Original Article

Assessment of serum levels of soluble CD40L in Egyptian children and adolescents with type 1 diabetes mellitus: Relationship to microalbuminuria and glycemic control

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ABSTRACT

Context: Soluble CD40 ligand (sCD40L) is known to be elevated in different clinical situations including hypercholesterolemia, acute coronary syndromes, and type 2 diabetes mellitus (T2DM), Data about the relationship between type 1 diabetes mellitus (T1DM) and sCD40L is limited. In addition, the potential role ofsCD40Lin the pathogenesis of vascular complications in children and adolescents with T1DM is to be clarified. Hence, the study aimed at assessment of sCD40L levels in children and adolescents with T1DM and correlation of these levels with glycemic control and microalbuminuria. **Settings and Design:** Cross-sectional controlled study. **Materials and Methods:** The study was performed in the Pediatric Endocrinology and Diabetes Unit, Assuit University Children Hospital, Assiut, Egypt. It included 70 children and adolescents with T1DM (mean age 14. 76 ± 2.21 years). Cases were further subdivided into 43 cases with normoalbuminuria and 27 cases with microalbuminuria according to presence or absence or microalbuminuria in fresh urine samples. Twentyfive healthy subjects, age- and sex-matched were included as control group (mean age = 13.62 ± 2.11 years). Studied cases were subjected to medical history, clinical examination, and laboratory assessment of fasting blood glucose (FBG), lipid profile, glycosylated hemoglobin (HbA1c), and sCD40L were performed. **Results:** Mean HbA1c and sCD40L were significantly higher in diabetic children ($n = 70$) compared to control ($n = 25$) ($P < 0.001$ for each). Mean HbA1c and sCD40L levels were significantly higher in microalbuminuric cases ($n = 27$) compared to normoalbuminuric cases ($n = 43$) ($P < 0.05$ and <0.01, respectively).We also observed a significant positive correlation between sCD40L levels and the age, diabetes duration, HbA1c, and urinary albumin creatinine ratio. **Conclusions:** The high serum sCD40L levels in children and adolescents with T1DM particularly in those with microalbminuria and its positive correlation with diabetes duration, urinary albumin excretion, and glycemic control may reflect the role of sCD40L in diabetic vasculopathy in the pediatric age group. Moreover, measurement of serum sCD40L levels in poorly controlled patients would help to identify those at high risk of developing nephropathy.

Key words: Glycemic control, microalbuminuria, sCD40L, type 1 diabetes mellitus

Introduction

CD40 ligand (CD40L) is a transmembrane protein found on cells of the immune system, including platelets, and is rapidly presented to the platelet surface after stimulation.[1] The surface-expressed CD40L is subsequently cleaved, generating a soluble fragment termed "sCD40L".[2] Both soluble and membrane-bound forms of this ligand may interact with CD40, which is constitutively expressed on macrophages, endothelial cells (ECs), vascular smooth muscle cells, and B cells, as well as on glomerular mesangial cells.[3] There is *in vitro* and *in vivo* evidence of their participation in atherothrombosis.[4] Elevated sCD40L levels have been reported in hypercholesterolemia, unstable angina, type 2 diabetes mellitus (T2DM), and acute coronary syndromes and are predictive of increased risk of cardiovascular events in clinically healthy individuals.[5] Microalbuminuria is

Access this article online	
Quick Response Code:	Website: www.ijem.in
	DOI: 10.4103/2230-8210.122617

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recognized as a risk factor for increased mortality and renal dysfunction in type 1 diabetes mellitus (T1DM). Prevention of long-term chronic complications has now become one of the main goals of modern treatment in T1DM in children.[6] The inflammatory process seems to play an important role in the development of both diabetes and its late complications.[7] Data about the relationship between T1DM and sCD40L is limited. In addition, the potential role of sCD40L in the pathogenesis of vascular complications in diabetic children and adolescents is to be clarified. Hence, the study aimed at assessment of sCD40L levels in children and adolescents with T1DM and correlation of these levels with glycemic control and microalbminuria.

Materials and Methods

We conducted a cross-sectional and case-control study. It included 70 children with T1DM (group 1). In addition, 25 apparently healthy age- and sex-matched children were studied as a control (group 2). To be included in the study, the control subjects had to be without any acute disease and without clinical conditions involving the endocrine-metabolic system. Both patients and controls were recruited from Pediatric Endocrinocriology Outpatients Clinic in Assiut University Children Hospital. The study protocol was approved by the Ethical Committees of Assiut University Children Hospital, Egypt. Written informed consents were obtained from the parents of both patients and controls.

Inclusion criteria were

- Definite diagnosis of T1DM according to the criteria of American Diabetes Association (ADA)[8]
- Age between 5 and 18 years
- Duration of insulin-treated diabetes of more than 1 year.

Exclusion criteria were

- Intercurrent illness or surgery within the previous 2 months
- Systemic inflammatory disorder or malignancy
- Treatment with medication other than insulin and captopril. Captopril was given only for patients with microalbuminuria as a treatment for nephropathy.

All cases were subjected to

- Detailed history which included: Age of onset, duration of diabetes, type, dose of insulin, frequency of diabetic ketoacidosis (DKA) or hypoglycemic attacks
- Hospital records were reviewed for the presence of microvascular complications
- Clinical examination which included:
  - Blood pressure measurement using conventional sphygmomanometer in the seated position after minutes of rest. If it was greater than 90th percentile for age and sex, the blood pressure was repeated twice for the validity of the reading[9]
  - Anthropometric measurements: Height and weight were measured using a wall-mounted stadiometer and a calibrated weight scale, respectively with subjects wearing underwear only. Body mass index (BMI) of children and parents was calculated by using the formula; BMI = weight (kg)/height (m^2). Children's BMI above 85th percentile were considered overweight.[9] The reference value of height, weight, and BMI of children were the Egyptian growth charts of Cairo University.[10] Pubertal Tanner staging was performed by standardized methods (Tanner stages)[11]
  - Full neurological examination for the cases that are not known to have diabetic neuropathy to detect any evidence of peripheral neuropathy and confirmed by nerve conduction velocity to provide accurate diagnosis[12]
  - Fundus examination was performed by an ophthalmologist after maximum papillary dilatation using indirect ophthalmoscope to identify diabetic retinopathic changes[13] according to the guidelines of International Society for Pediatric and Adolescent Diabetes.[14]
- Laboratory investigations:
  - Serum fasting glucose level was measured using the glucose oxidase technique (Beckman Coulter's L × 20)
  - Mean 2-h postprandial blood glucose (PPBG) levels in the last 3 months, prior to the study was calculated based on the patients' glucose meter records
  - Levels of serum total cholesterol, high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), triglyceride (TG) were measured using the commercially available enzymatic *in vitro* tests by Roche (Cholesterol CHOD-PAP, HDL-C Plus, LDL-C Plus, Triglycerides GPO-PAP, Hitachi, Roche Diagnostics)
  - Assessment of glycemic control by calculating the mean glycosylated hemoglobin (HbA1c) over the last year was performed using high performance liquid chromatography (HPLC) technique.[15] Patients were considered under optimal glycemic control when their HbA1c was <7.5%[16]
  - Microalbuminuria was assayed using SERAPAK immuno-microalbumin Kit (Bayer Corporation, Benedict Ave, Tarry town, NY, USA). Persistent microalbuminuria was defined when two out of three early morning urine samples ,2 months apart showed urinary albumin excretion rate

of 30-300 μg/mg creatinine.[17] Potential factors affecting urinary albumin excretion as exercise, fever, and posture were excluded.[18] Patients were further subdivided into two subgroups: Normoalbuminuric and microalbuminuric according to presence or absence or microalbuminuria in the fresh urine samples

- Measurement of serum sCD40L levels was carried out using a specific ELISA (Biosource Int., CA, USA) according to the manufacturer's instructions. The normal range of sCD40L level is 0.16-10 ng/ml.[19]

Statistical analysis

Analysis was carried out using SPSS (version 16). The numerical data were represented as mean ± SD. For comparison of the two groups, Student's *t*-test was used for parametric data and the Mann-Whitney U-test was used for nonparametric data. Linear correlations were performed by Spearman's or Pearson's test. For all analyses, *P* value of <0.05 provides statistical significance.

Results

- The rate of microalbuminuria in our studied cases was 27/70 (38.5%), while the rate of neuropathy and retinopathy was 6/70 (8.5%) and 5/70 (7%), respectively
- Diabetic patients (n = 70) and controls (n = 25) were comparable with regards to age, gender, systolic blood preasure (SBP), diastolic blood preasure (DBP), BMI, and lipid profile; while sCD40L and HbA1c were significantly higher in subjects with T1DM diabetes compared to controls ($P < 0.001$ for each) [Table 1]
- 68.5% of the studied diabetic cases were on two insulin injections per day, while 31.5 % were on three injections or more (multiple) injections per day
- Patients with microalbuminuria were older with longer disease duration. They had significantly higher mean systolic and diastolic blood pressures, HbA1c, urinary albumin excretion ratio, serum lipids (except HDL cholesterol), and insulin dose compared with normoalbuminuric cases. Microalbuminuric T1DM subjects showed a significant increase in sCD40L as compared to normoalbuminuric ($P < 0.01$) [Table 2]
- Serum levels of sCD40L have significant positive correlations to age (r = 0.70, $P < 0.001$), diabetes duration (r = 0.876, $P < 0.001$), daily insulin dose (r = 0.432, $P < 0.05$), FBS (r = 0.467, $P < 0.05$), HbA1c (r = 0.765, $P < 0.001$), urinary albumin creatinine ratio (UACR) (μg/mg) (r = 0.554, $P < 0.01$), and serum cholesterol (r = 0.653, $P < 0.001$) [Table 3]. None of the other assessed variables, including BMI, systolic and diastolic blood pressure, and triglycerides had significant correlations with sCD40L.

Table 1: Demographic, clinical, and metabolic characteristics of cases with type 1 diabetes and controls

	Diabetic cases (70)	Control (25)	*P* value
Age (years)	14.76±2.21	13.62±2.11	NS
Male/Female	44/26	14/11	NS
BMI	17.8±5.3	16.8±4.6	NS
Diabetes duration in years	8.66±2.8	-	-
Systolic blood pressure (mmHg)	108.5±25	105.6±34	NS
Diastolic blood pressure (mmHg)	69.0±13	68.9±12	NS
Hb1Ac (%)	9.2±4.21	3.9±1.2	<0.001
Triglycerides(mg/dL)	111.5±34.5	101.2±29.6	NS
Total cholesterol (mg/dL)	129.7±32.5	125±34.7	NS
LDL cholesterol (mg/dL)	92.5±23.5	89.3±18.4	NS
HDL cholesterol (mg/dL)	56.4±11.3	60.2±12.6	NS
FBG (mg/dL)	109±16.2	68.2.1±7.2.1	<0.05
2-h PPBG (mg/dl) mean±SD	209.2±49	108.5±37	<0.001
sCD40L ng/ml	12.2±3.5	3.66±1.2	<0.001

Data are given as mean±SD unless otherwise specified. BMI: Body mass index, FBG: Fasting blood glucose, HbA1c: Hemoglobin A1c, HDL: High density lipoprotein, LDL: Low density lipoprotein, PPBG: Postprandial blood glucose, sCD40L: Soluble CD40 ligand, NS: Non significant, SD: Standard deviation

Discussion

Diabetes is a major risk factor for cardiovascular disease (CVD). In patients with T1DM, atherosclerosis occurs earlier in life, leading to increased morbidity and mortality compared with those in the general population.[20] In the present study, we have shown that children with T1DM is associated with increased serum sCD40L concentrations compared to controls. This is in agreement with Harding *et al.*,[21] who reported that diabetes mellitus is associated with increased serum sCD40L concentrations and platelet surface expression of CD40L. The increase in inflammatory markers may have major implications on our understanding of the role of inflammation in vasculopathies related to this disease.[22] The effects are related to the ability of sCD40L to activate circulating leucocytes, enhance the release of both proinflammatory cytokines and chemokines by mononuclear cells, increase the expression of adhesion molecules at the endothelium, and induce the production of tissue factors.[23] sCD40L is differentiated from other inflammatory markers by its widespread distribution on atheroma associated cells and its ability to mediate broad range of functions considered to be important in atherogenesis and the development of acute coronary syndromes.[3]

The presence of microalbuminuria is observed to precede and predict overt diabetic nephropathy and is the most commonly used clinical marker and can be seen in stage III of diabetic nephropathy. Several studies suggest that at these early stages progression of diabetic nephropathy can be prevented.[24] In the present study, the rate of

Table 2: Clinical and laboratory variables of normalbuminuric diabetic children compared with microalbuminuric

	Normoalbuminuric (*n*=43)	Microalbuminuric (*n*=27)	*P* value
Age	13.5±3.4	16.9±5.2	NS
Sex, male/female	27/16	18/9	NS
Diabetic duration	8.1±3.1	10.2±4.3	<0.05
FBG (mg/dL), mean±SD	97.2±24	134.4±34	<0.001
2-h PPBG (mg/dL), mean±SD	170±67.76	211.21±56.2	<0.05
Mean Hb1Ac (%)	8.5	11.2	<0.05
Insulin dose (U/kg per 24 h)	0.8±0.1	1.6±0.3	<0.05
BMI	16.3±4.54	18.5±5.3	NS
Systolic blood pressure (mmHg)	103.1±29.1	117.1±20.3	<0.05
Diastolic blood pressure (mmHg)	68.8±14.7	79.7±12.1	<0.05
Triglycerides (mg/dL)	101.2±21.6	149.8±33.5	<0.001
Total cholesterol (mg/dL)	123.8±22.8	183.7±18.6	<0.001
LDL cholesterol (mg/dL)	88.3±26.5	96.7±33.5	< 0.05
HDL cholesterol (mg/dL)	61.7±15.9	51.2±12.91	< 0.05
UACR (µg/mg)	19.1±6.4	244.6±65.1	<0.001
sCD40L (ng/ml)	11±4.9	18.4±5.1	<0.01

Data are given as mean±SD unless otherwise specified. BMI: Body mass index, FBG: Fasting blood glucose, HbA1c: Hemoglobin A1c, HDL: High density lipoprotein, LDL: Low density lipoprotein, PPBG: Postprandial blood glucose, sCD40L: Soluble CD40 ligand, UACR: Urinary albumin creatinine ratio, NS: Nonsignificant, SD: Standard deviation

Table 3: Correlation coefficient between serum sCD40L level and clinical and biochemical data of studied cases

	Age	Diabetes duration	Daily insulin dose	FBG (mg/dL)	HbA1c	UACR; µg/mg	Cholesterol
sCD40L	r=0.70	r=0.876	r=0.432	r=0.467	r=0.765	r=0.554	r=0.653
	P<0.001	*P*<0.001	*P*<0.05	*P*<0.05	*P*<0.001	*P*=0.01	*P*<0.001

FBG: Fasting blood glucose, HbA1c: Hemoglobin A1c, sCD40L: Soluble CD40 ligand, UACR: Urinary albumin creatinine ratio

microalbuminuria among our studied caseswas reported to be 38.5%. Estimates of the prevalence of microalbuminuria in children vary between 7-28.2% in different studies.[25] The wide range of prevalence in various studies may be due to difference in ethnic groups (genetic factors are believed to be responsible for the development of diabetic nephropathy), methodology and definition of microalbuminuria, population size, length of follow-up, and mean age of study population.

In present study, sCD40L was significantly elevated in microalbuminuric T1DM subjects as compared to normoalbuminuric T1DM. Besides, sCD40L showed a significant strong positive correlation with microalbuminuria. This is in agreement with El-Asrar *et al.*,[26] in a cross-sectional controlled study enrolling 60 children with T1DM documented elevated levels of sCD40L in microalbuminuric type 1 diabetic subjects as compared to normoalbuminuric type 1 diabetics. This indicates that microalbuminuria is associated with early platelet activation, which may contribute to the atherosclerotic process via CD40L upregulation. CD40 is expressed on mesangial cells and its activation by binding of CD40L results in matrix expansion and mesangial proliferation. Recent data suggest that sCD40L may be involved in the development of diabetic nephropathy rather than its progression.[27] Another possibility could be that the state of microalbuminuria is accompanied by a certain degree of renal insufficiency that may further stimulate sCD40L production or alternatively leads to a defect in the clearance of sCD40L.[28]

The present study demonstrated that patients with microalbuminuria had significantly higher mean cholesterol and TG levels and received higher doses of insulin compared to patients with normal albuminuria. In addition, sCD40L was positively correlated with fasting plasma glucose concentration and HbA1c. These findings suggest that the degree of glycemic control is important in modulating the inflammatory response and platelet activation. This finding is in agreement with previous reports that have demonstrated that chronic hyperglycemia could increase CD40L synthesis.[28] This concept is supported by studies demonstrating that acute hyperglycemia *in vivo* leads to increases in plasma cytokine concentrations and platelet activation.[21]

In the present study, mean systolic and diastolic blood pressures were significantly higher in microalbuminuric patients compared to normoalbuminuric diabetic group [Table 2]. There is controversy as to whether an increase in blood pressure precedes or is a result of the development of microalbuminuria.[29] The association of microalbuminuria and hypertension in childhood has been demonstrated in some studies but not in others.[30] Angiotensin converting enzyme (ACE) inhibitors delay the progression of diabetic nephropathy by normalizing

glomerular capillary pressure independent of their antihypertensive effect in hypertensive and normotensive adults with diabetes.[31] Data on the use of ACE inhibitors in childhood diabetes have been shown to halt or reverse the progression of microalbuminuria in a group of normotensive children with microalbuminuria.[32] It is important to note that although our patients with microalbuminuria were on captopril therapy, yet, sCD40L serum levels were elevated documenting the relationship between this marker and the development of microvascular complications.

Limitations of the Study

First, we measured levels of sCD40L from serum that was clotted on ice to minimize the release of sCD40L *ex vivo*. Consequently, our data does not directly assess the *in vivo* levels of sCD40L.

Second, insulin has been demonstrated to have Anti- inflammatory effects.[33] Therefore, it seems that insulin therapy may contributes to the upregulation of CD40L and platelet-monocyte aggregates in the present study.

Conclusions

The high serum sCD40L levels in children and adolescents with T1DM particularly in those with microalbminuria and its positive correlation with diabetes duration, urinary albumin excretion, and glycemic control may reflect the role of sCD40L in diabetic vasculopathy in the pediatric age group. Moreover, measurement of serum sCD40L levels in poorly controlled patients would help to identify those at high risk of developing nephropathy.

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Cite this article as: Metwalley KA, Farghaly HS, El-Saied AA. Assessment of serum levels of soluble CD40L in Egyptian children and adolescents with type 1 diabetes mellitus: Relationship to microalbuminuria and glycemic control. Indian J Endocr Metab 2013;17:1024-9.

Source of Support: Nil, **Conflict of Interest:** None declared.