy in type 2 diabetes mellitus: Role of specific angiogenic modulators

Article *in* Journal of Physiology and Biochemistry · February 2011

DOI: 10.1007/s13105-011-0080-8 · Source: PubMed

4 authors, including:

Enas Hamed
Assiut University
47 PUBLICATIONS **1,270** CITATIONS

SEE PROFILE

Vasculopathy in type 2 diabetes mellitus: role of specific angiogenic modulators

Enas A. Hamed, Madeha M. Zakary, Reffat M. Abdelal & Effat M. Abdel Moneim

Journal of Physiology and Biochemistry
Official Journal of the University of Navarra, Spain

ISSN 1138-7548
Volume 67
Number 3

J Physiol Biochem (2011) 67:339-349
DOI 10.1007/s13105-011-0080-8

Springer

J Physiol Biochem (2011) 67:339–349
DOI 10.1007/s13105-011-0080-8

ORIGINAL PAPER

Vasculopathy in type 2 diabetes mellitus: role of specific angiogenic modulators

Enas A. Hamed · Madeha M. Zakary · Reffat M. Abdelal · Effat M. Abdel Moneim

Received: 21 September 2010 / Accepted: 4 February 2011 / Published online: 19 February 2011

Abstract Type 2 diabetes mellitus (T2DM) is largely defined by hyperglycemia that promotes vascular complications. Abnormal angiogenesis has been claimed to have a role in this disease. This study aimed to investigate serum levels of both conventional and other markers of angiogenesis not well studied before in diabetes, and to correlate findings with age of the patients, glycemic control, presence of microvascular complications, and oxidative stress. Thirty-eight patients with T2DM and 13 age- and sex-matched healthy persons representing controls were recruited. Serum levels of basic fibroblast growth factor (b-FGF) was measured by immunosorbent assay kit; advanced glycosylation end products, platelet-derived endothelial cell growth factor (PD-ECGF), cathepsin-D (CD), gangliosides, hyaluronic acid (HA), nitric oxide (NO), lipid peroxides (LPER), superoxide dismutase, and total thiols by chemical methods; and copper (Cu) by atomic absorption flame photometry. Advanced glycosylation end products and angiogenic factors (b-FGF, PD-ECGF, CD, gangliosides, HA, and Cu) were significantly higher in patients than controls. Oxidative stress markers, NO, and LPER were significantly higher while total thiols were significantly lower in patients than controls. These changes were more pronounced with age, poor glycemic control, and presence of microvascular complications. Angiogenesis dysfunction coinciding with elevated levels of many angiogenic growth factors may point to their malfunctioning due to oxidative stress and/or protein glycation at the factor and the receptor levels. This necessitates further investigations.

Keywords Advanced glycosylation end products · Angiogenesis · Basic fibroblast growth factor · Oxidative stress · Type 2 diabetes mellitus

E. A. Hamed (✉) · E. M. Abdel Moneim
Department of Physiology, Assiut University,
P.O. Box: 71526, Assiut, Egypt
e-mail: eah3a2010@yahoo.com

M. M. Zakary
Department of Biochemistry, Assiut University,
Assiut, Egypt

R. M. Abdelal
Department of Internal medicine, Faculty of Medicine,
Assiut University,
Assiut, Egypt

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by and responsible for alterations in micro- and macrovascular beds. This implicates impaired angiogenic mechanisms and vascular homeostasis. Microvascular complications include retinopathy, nephropathy, and neuropathy. Macrovascular complications comprise peripheral vascular diseases and cardiovascular complications, such as ischemic heart disease and hypertension [20]. In order for the complex angiogenic

process to function, a huge number of anti- and proangiogenic matrix and soluble factors must act in a coordinate and synergistic manner to assemble functional blood vessels [12]. However, angiogenic defects in diabetic patients are still not completely clarified.

Diabetic hyperglycemia results in an increase in free-radical production by a mechanism involving glucose oxidation followed by non-enzymatic protein glycation and oxidative degeneration. Glycation involves the condensation of glucose with the ε-amino group of lysine, the α-amino group of an N terminal amino acid, or the amines of nucleic acids. The first reaction is the formation of an unstable Schiff base, which reaches a steady state within hours and is reversible. Rearrangement of the Schiff base into an Amadori product reaches a steady state in approximately 28 days and is also reversible. When molecules have slow turnover rates, these Amadori products undergo multiple dehydration reactions and rearrangements to irreversibly form advanced glycation end products (AGEs) [9]. The latter is believed to be involved in the genesis of many of the irreversible complications of diabetes, including expanded extracellular matrix (ECM), cellular hypertrophy, hyperplasia, and vascular complications [1].

Basic fibroblast growth factor (b-FGF) is a multifunctional single-chain polypeptide (146 amino acids) with angiogenic, antiapoptotic, and mitogenic effects on various kinds of cells [54]. Platelet-derived endothelial cell growth factor (PD-ECGF)/thymidine phosphorylase (TP) is a classical growth factor with angiogenic, endothelial cell chemotactic, hypoxia-induced antiapoptotic, and antiproliferative activities in vascular smooth muscle cells. TP releases 2-deoxy-D-ribose-1-phosphate (2-dR-1-P) from thymidine [13]. dR and 2-dR-1-P are highly reactive and involved in non-enzymatic glycosylation of proteins due to their reducing nature. Deoxyribose released by TP was associated with induction of reactive oxygen species (ROS). However, ribose and deoxyribose are highly angiogenic [8]. The role of this factor is not well studied in T2DM.

Cathepsins are papain-like cysteine endopeptidases. Cathepsin-D (CD) plays an important role in protein degradation, generation of bio-active proteins, regulating cell growth, and tissue homeostasis. Several reports have indicated that CD stimulates cell proliferation and tumor angiogenesis [6]. Gangliosides are sialylated glycosphingolipids abundant in neuronal membranes within the central nervous system. They have been implicated in cell–cell recognition, transmembrane signaling and adhesion, and in modulation of cell proliferation and differentiation induced by polypeptide growth factors [29]. Copper (Cu) is cytoprotectant by activating oxygen transport and cellular energy production, immune function, wound healing, and antioxidation. Moreover, Cu is angiogenic through activating several proangiogenic factors, e.g., vascular endothelial growth factor (VEGF), b-FGF, tumor necrosis factor alpha (TNF-α), and angiogenin [23]. Hyaluronic acid (HA) is a naturally occurring non-sulfated glycosaminoglycan component of extracellular matrix. In vivo and in vitro studies indicated that native, long-chain HA at physiologic concentrations has anti-inflammatory, anti-angiogenic, and antiproliferative properties. The later is through HA interactions with cellular CD44 receptors. Differently, HA fragment, particularly oligomers, are angiogenic, although these effects are often influenced by concomitant signaling by other growth factors [32]. The serum levels of CD activity, gangliosides, Cu, and HA need to be further studied in T2DM.

Oxidative stress has been implicated in both microvascular and macrovascular diseases. Hyperglycemia promotes formation of ROS, which can interact with both deoxyribonucleic acid (DNA) and proteins, causing damage. Mitochondrial DNA may be an especially relevant target. Interestingly, ROS-mediated cellular damage may be in the form of pathologic "memory" in the microvasculature that persists even after glucose normalization, as suggested in human retinal vasculature. T2DM is associated with oxidative stress due to the imbalance between the normal levels of pro- and antioxidative agents. Oxidative stress may link hyperglycemia with other pathways implicated in diabetic vascular complications, including AGEs formation, protein kinase C (PKC) activation, increased polyol flux, and hexosamine formation [9].

Despite the increasing awareness of diabetes-associated angiogenic disorders, the molecules' angiogenic abnormalities need further studies. The present study was conducted to determine the serum levels of angiogenic factors either previously well evaluated in T2DM patients as b-FGF together with angiogenic factors needing further evaluation in these patients as PD-ECGF, CD, gangliosides, HA, and Cu. The status of oxidative stress in the form of AGEs,

nitric oxide (NO), and lipid peroxides (LPER) as well as the antioxidants as superoxide dismutase (SOD) and total thiols would be evaluated. The levels of these factors would be correlated with indices of glycemic control, age of the patients, and presence of microvascular complications that reflect abnormal angiogenesis.

Patients and methods

Patient's characteristics and protocol

Informed consented 38 patients with T2DM [29 (76.30%) males and nine (23.70%) females] with mean ± standard deviation (SD) of age 47.53± 8.55 years were recruited voluntarily from those regularly visiting the Diabetes Outpatient Clinic, Assiut University Hospital, Assiut, Egypt, during March 2008 to December 2009 period. All patients were on diet control and/or antidiabetic medications [including oral agents (gliclazide and/or metformin) and/or insulin] at baseline. Informed consented 13 apparently healthy control volunteers [nine males (69.20%) and four (30.80%) females] with mean ± SD of age 49.62±10.70 years recruited among patients' relatives or neighbors.

Each participant was subjected to detailed history taking, thorough clinical examination, and routine laboratory investigations, e.g., random blood sugar, % glycosylated hemoglobin (HbA1c), complete blood counts, kidney and liver function tests, and fasting lipograms. Radiological examination was also done when indicated. Among diabetic patients recruited, 24 (63.20%) patients had microvascular complications in the form of retinopathy (n=4, 10.50%), nephropathy (n=5, 13.20%), neuropathy (n=10, 26.30%), and combined complications (n=5, 13.20%) [n=1, 2.60% retinopathy and nephropathy; n=3, 7.90% retinopathy and neuropathy; n=1, 2.60% nephropathy and neuropathy].

Diabetic retinopathy was diagnosed by fundoscopic examination, neuropathy by clinical history and neurological examinations and nephropathy by determination of 24 h urine albumin >30 mg/day. For comparison, the patients were subdivided into two groups according to their age (<50 and ≥50 years), multiple readings of random blood glucose levels (<140 and ≥140 mg/dl), and presence or absence of microvascular complications. The patient exclusion criteria were severe neurological diseases and neuropathy due to causes other than diabetes, symptoms, or sequel of cerebral vascular diseases, peripheral vascular diseases, presence of autoimmune diseases and/or organ-specific autoantibodies, renal insufficiency, congestive heart failure, chronic treatment with analgesics, and/or steroidal and non-steroidal anti-inflammatory drugs for conditions other than neuropathy and/or treatment with antioxidants. The research had been carried out in accordance with the Declaration of Helsinki of the World Medical Association, and had been approved by Ethics Committee of Faculty of Medicine, Assiut University, Egypt.

Laboratory assays

Venous blood samples (10 ml) were collected by venipuncture between 8 and 10 a.m. after an overnight fasting, allowed to clot and centrifuged at 3,000 rpm for 5 min. Serum was recovered and aliquot stored at −70°C until analysis. AGEs level was determined chemically by using 2,4-dinitrophenyl hydrazine as a reagent [5]. Level of b-FGF was determined using ELISA assay (R&D Systems, Minneapolis, MN, USA). Levels of PD-ECGF were determined chemically as described by Scocca [38] as 1 nmol/ml/h. The proteolytic activity of CD was assayed as described by Barrett [4] using acid denatured hemoglobin as substrate. Gangliosides were determined chemically as described previously [33]. Cu was determined by atomic absorption flame photometry (Schimadzu Seisakusho LTD, model AA-630-02, Japan). HA concentration was determined as described by Greiling [19] using HA lyase and Morgan-Elson reaction to colorimetrically detect the liberated *N*-acetyl glucosamine. NO was determined colorimetric as total nitrite + nitrate [43]. LPER was determined as malondialdehyde according to the colorimetric method described by Grau et al. [18]. SOD was determined according to colorimetric method of Misra and Fridovich [27]. Total thiols were determined by colorimetric method of Ellman [15].

Statistical analysis

Statistical Package for the Social Science (SPSS, Chicago, IL, USA) version 12 was used for data analysis. Data were presented as mean (SD), or number

(%) as appropriate. For comparison of two independent variables, student "*t* test" for normally distributed data and "Mann–Whitney test" for kurtotic and skewed data were used for data analysis. Comparisons of multiple groups were done using "one-way ANOVA test" for parametric variable. Pearson's correlation test was used for correlating parametric variable. For all tests, a probability (P) <0.05 was considered significant.

Results

Table 1 shows the serum levels of the bioindices studied in diabetic patients compared with healthy controls. Serum levels of glucose, HbA1c, AGEs, b-FGF, PD-ECGF, HA, and LPER were significantly higher, while total thiols were significantly lower in diabetic patients than controls.

Serum levels of glucose, HbA1c, AGEs, b-FGF, PD-ECGF, CD, gangliosides, Cu, HA, NO, and LPER were significantly higher, while SOD and total thiols were significantly lower in both age-dependent subgroups of diabetic patients (<50 years and ≥50 years) than controls. In patients ≥50 years old, serum levels of glucose, HbA1c, AGEs, PD-ECGF, CD, gangliosides, Cu, and HA were significantly higher than patients <50 years old. Serum levels of b-FGF, PD-ECGF, Cu, and LPER were significantly higher while SOD and total thiols were significantly lower in both blood glucose-dependent subgroups of diabetic patients (<140 mg/dl and ≥140 mg/dl) than controls. In patients with blood glucose (≥140 mg/dl), serum levels of glucose, HbA1c, AGEs, b-FGF, PD-ECGF, Cu, and LPER were significantly higher; while SOD and total thiols were significantly lower than in patients with blood glucose (<140 mg/dl). In diabetic patients with and without microvascular complications, serum levels of glucose, HbA1c, AGEs, b-FGF, PD-ECGF, Cu, HA, and LPER were significantly higher while SOD and total thiols were significantly lower than controls. Also, serum levels of CD, gangliosides, and NO were significantly higher in patients with microvascular complications than controls. Serum levels of glucose, HbA1c, AGEs, b-FGF, PD-ECGF, CD, gangliosides, Cu, HA, NO, and LPER were significantly higher, while SOD and total thiols were significantly lower in patients with microvascular complications than those without microvascular complications (Table 2).

Table 1 Diabetic control and angiogenic and oxidative stress indices investigated in type 2 diabetes mellitus patients and healthy control subjects

Indices	Patients (n=38)	Controls (n=13)	P value
Glucose (mg/dl)	225.61±67.7	104.85±13.8	0.0001
HbA1c (%)	7.13±0.95	5.41±0.31	0.0001
AGEs (nmol/l)	139.69±57.26	60.12±4.93	0.0001
b-FGF (pg/ml)	578.58±163.95	111.95±29.84	0.0001
PD-ECGF (nmol/ml)	188.08±149.45	15.12±3.24	0.002
Cathepsin-D (nmol/l)	17.25±12.27	5.86±1.33	0.072
Gangliosides (mg/dl)	30.17±22.11	9.42±2.49	0.067
Copper (μg/dl)	189.7±31.48	115.15±24.98	0.762
Hyaluronic acid (μg/l)	75.48±51.98	21.84±5.22	0.042
NO (μmol/l)	11.34±6.59	4.53±0.89	0.131
LPER (μmol/l)	6.20±2.14	1.2±0.52	0.0001
SOD (unit/ml)	1.57±0.85	4.52±1.18	0.080
Total thiols (mmol/l)	2.53±1.27	8.11±1.75	0.045

Data are presented as the mean +/- SD; *P*, versus controls

HbA1c glycosylated hemoglobin, *AGES* advanced glycation end products, b-*FGF* basic fibroblast growth factor, *PD-ECGF* platelet-derived endothelial cell growth factor, *NO* nitric oxide, *LPER* lipid peroxides, *SOD* superoxide dismutase

In diabetics, SOD and total thiols were significantly negatively correlated with the other parameters investigated (glucose, HbA1c, AGEs, b-FGF, PD-ECGF, CD, gangliosides, Cu, HA, NO, and LPER). Meanwhile, there were significant positive correlations among the angiogenic and oxidative stress indices (glucose and HbA1c, AGEs, b-FGF, PD-ECGF, CD, Cu, gangliosides, HA, NO, and LPER; Table 3).

Discussion

Angiogenesis is a pathophysiological process involving formation of blood vessels from preexisting vessels. It is essential for ontogenic growth and physiological tissue remodeling or repair, but its dysfunctioning underlies a wide range of pathogenesis [52]. Many patients who have T2DM develop vascular complications despite a variety of available antidiabetic medications and improved methods of assessing disease progression. In this study, 63.20% of diabetic patients showed microvascular complica-

Table 2 Investigated indices in type 2 diabetes mellitus patients subdivided according to age, diabetic control, and presence of complications

Indices	Controls	Age		Blood glucose		Complications	
	(n=13)	<50 years (n=24, 63.16%)	≥50 years (n=14, 36.84%)	Glucose <140 mg/dl (n=4, 10.53%)	Glucose ≥140 mg/dl (n=34, 89.47%)	Without complications (n=14, 36.84%)	With complications (n=24, 63.16%)
Glucose (mg/dl)	104.85±13.8	205.67±66.02	259.79±57.81	134.25±3.77	236.35±63.30	170.21±32.69	257.92±61.74
		P<0.0001	P<0.0001, *P<0.005	P<0.336	P<0.0001, *P<0.001	P<0.001	P<0.0001, *P<0.0001
HbA1c (%)	5.41±0.31	6.86±0.97	7.59±0.74	5.95±0.06	7.27±0.91	6.41±0.43	7.55±0.92
		P<0.0001	P<0.0001, *P<0.009	P<0.223	P<0.0001, *P<0.002	P<0.0001	P<0.0001, *P<0.0001
AGEs (nmol/l)	60.12±4.93	121.3±58.23	171.23±40.48	72.55±5.92	147.59±55.34	98.41±34.87	163.78±54.27
		P<0.0001	P<0.0001, *P<0.002	P<0.639	P<0.0001, *P<0.003	P<0.021	P<0.001, *P<0.0001
b-FGF (pg/ml)	111.95±29.84	539.72±167.36	618.06±151.03	398.7±33.14	588.57±161.64	462.24±60.4	630.62±174.01
		P<0.0001	P<0.0001, *P< *0.124*	P<0.001	P<0.0001, *P<0.009	P<0.0001	P<0.0001, *P <0.0001
PD-ECGF (nmol/ml)	15.12±3.24	173.88±43.35	212.43±51.22	124.57±27.99	195.55±46.08	156.09±31.82	206.75±48.73
		P<0.0001	P<0.0001, *P<0.006	P<0.0001	P<0.0001, *P<0.001	P<0.0001	P<0.0001, *P<0.0001
Cathepsin-D (nmol/l)	5.86±1.33	14.6±5.7	21.79±18.32	9.18±1.94	18.20±12.63	12.06±3.21	20.28±14.50
		P<0.018	P<0.0001, *P<0.044	P<0.584	P<0.001, *P<0.111	P<0.121	P<0.0001, *P<0.020
Gangliosides (mg/dl)	9.42±2.49	25.42±9.66	38.31±33.36	15.93±3.8	31.84±22.79	20.95±5.35	35.54±26.23
		P<0.016	P<0.0001, *P<0.045	P<0.551	P<0.001, *P<0.119	P<0.110	P<0.0001, *P<0.023
Copper (μg/dl)	115.15±24.98	182.28±27.63	202.41±37.56	152.55±14.38	194.07±30.09	169.09±16.96	201.72±31.97
		P<0.0001	P<0.0001, *P<0.045	P<0.024	P<0.0001, *P<0.007	P<0.0001	P<0.0001, *P<0.001
Hyaluronic acid (μg/l)	21.84±5.22	61.23±32.98	95.62±76.98	51.25±32.65	78.33±53.41	51.41±16.13	87.01±61.20
		P<0.0001	P<0.008, *P<0.035	P<0.260	P<0.0001, *P<0.262	P<0.050	P<0.0001, *P<0.038
NO (μmol/l)	4.53±0.89	9.96±2.93	13.61±9.94	6.95±1.69	11.85±6.77	8.75±2.23	12.85±7.79
		P<0.007	P<0.0001, *P< *0.053*	P<0.458	P<0.0001, *P<0.170	P<0.054	P<0.0001, *P<0.033
LPER (μmol/l)	1.2±0.52	5.98±2.22	6.58±2.01	3.9±0.96	6.47±2.08	5.06±1.66	6.87±2.13
		P<0.0001	P<0.0001, *P< *0.347*	P<0.010	P<0.0001, *P<0.008	P<0.0001	P<0.0001, *P<0.003
SOD (unit/ml)	4.52±1.18	1.68 ±0.91	1.39±0.74	4.33±1.08	2.23±1.12	2.04±0.79	1.29±0.77
		P<0.0001	P<0.0001, *P <*0.339*	P<0.0001	P<0.0001, *P<0.005	P<0.0001	P<0.0001, *P<0.016
Total thiols (mmol/l)	8.11±1.75	2.7 ±1.37	2.24±1.04	2.73±0.56	1.43±0.77	3.22±1.20	2.13±1.14
		P<0.0001	P<0.0001, *P< *0.329*	P<0.001	P<0.0001, *P<0.008	P<0.0001	P<0.0001, *P<0.018

Data are presented as the mean +/- SD; P, versus controls

*P significance between subgroups of patients, *HbA1c* glycosylated hemoglobin, *AGES* advanced glycation end products, *b-FGF* basic fibroblast growth factor, *PD-ECGF* platelet-derived endothelial cell growth factor, *NO* nitric oxide, *LPER* lipid peroxides, *SOD* superoxide dismutase

Table 3 Correlation between measured parameters in type 2 diabetes mellitus patients

Parameters	NO	LPER	Total thiols	SOD	Cu	AGEs	Glucose	HbA1c	b-FGF	PD-ECGF	Gangliosides	Hyaluronic acid	Cathepsin-D
LPER (r)	0.644												
P value	0.000												
Total thiols (r)	−0.5860	−0.916											
P value	0.0001	0.0001											
SOD (r)	−0.569	−0.880	0.973										
P value	0.0001	0.0001	0.0001										
Copper (r)	0.416	0.601	−0.592	−0.557									
P value	0.009	0.0001	0.0001	0.0001									
AGEs (r)	0.604	0.765	−0.679	−0.675	0.639								
P value	0.0001	0.0001	0.0001	0.0001	0.0001								
Glucose (r)	0.589	0.664	−0.597	−0.580	0.775	0.876							
P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001							
HbA1c (r)	0.598	0.821	−0.727	−0.715	0.748	0.940	0.915						
P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001						
b-FGF (r)	0.454	0.745	−0.663	−0.634	0.832	0.780	0.868	0.894					
P value	0.004	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001					
PD-ECGF (r)	0.790	0.778	−0.725	−0.706	0.610	0.851	0.811	0.885	0.719				
P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001				
Gangliosides (r)	0.985	0.650	−0.577	−0,560	0.427	0.640	0.635	0.649	0.496	0.832			
P value	0.0001	0.0001	0.0001	0.0001	0.008	0.0001	0.0001	0.0001	0.002	0.0001			
Hyaluronic acid (r)	0.924	0.661	−0.591	−0.565	0.476	0.645	0.655	0.673	0.536	0.877	0.957		
P value	0.0001	0.0001	0.0001	0.0001	0.003	0.0001	0.0001	0.0001	0.001	0.0001	0.0001		
Cathepsin-D (r)	0.990	0.662	−0.585	−0,570	0.425	0.653	0.641	0.656	0.506	0.829	0.998	0.947	
P value	0.0001	0.0001	0.0001	0.0001	0.008	0.001	0.0001	0.0001	0.001	0.0001	0.0001	0.0001	
Age (r)	0.444	0.310	−0.278	−0.296	0.436	0.540	0.491	0.497	0.337	0.498	0.450	0.448	0.449
P value	0.005	0.068	0.091	0.071	0.006	0.0001	0.002	0.002	0.039	0.001	0.005	0.005	0.005

NO nitric oxide, *LPER* lipid peroxides, *SOD* superoxide dismutase, *AGES* advanced glycation end products, *HbA1c* glycosylated hemoglobin, *b-FGF* basic fibroblast growth factor, *PD-ECGF* platelet-derived endothelial cell growth factor

tions, 10.50% had retinopathy, 13.20% had nephropathy, 26.30% had neuropathy, and 13.20% had combined complications. Multiple random blood glucose levels were used to analyze glycemic control in this study. Poor glycemic control (≥140 mg/dl) was found in 89.47% of our diabetic patients.

Diabetes-induced glycation of proteins is a major cause of spontaneous damage to the proteome and chronic morbidity. In consistence with others [1], the present study revealed significantly increased AGEs in diabetic patients compared to healthy controls. Moreover, in accordance with others [25, 34, 48], AGEs were significantly increased with poor glycemic control, age, and presence of microvascular complications. Similarly, others reported a significant increase in AGE with age [34], which is also increased in diabetics [48]. AGEs are involved in pericyte loss and dysfunction, retinal inflammation, and thrombosis [52]. Others reported correlation of serum AGEs levels with severity of diabetic retinopathy in either type 1 diabetes mellitus (T1DM) or T2DM patients [22]. In diabetic neuropathy, AGEs were reported to be present in the perineurium, ECM, and pericytes of endothelial microvessels and in myelinated and unmylinated fibers [42]. In our diabetic patients, AGEs showed positive correlation with measured angiogenic and oxidative stress markers and negative correlation with antioxidants. AGEs increase vascular permeability; inhibit vascular dilatation by interfering with endothelial and mesangial cells secretion of cytokines and by enhancing oxidative stress [53]. AGEs increase ECM due to discordant matrix metalloproteinases (MMPs) expression and reduced turnover [44]. Also, AGEs causes glycation of growth factors including b-FGF [47].

The b-FGF is a pluripotent angiogenic effector [14]. Moreover, b-FGF is involved in development, differentiation, and survival of the neural retina [41]. In this study, marked increase in serum levels of b-FGF was found in T2DM patients compared with healthy controls. The level was higher in patients with poor glycemic control and those with microvascular complications which might reflect widespread endothelial injury in them. In agreement with the present study, Zimering et al. [59] reported elevated plasma b-FGF in a subgroup of non-Hispanic white patients with advanced T2DM. Angiogenic growth factors unresponsiveness of diabetic patients could be attributed at least partially to known glycation of them and their receptors [47]. Differential measurement of glycated vs. non-glycated b-FGF in diabetics warrants further studies. In this study, b-FGF positively correlated with other measured angiogenic factors and oxidative stress markers but negatively correlated with antioxidants. This indicates that b-FGF exerts its angiogenic effects in concert with other angiogenic factors. The effects of b-FGF are mediated through its tyrosine kinase receptor FGFR1 utilizing PKC (α and ε isoforms), syndecan-4, and NO release as a downstream mediators [40, 58]. Additionally, b-FGF stimulates endothelial cells to produce a variety of proteases, including plasminogen activator and MMP-2 [46].

In this study, cathepsin-D was significantly elevated in patients with old age and those with microvascular complications. Witek et al. [51] and Nayeemunnisa [30] reported significant increase in CD activity in alloxan diabetic rats. In this study, a positive correlation was found between CD and measured angiogenic and oxidative stress markers. Increased CD leakage into the blood could be due to the ROS oxidative damage of cell membranes [39]. CD might favor angiogenesis by release of ECM-bound b-FGF [7]. Moreover, it is presumed that CD may stimulate endothelial cell growth via a paracrine loop, acting as a protein ligand, by directly or indirectly triggering a yet unidentified cell surface receptor [6].

In this investigation, PD-ECGF increased markedly in diabetic patients compared with healthy controls. Also, significant increase in PD-ECGF was found in patients with increased age, poor glycemic control, and those with microvascular complications. PD-ECGF has been implicated in the stabilization of blood vessels during angiogenesis through the investment of newly formed vessels by mural pericytes/smooth muscle cells [36]. This study showed that serum levels of gangliosides increased markedly in diabetic patients with poor glycemic control and those with complications than healthy controls and also in patients with old age (≥50 years), and those with microvascular complications than those <50 years old, and uncomplicated cases, respectively. In vitro and in vivo studies suggested that gangliosides exhibit neuroprotective effects against retinal ischemia [49]. Also in this study, a positive correlation was found between PD-ECGF and gangliosides and other measured angiogenic factors and oxidative stress markers, which may suggest a synergistic protective feedback

induction in diabetics. The studies conducted on serum levels of PD-ECGF and gangliosides levels in diabetic patients are rather limited. To our knowledge, this is the first study that reported elevated serum levels of PD-ECGF and gangliosides in T2DM.

The role of copper in angiogenesis was first noted in 1980 during trying to isolate a peptide "endothelial stimulating growth factor", McAuslan and Reilly [26] found that copper salts were the simplest angiogenic components of tumor extracts, stimulating the migration of endothelial cells in vitro. Confirmed later by Hu [21] who showed that Cu stimulates the proliferation and migration of endothelial cells and induces their fibronectin production. In this study, Cu increased markedly in diabetic patients with old age, those with poor glycemic control, and those with microvascular complications. Increased serum levels of Cu in patients with T1DM and T2DM [45, 57] implicated as an additional factor for atherogenicity. Impaired homeostasis of Cu could be due to glycation of its plasma carrier proteins.

This study revealed significant increased HA in diabetic patients compared to healthy controls which were more advanced with old age, poor glycemic controls, and with the presence of microvascular complications. Hyaluronan oligomers compete with native long-chain HA in binding to HA receptor CD44 present on endothelial cells [32]. CD44 activation is mitogenic and induces MMP-2 and MMP-9 production that activate transforming growth factor-beta (TGF-β) and cell invasion through ECM to facilitate vessel sprouting and outgrowth [56]. In diseases such as diabetic retinopathy, long-chain hyaluronan is broken down into hyaluronan oligomers to locally induce angiogenesis within the normally unvascularized vitreous, and result in hemorrhage and blurred vision [50]. Furthermore, Murphy et al. [28] showed that activation of CD44 by depolymerized hyaluronan induces endogenous release of the angiogenic VEGF.

Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications [11]. In this study, LPER was significantly increased while total thiols were significantly decreased in diabetic patients compared to healthy controls. Also, NO and LPER were significantly elevated, while SOD and total thiols were significantly decreased in patients with blood glucose levels ≥140 mg/dl and patients with microvascular complications than those with blood glucose levels <140 mg/dl and uncomplicated cases, respectively. Fukui et al. [17] found that troglitazone prevented increased levels of lipid hydroperoxide and SOD activity in the OLETF rat, an animal model of T2DM. Many clinical studies suggest that patients with T2DM are subjected to chronic oxidative stress [37]. Diabetes and high-glucose conditions can increase the formation of ROS by multiple biochemical pathways, including increased leakage of superoxide from mitochondrial electron-transport chain, glucose auto-oxidation, activation of the polyol pathway that reduces glutathione, and/or increase AGEs and activation of PKC which activates nicotinamide adenine dinucleotide phosphate oxidase [9, 10]. Oxidative stress has been linked to increased production of VEGF upon high-glucose treatment in vitro and in the diabetic retina [16]. It is also known that oxidative stress induces glomerular hyperfiltration, proteinuria, glomerula, and interstitial fibrosis by expression of transcription factor kappa-B (NF-κB), TGF-β, and VEGF [31].

In this study, oxidative stress markers (NO and LPER) were significantly correlated with the measured angiogenic parameters which suggest the important role of oxidative stress in promoting angiogenesis. Increasing evidence suggests that oxidative stress with increased NO and peroxyl nitrite radical formation play major roles in the onset of diabetic complications. Generally, low concentrations of NO produced in response to angiogenic factors stimulate angiogenesis, whereas higher concentrations typical of inflammatory responses inhibit angiogenesis and contribute to severe medical problems [35]. Increased oxidative stress and subsequent activation of NF-κB enhance NO production and islet beta-cell damage [24]. The b-FGF induces NO production through endothelial NO synthase (eNOS) activation, resulting in vasodilatation [53]. NO likely acts as an important signal in the angiogenic response to b-FGF, presumably by terminating its proliferative actions and promoting the differentiation of endothelial cells into vascular tubes [2].

Aging is a biological process that causes progressive deterioration of cellular structure and function over time. Aging-associated impairment of angiogenesis might relate to an inappropriate production and/or function of angiogenic cytokines. In vivo studies have shown that the reduced number of capillary and vascular structure are associated with decreased

mRNA and/or protein expression of some key angiogenic growth factors, such as VEGF, b-FGF, platelet-derived growth factor, TGF-β, neuropeptide Y (NPY), and hypoxia-induced factor-1 (HIF-1). Moreover, the aging process may increase expression of anti-angiogenic factors, e.g., TNF-α and thrombospondin [55]. The impaired eNOS function found in the aged vascular tissues may be a major factor responsible for the reduction of angiogenic capacity in the elderly [3]. In this study, the serum levels of measured angiogenic factors as PD-ECGF, CD, gangliosides, copper, and HA were significantly higher in patients ≥50 years old compared to those <50 years old, meanwhile, oxidative stress markers (NO, LPER, SOD, and total thiols) and b-FGF showed no significant changes between age subgroups. These data suggest defect in balance of secretion of angiogenic factors occur in aged diabetic patients.

In conclusion, vascular complications are key players in diabetes as various angiogenic factors increased markedly in the serum of diabetic patients. Various factors can be postulated to explain the impaired angiogenic response in T2DM: First, the exposure to chronic hyperglycemia leads to the non-enzymatic glycation of proteins and dysangiogenesis leading to increase oxidative stress. Secondly, a feedback reparative process increases the angiogenic responses that could also indicates abnormalities in growth factor signaling and/or expression. The vascular dysfunctions increased with old age, poor glycemic control, and presence of microvascular complications. This raises many questions regarding the use of angiogenesis as a therapeutic approach in diabetes. Translation and clinical researches are critical in the near future to elucidate the inherent molecular mechanisms that hold the angiogenic paradox, and to predict which patients could benefit from each therapeutic approach. In this context, antioxidants and anti-AGEs appear to be more crucial.

Conflicts of interest There are no conflicts of interest.

Limitation of the study The small number of patients and study being cross-sectional rather than longitudinal are the major limitations of this study. The major cause was the limitation of the local research resources and budget to cover a few hundred patients required for proper sample size and an elaborate longitudinal and may be interference study.

References

1. Ahmed N, Thornalley PJ (2007) Advanced glycation end products: what is their relevance to diabetic complications. Diab Obes Metab 9:233–245
2. Babaei S, Teichert-Kuliszewska K, Monge JC, Mohamed F, Bendeck MP, Stewart DJ (1998) Role of nitric oxide in the angiogenic response in vitro to basic fibroblast growth factor. Circ Res 82:1007–1015
3. Bach MH, Sadoun E, Reed MJ (2005) Defects in activation of nitric oxide synthases occur during delayed angiogenesis in aging. Mech Ageing Dev 126:467–473
4. Barrett AJ (1970) Cathepsin D. Purification of isoenzymes from human and chicken liver. Biochem J 117(3):601–607
5. Bejma J, Ji LL (1999) Aging and acute exercise enhance free radical generation in rat skeletal muscle. J Appl Physiol 87(1):465–470
6. Berchem G, Glondu M, Gleizes M, Brouillet JP, Vignon F, Garcia M, Liaudet-Coopman E (2002) Cathepsin-D affects multiple tumor progression steps in vivo: proliferation, angiogenesis and apoptosis. Oncogene 21(38):5951–5955
7. Briozzo P, Badet J, Capony F, Pieri I, Montcourrier P, Barritault D, Rochefort H (1991) MCF7 mammary cancer cells respond to bFGF and internalize it following its release from extracellular matrix: a permissive role of cathepsin D. Exp Cell Res 194(2):252–259
8. Brown NS, Jones A, Fujiyama C, Harris AL, Bicknell R (2000) Thymidine phosphorylase induces carcinoma cell oxidative stress and promotes secretion of angiogenic factors. Cancer Res 60(22):6298–6302
9. Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414(6865):813–820
10. Brownlee M (2005) The pathobiology of diabetic complications: a unifying mechanism. Diabetes 54:1615–1625
11. Calhau C, Santos A (2009) Oxidative stress in the metabolic syndrome. In: Soares R, Costa C (eds) Oxidative stress, inflammation and angiogenesis in metabolic syndrome. Springer, The Netherlands, pp 33–63
12. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257
13. de Bruin M, Temmink OH, Hoekman K, Pinedo HM, Peters GJ (2006) Role of platelet derived endothelial cell growth factor/thymidine phosphorylase in health and disease. Cancer Ther 4:99–124
14. Detillieux KA, Sheikh F, Kardami E, Cattini PA (2003) Biological activities of fibroblast growth factor-2 in the adult myocardium. Cardiovasc Res 57:8–19
15. Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 2:70–77
16. El-Remessy A, Abou-Mohamed G, Caldwell RW, Caldwell RB (2003) High glucose increases tyrosine nitration and superoxide anion formation in endothelial cells: role of eNOS uncoupling and aldose reductase activation. Invest Ophthalmol Vis Sci 44:3135–3143
17. Fukui T, Noma T, Mizushige K, Aki Y, Kimura S, Abe Y (2000) Dietary troglitazone decreases oxidative stress in early stage type II diabetic rats. Life Sci 66:2043–2049
18. Grau A, Codony R, Rafecas M, Barroeta AC, Guardiola F (2000) Lipid hydroperoxide determination in dark chicken

meat through a ferrous oxidation-xylenol orange method. J Agric Food Chem 48(9):4136–4143
19. Greiling H (1961) A new colorimetric method for enzymatic determination of hyaluronic acid. Z Rheumaforsch 20:298–302
20. Hameed A, Malik S, Sharif FR, Ahmed N, Nurjis F, Ali S, Qureshi JA (2002) Diabetic complications: influence of age, sex, family history, duration, glycemic control and obesity. Online J Biol Sci 2(10):710–714
21. Hu GF (1998) Copper stimulates proliferation of human endothelial cells under culture. J Cell Biochem 69(3):326–335
22. Koga K, Yamagishi S, Okamoto T, Inagaki Y, Amano S, Takeuchi M, Makita Z (2002) Serum levels of glucose-derived advanced glycation end products are associated with the severity of diabetic retinopathy in type 2 diabetic patients without renal dysfunction. Int J Clin Pharmacol Res 22(1):13–17
23. Larke RP, Froese GJ, Devine RD, Petruk MW (1988) Comprehensive seroepidemiologic study of hepatitis B virus infection in the Northwest Territories of Canada. Arct Med Res 47(1):711–713
24. Maritim AC, Sanders RA, Watkins JB III (2003) Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol 17:24–38
25. Martin A, Komada MR, Sane DC (2003) Abnormal angiogenesis in diabetes mellitus. Med Res Rev 23:117–145
26. McAuslan BR, Reilly W (1980) Endothelial cell phagokinesis in response to specific metal ions. Exp Cell Res 130 (1):147–157
27. Misra HP, Fridovich I (1972) The generation of superoxide radical during the autoxidation of hemoglobin. J Biol Chem 247(21):6960–6962
28. Murphy JF, Lennon F, Steele C, Kelleher D, Fitzgerald D, Long AC (2005) Engagement of CD44 modulates cyclooxygenase induction, VEGF generation and proliferation in human vascular endothelial cells. FASEB J 19:446–448
29. Nagai Y (1995) Functional roles of gangliosides in biosignaling. Behav Brain Res 66:99–104
30. Nayeemunnisa (2008) Proteolytic activity in the brain of alloxan diabetic rats: presence of a proteolytic activator in the cerebral extract. Int J Diabetes Dev Ctries 28(3):83–85
31. Ohshiro Y, Takasu N (2006) Molecular mechanism of diabetic nephropathy. Nippon Rinsho 64:997–1003
32. Pardue EL, Ibrahim S, Ramamurthi A (2008) Role of hyaluronan in angiogenesis and its utility to angiogenic tissue engineering. Organogenesis 4(4):203–214
33. Plucinsky MC, Prorok JJ, Alhadeff JA (1986) Variant serum beta-hexosaminidase as a biochemical marker of malignancy. Cancer 58(7):1484–1487
34. Poggioli S, Hilaire B, Friguet B (2002) Age-related increase of proteins glycation in peripheral blood lymphocytes is restricted to preferential target proteins. Exp Geront 37:1207–1215
35. Roberts DD, Isenberg JS, Ridnour LA, Wink DA (2007) Nitric oxide and its gatekeeper thrombospondin-1 in tumor angiogenesis. Clin Cancer Res 13(3):795–798
36. Saharinen P, Alitalo K (2003) Double target for tumor mass destruction. J Clin Invest 111:1277–1280
37. Sakuaba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S (2002) Reduced β-cell mass and expression of oxidative stress related DNA damage in the islet of Japanese type II diabetic patients. Diabetologia 45:85–96
38. Scocca JJ (1978) Pyrimidine nucleoside phosphorylase from *Haemophilus influenzae*. Meth Enzymol 51:432–437
39. Skrzydlewska E, Sulkowska M, Wincewicz A, Koda M, Sulkowski S (2005) Evaluation of serum cathepsin B and D in relation to clinicopathological staging of colorectal cancer. World J Gastroenterol 11(27):4225–4229
40. Slavin J (1995) Fibroblast growth factors: at the heart of angiogenesis. Cell Biol Int 19:431–444
41. Tanihara H, Inatani M, Honda Y (1997) Growth factors and their receptors in the retina and pigmented epithelium. In: Osborne N, Chader G (eds) Progress in retinal and eye research. Pergamon, Oxford, pp 271–301
42. Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, Dawnay A (2003) Quantitative screening of advanced glycation end-products in cellular and extracellular proteins by tandem mass spectrometry. Biochem J 375:581–592
43. van Bezooijen RL, Que I, Ederveen AG, Kloosterboer HJ, Papapoulos SE, Lowik CW (1998) Plasma nitrate+nitrite level are regulated by ovarian steroids but do not correlate with trabecular bone mineral density in rats. J Endocrinol 159:27–34
44. Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinses—structure, function, and biochemistry. Circ Res 92:827–839
45. Walter RM Jr, Uriu-Hare JY, Olin KL, Oster MH, Anawalt BD, Critchfield JW, Keen CL (1991) Copper, zinc, manganese, and magnesium status and complications of diabetes mellitus. Diab Care 14(11):1050–1056
46. Wang SY, Friedman M, Johnson RG, Weintraub RM, Sellke FW (1994) Adrenergic regulation of coronary microcirculation after extracorporeal circulation and crystalloid cardioplegia. Am J Physiol 267:H2462–H2470
47. Wang XH, Chen SF, Jin HM, Hu RM (2009) Differential analyses of angiogenesis and expression of growth factors in micro- and macrovascular endothelial cells of type 2 diabetic rats. Life Sci 84:240–249
48. Wautier JL, Schmidt AM (2004) Protein glycation. Circ Res 95:233–238
49. Weber M, Mohand-Said S, Hicks D, Dreyfus H, Sahel JA (1996) Monosialoganglioside GM1 reduces ischemia-reperfusion-induced injury in the rat retina. Invest Ophthalmol Vis Sci 37:267–273
50. West DC, Kumar S (1988) Endothelial cell proliferation and diabetic retinopathy. Lancet 1:715–716
51. Witek B, Król T, Kołataj A, Ochwanowska E, Stanisławska I, Slewa A (2001) The insulin, glucose and cholesterol level and activity of lysosomal enzymes in the course of the model alloxan diabetes. Neuro Endocrinol Lett 22(4):238–242
52. Yamagishi S, Nakamura K, Imaizumi T (2005) Advanced glycation end products (AGEs) and diabetic vascular complications. Curr Diab Rev 1(1):93–106
53. Yan HD, Li XZ, Xie JM, Li M (2007) Effects of advanced glycation end products on renal fibrosis and oxidative stress in cultured NRK-49F cells. Chin Med J (Engl) 120(9):787–793
54. Yang HT, Yan Z, Abraham JA, Terjung RL (2001) VEGF121- and bFGF-induced increase in collateral

blood flow requires normal nitric oxide production. Am J Physiol Heart Circ Physiol 280:H1097–H1104
55. Yang HT (2007) Effect of aging on angiogenesis and arteriogenesis. Curr Cardiol Rev 3:65–74
56. Yu Q, Stamenkovic I (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF beta and promotes tumor invasion and angiogenesis. Genes Dev 14:163–176
57. Zargar AH, Shah NA, Masoodi SR, Masoodi SR, Laway BA, Wani AI, Dar FA (1998) Copper, zinc and magnesium levels in non insulin dependent diabetes mellitus. Postgrad Med J 74:665–668
58. Zhang Y, Li J, Partovian C, Sellke FW, Simons M (2003) Syndecan-4 modulates basic fibroblast growth factor 2 signaling in vivo. Am J Physiol Heart Circ Physiol 284: H2078–H2082
59. Zimering MB, Andersonc RJ, Luoc P, Moritz TE (2008) Plasma basic fibroblast growth factor is correlated with plasminogen activator inhibitor-1 concentration in adults from the Veterans Affairs Diabetes Trial. Metab Clin Exp 57:1563–1569