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Association of soluble CD40 ligand with carotid atherosclerosis in Japanese type 1 diabetic patients

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Abstract *Aims/hypothesis:* It has recently been shown that the soluble form of CD40 ligand (sCD40L) interacts with CD40 on vascular cells, leading to a variety of proinflammatory responses, and that serum sCD40L levels can be a predictive marker of cardiovascular events. The aim of this study was to estimate sCD40L levels in type 1 diabetic patients to examine a possible association with carotid atherosclerosis. *Subjects and methods:* Human sCD40L levels in serum and intima-media thickness (IMT) of carotid artery were examined in 80 Japanese type 1 diabetic patients (27 men and 53 women, age 22.8 ± 3.4 years (mean $\pm$ SD), duration of diabetes 13.2 ± 6.1 years) and 20 healthy age-matched non-diabetic individuals. *Results:* Serum sCD40L levels were significantly ($p=0.0185$) higher in subjects with type 1 diabetes (2.10 ± 1.33 ng/ml) compared with non-diabetic subjects (1.35 ± 0.88 ng/ml). The greatest IMT (Max-IMT) and averaged IMT (Mean-IMT) were also significantly greater in patients with type 1 diabetes than in control subjects (0.73 ± 0.14 vs 0.64 ± 0.07 mm, $p=0.0041$, 0.63 ± 0.09 vs 0.57 ± 0.06 mm, $p=0.0066$, respectively). Levels of sCD40L were statistically significantly associated with Max-IMT ($r=0.383$, $p<0.001$) and Mean-IMT ($r=0.275$, $p=0.0058$). Furthermore, stepwise multivariate regression analyses demonstrated that sCD40L is a determinant of both Max- and Mean-IMT, independently of conventional risk

factors. *Conclusions/interpretation:* It is suggested that increased levels of serum sCD40L are associated with accelerated atherosclerotic change observed in young patients with type 1 diabetes.

Keywords Atherosclerosis · Carotid artery · CD40 ligand · IMT · Intima-media thickness · Type 1 diabetes

Abbreviations hs-CRP: high-sensitivity C-reactive protein · IMT: intima-media thickness · sCD40L: soluble CD40 ligand

Introduction

Platelet hyperactivity plays an important role in the pathogenesis of atherosclerosis. Although CD40 ligand (CD40L) was originally identified on CD4⁺ T cells, it has recently been found on activated platelets. Membrane-bound CD40L interacts with CD40 on vascular cells, which leads to a variety of proinflammatory and prothrombotic responses, including the release of cytokines and induction of adhesion molecule expression [1]. Adhesion molecules mediate leucocyte adhesion to the endothelium and transendothelial migration, which is an important early step in the atherosclerotic process [2, 3]. Furthermore, disruption of CD40-CD40L interactions reduces atherosclerotic lesions in hypercholesterolaemic mice [4, 5]. The soluble form of CD40L (sCD40L) is similar to membrane-bound CD40L in terms of its actions on monocytes and endothelial cells, and is involved in inflammation and thrombosis [6, 7]. It has been suggested that increased levels of sCD40L are associated with atherosclerotic diseases [8–10] and can be a predictive marker of cardiovascular events [11, 12].

In type 1 diabetic patients, atherosclerosis is often observed clinically and platelet activity is thought to be increased [13–15]. It has not yet been fully evaluated, however, whether the platelet hyperactivity is actually associated with the progression of atherosclerosis in this group of patients. Thus, the aim of this study, was to

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examine whether platelet hyperactivity indicated by serum sCD40L levels is associated with carotid atherosclerosis in type 1 diabetic patients.

Subjects and methods

Study population A total of 80 Japanese type 1 diabetic patients (27 men and 53 women, age 22.8 ± 3.4 years (mean $\pm$ SD), duration of diabetes 13.2 ± 6.1 years, daily net urinary C-peptide 1.8 ± 2.9 nmol/day [5.3 ± 8.7 μ g/day]) undergoing periodic follow-up examinations at the Diabetes Clinic of Osaka University Hospital and the Osaka Police Hospital were enrolled in this study. All diabetic patients were treated with at least three or four daily insulin injections or a continuous subcutaneous insulin infusion. The daily insulin dose was 0.88 ± 0.26 U/kg. We enrolled 20 age-matched healthy non-diabetic individuals (9 men and 11 women, age 24.2 ± 1.5 years) as control subjects. The subjects did not have any clinical evidence of infection, connective tissue disease, liver dysfunction or angiopathy. None of the subjects received antihypertensive, antiplatelet or lipid-lowering medication during this study. After a full explanation of the study, which was approved by the Ethical Committee for Human Studies at Osaka University Graduate School of Medicine, written informed consent was obtained from each subject.

Laboratory procedures Fasting blood samples were collected and the following laboratory analyses were performed by SRL (Tokyo, Japan). Serum total cholesterol

and HDL cholesterol, serum triglyceride and HbA_{1c} levels were measured using standard laboratory protocols. LDL cholesterol was calculated using the Friedewald formula [16]. Serum high-sensitivity C-reactive protein (hs-CRP) was measured by ELISA. BMI, systolic BP and diastolic BP were also measured.

The subjects submitted urine samples that had been collected at home over the previous 24 h. Written instructions and careful explanation regarding the procedure for urine collection were given to each subject. Most of the patients with diabetes were familiar with the method for collecting urine at home. Nevertheless, a urine sample was discarded if there was any doubt with regard to its collection. Each participant's daily urinary AER was calculated by multiplying their 24-h urine volume by their urinary albumin concentration measured using standard laboratory protocols. Patients were classified according to urinary AER into the normoalbuminuria group (urinary AER < 30 mg/day, $n=68$), microalbuminuria group (urinary AER $30-300$ mg/day, $n=7$) or the macroalbuminuria group (urinary AER > 300 mg/day, $n=1$). The mean urinary AER was 18.3 ± 43.1 mg/day. Presence of retinopathy was diagnosed by ophthalmologists based on the findings of funduscopy; there were 53 patients without retinopathy, 21 with simple retinopathy and four with proliferative retinopathy.

Measurement of serum levels of sCD40L The BioSource Human sCD40L Immunoassay Kit (Camarillo, CA, USA) was used to assess serum sCD40L levels. Measurements were performed according to the manufacturer's instruc-

Table 1 Baseline characteristics

	Control subjects	Type 1 diabetes patients	<i>p</i> value
<i>n</i>	20	80	–
Sex (male/female)	9/11	27/53	NS
Age (years)	24.2 ± 1.5	22.8 ± 3.4	NS
Duration of diabetes (years)	–	13.2 ± 6.1	–
Smoking (yes/no)	2/18	10/70	NS ^a
BMI (kg/m ²)	20.6 ± 1.9	22.2 ± 2.7	$p=0.0097$
Systolic BP (mmHg)	114 ± 11	117 ± 13	NS
Diastolic BP (mmHg)	72 ± 8	72 ± 9	NS
Plasma glucose (mmol/l)	5.00 ± 0.33	10.70 ± 5.61	$p=0.0156$
HbA _{1c} (%)	4.62 ± 0.32	7.82 ± 1.44	$p<0.0001$
Total cholesterol (mmol/l)	4.66 ± 0.75	4.92 ± 0.78	NS
HDL cholesterol (mmol/l)	1.73 ± 0.34	1.84 ± 0.36	NS
Triglyceride (mmol/l)	0.75 ± 0.28	1.14 ± 0.72	$p=0.0202$
Calculated LDL cholesterol (mmol/l)	2.59 ± 0.67	2.54 ± 0.70	NS
hsCRP (mg/l)	0.152 ± 0.084 (0.05–0.394)	0.912 ± 1.669 (0.05–10.5)	$p<0.0001^b$
sCD40L (ng/ml)	1.35 ± 0.88	2.10 ± 1.33	$p=0.0185$
Urinary AER (mg/day)	–	18.3 ± 43.1	–
Retinopathy (NDR/SDR/PDR)	–	53/21/4	–
Mean-IMT (mm)	0.57 ± 0.06	0.63 ± 0.09	$p=0.0066$
Max-IMT (mm)	0.64 ± 0.07	0.73 ± 0.14	$p=0.0041$

Data are means $\pm$ SD. The Student's *t*-test was performed. ^a χ^2 test; ^b Mann–Whitney *U*-test
NDR No diabetic retinopathy, SDR simple diabetic retinopathy, PDR proliferative diabetic retinopathy

Fig. 1 Serum levels of sCD40L were significantly ($p=0.0185$) higher in subjects with type 1 diabetes (2.10 ± 1.33 ng/ml) than in age-matched healthy control subjects (1.35 ± 0.88 ng/ml). * $p=0.0185$ vs control subjects

tions. The intra-assay CV was 4.0%.

Measurement of IMT To identify early stages of atherosclerosis, ultrasonographic scanning of the carotid artery was performed using an echotomographic system (SSA-340A, Toshiba, Tokyo, Japan) with an electrical linear transducer (midfrequency 7.5 MHz). Scanning of the extracranial common carotid artery, the carotid bulb and the internal carotid artery in the neck was performed bilaterally from three different longitudinal projections (i.e. anterior oblique, lateral and posterior oblique), as well as the transverse projections, as reported in our previous studies [15–18]. All of the images were photocopied. The detection limit of this echo system, using 7.5 MHz, was

0.1 mm. The IMT was measured as the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line [19]. The first line represents the lumen–intima interface, and the second line is produced by the collagen-containing upper layer of the tunica adventitia. At each longitudinal projection, the site of the greatest thickness including a plaque lesion was sought along the arterial walls. Three determinations of IMT were conducted at the site of the thickest point—maximum IMT (Max-IMT)—and two adjacent points (located 1 cm upstream and 1 cm downstream from this site). These three determinations were averaged (Mean-IMT). The greatest value among the six Max- or Mean-IMTs (three from the left and three from right) was used as the representative value for each individual. All ultrasound scans were performed by an experienced sonographer (K. Sakamoto), and an experienced physician (N. Katakami) determined IMT from the photograph; both were unaware of the subject's study group and clinical characteristics. Reproducibility of the Mean-IMT measurement was examined 1 week later in 30 participants with type 1 diabetes by the same sonographer and the same physician. The mean difference in mean-IMT between these two determinations was 0.04 mm and the standard deviation was 0.07 mm, demonstrating good reproducibility for repeated measurements.

Statistical analysis Data are given as means $\pm$ SD. Means or proportions for clinical characteristics were computed for the case and control subjects, and the laboratory data were compared using unpaired *t*-tests. Differences in proportions were tested using the χ^2 test. Because the CRP distributions were skewed to the left, the significance of any differences between the patients and control subjects was determined using the Mann–Whitney *U*-test. For the

Table 2 Correlation between sCD40L and variables in the subjects

	Univariate analysis ^a		Multivariate analysis		
	<i>r</i> value	<i>p</i> value	β coefficient	<i>F</i> value	<i>p</i> value
Age (years)	−0.070	NS	NI	0.1	—
Sex (male/female)	—	NS	NI	1.1	—
Duration of diabetes (years)	−0.023	NS	NI	0.1	—
Smoking (yes/no)	−0.043	NS	NI	0.1	—
BMI (kg/m ²)	0.190	NS	NI	0.6	—
Systolic BP (mmHg)	0.140	NS	NI	0.6	—
Diastolic BP (mmHg)	0.223	0.0269	NI	2.0	—
Plasma glucose (mmol/l)	0.058	NS	NI	0.1	—
HbA _{1c} (%)	0.117	NS	NI	0.1	—
Total cholesterol (mmol/l)	0.008	NS	NI	0.1	—
HDL cholesterol (mmol/l)	0.018	NS	NI	0.0	—
Triglyceride (mmol/l)	0.072	NS	NI	0.4	—
LDL cholesterol (mmol/l)	−0.051	NS	NI	1.0	—
log ₁₀ hs-CRP (mg/l)	0.285	0.0043	0.684	8.1	0.006
<i>R</i> ²	—	—	0.078	—	—

^aPearson's univariate correlation coefficients. A stepwise multivariate regression analysis was performed. Sex: M (male)=1, F (female)=0; Smoking: yes=1, no=0

β Partial regression coefficient, NI not included in the model because variable was not significant

forward and backward stepwise multivariate regression analyses, the F value for the inclusion and exclusion of variables was set at 4.0. These statistical analyses were performed using Stat-View statistical software (Version 5.0 for Windows; Abacus Concepts, Berkeley, CA, USA) and HALBOU statistical software (Gendai Sugaku-sha, Kyoto, Japan) on a personal computer. The threshold of statistical significance was defined as $p < 0.05$.

Results

Serum levels of sCD40L are higher in type 1 diabetic patients than in non-diabetic subjects The clinical and biochemical characteristics of the study subjects are presented in Table 1. BMI, plasma glucose, HbA_{1c}, serum triglyceride, and hs-CRP levels were significantly higher in subjects with type 1 diabetes than in non-diabetic subjects ($p < 0.05$). There was no significant difference between the two groups regarding the other clinical parameters, such as age, sex, current smoking habit, sBP, dBP, total cholesterol, LDL cholesterol and HDL cholesterol.

Levels of sCD40L were significantly ($p = 0.0185$) higher in subjects with type 1 diabetes compared with non-diabetic subjects (2.10 ± 1.33 vs 1.35 ± 0.88 ng/ml, respectively) (Fig. 1). Although mean overall sCD40L levels (20 control subjects, 80 diabetic patients) were correlated with dBP ($r = 0.223$, $p = 0.0269$) and common logarithm of hs-CRP ($r = 0.285$, $p = 0.0043$), sCD40L levels were not significantly correlated with plasma glucose, HbA_{1c}, duration of diabetes or LDL cholesterol levels (Table 2). When only the diabetic subjects were analysed, there were still statistically significant correlations between sCD40L and diastolic BP ($r = 0.276$, $p = 0.0141$) or log₁₀ hs-CRP ($r = 0.249$, $p = 0.0275$). Furthermore, a stepwise multivariate regression analysis including age, duration of diabetes, sex, current smoking habit, BMI, systolic BP, diastolic BP, plasma glucose, HbA_{1c}, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride and log₁₀ hs-CRP revealed that log₁₀ hs-CRP ($F = 8.1$, $p = 0.006$) was an independent determinant of a high sCD40L value (Table 2). Additionally, we performed a stepwise multivariate regression analysis including the existence of type 1 diabetes, sex, BMI, plasma glucose, HbA_{1c} and triglyceride as independent variables, and sCD40L levels as an induced variable. This analysis demonstrated that type 1 diabetes is the independent determinant ($F = 5.4$, $p = 0.0228$) for high sCD40L levels, suggesting that the presence of type 1 diabetes affects sCD40L levels independently of sex and BMI.

Univariate regression analysis also revealed that log₁₀ hs-CRP was significantly correlated with BMI ($r = 0.478$, $p < 0.0001$), sBP ($r = 0.375$, $p = 0.0004$), dBP ($r = 0.480$, $p < 0.0001$), HbA_{1c} ($r = 0.268$, $p = 0.0140$) and total cholesterol ($r = 0.246$, $p = 0.0245$) but not with the other parameters, including duration of diabetes. A stepwise multivariate regression analysis demonstrated that BMI ($F = 6.6$) and dBP ($F = 7.2$) were the independent determi-

nants for a high log₁₀ hs-CRP.

Association between sCD40L and carotid IMT Mean-IMT and Max-IMT were significantly greater in patients with type 1 diabetes than in control subjects (0.63 ± 0.09 vs 0.57 ± 0.06 mm, $p = 0.0066$; 0.73 ± 0.14 vs 0.64 ± 0.07 mm, $p = 0.0041$, respectively) (Table 1). Serum sCD40L was statistically significantly associated with Mean-IMT ($r = 0.275$, $p = 0.0058$) and Max-IMT ($r = 0.383$, $p < 0.001$) (Fig. 2). Mean-IMT was also correlated with BMI ($r = 0.279$, $p = 0.0047$), sBP ($r = 0.235$, $p = 0.0176$) and log₁₀ hs-CRP ($r = 0.334$, $p = 0.0006$). Max-IMT was also correlated with BMI ($r = 0.25$, $p = 0.0119$) and sBP ($r = 0.287$, $p = 0.0035$) (Table 3). However, plasma glucose levels and calculated LDL cholesterol levels were not correlated with Mean-IMT or Max-IMT. Analysis of the diabetic subjects alone showed statistically significant correlations between sCD40L and Mean-IMT ($r = 0.277$, $p = 0.0139$) and Max-IMT ($r = 0.376$, $p = 0.006$). To demonstrate that sCD40L is an independent determinant of Mean-IMT, we performed a stepwise multivariate regres-

Fig. 2 Levels of sCD40L showed statistically significant associations with (a) Mean-IMT ($r = 0.275$, $p = 0.0058$) and (b) Max-IMT ($r = 0.383$, $p < 0.001$)

Table 3 Correlation between IMT and variables in the subjects

	Mean-IMT					Max-IMT				
	Univariate analysis ^a		Multivariate analysis			Univariate analysis ^a		Multivariate analysis		
	<i>r</i> value	<i>p</i> value	β coefficient	<i>F</i> value	<i>p</i> value	<i>r</i> value	<i>p</i> value	β coefficient	<i>F</i> value	<i>p</i> value
Age (years)	0.051	NS	NI	1.5	–	0.134	NS	NI	2.3	–
Sex (male/female)	–	NS	NI	3.1	–	–	0.0137	NI	1.9	–
Duration of diabetes (years)	0.067	NS	NI	0.1	–	0.175	NS	NI	0.2	–
Smoking (yes/no)	–	NS	NI	0.3	–	–	NS	NI	0.5	–
BMI (kg/m ²)	0.279	0.0047	NI	1.6	–	0.250	0.0119	NI	0.8	–
Systolic BP (mmHg)	0.235	0.0176	NI	3.1	–	0.287	0.0035	0.002	6.8	0.011
Diastolic BP (mmHg)	0.189	NS	NI	0.7	–	0.176	NS	NI	0.5	–
Plasma glucose (mmol/l)	0.135	NS	NI	0.6	–	0.157	NS	NI	0.4	–
HbA _{1c} (%)	0.042	NS	NI	1.2	–	0.041	NS	NI	0.0	–
Total cholesterol (mmol/l)	0.076	NS	NI	0.1	–	0.099	NS	NI	1.5	–
HDL cholesterol (mmol/l)	0.036	NS	NI	0.1	–	0.033	NS	NI	2.1	–
Triglyceride (mmol/l)	0.045	NS	NI	0.0	–	0.100	NS	NI	0.2	–
LDL cholesterol (mmol/l)	0.171	NS	NI	0.0	–	0.149	NS	NI	0.7	–
log ₁₀ CRP (mg/l)	0.334	0.0006	0.046	6.8	0.011	0.159	NS	NI	0.1	–
sCD40L (ng/ml)	0.275	0.0058	0.014	4.3	0.041	0.383	<0.001	0.035	13.6	<0.001
<i>R</i> ²	–	–	0.141	–	–	–	–	0.203	–	–

^aPearson's univariate correlation coefficients. A stepwise multivariate regression analysis was performed. Sex: M (male)=1, F (female)=0; Smoking: yes=1, no=0

β Partial regression coefficient, *NI* not included in the model because variable was not significant

sion analysis including age, duration of diabetes, sex, current smoking habit, BMI, systolic BP, diastolic BP, plasma glucose, HbA_{1c}, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride and log₁₀ hs-CRP as independent variables and found that sCD40L ($F=4.3$, $p=0.041$) and log₁₀ hs-CRP ($F=6.8$, $p=0.011$) were variables that interacted independently with Mean-IMT in all subjects. A stepwise multivariate regression analysis also demonstrated that sCD40L ($F=13.6$, $p<0.001$) and sBP ($F=6.8$, $p=0.011$) were variables that interacted independently with Max-IMT in all subjects (Table 3).

Additionally, serum sCD40L levels in patients without retinopathy ($n=53$), with simple retinopathy ($n=21$), and with proliferative retinopathy ($n=4$) were 2.02 ± 1.24 , 2.38 ± 1.61 and 1.75 ± 0.91 ng/ml, respectively. There were no significant differences between the groups. There was no significant difference in serum sCD40L between patients with microalbuminuria or macroalbuminuria ($n=8$, 1.93 ± 1.33 ng/ml) and those without albuminuria ($n=68$, 2.13 ± 1.35 ng/ml). There was no correlation between serum sCD40L and daily urinary AER ($r=-0.041$, $p=0.726$).

Discussion

Serum levels of sCD40L are elevated in young patients with type 1 diabetes The results of this study indicate that serum sCD40L levels and carotid IMT were increased in subjects with type 1 diabetes and that there were statistically significant associations between sCD40L levels and carotid IMT.

In the present study, serum levels of sCD40L were higher in young type 1 diabetic patients than in age-matched healthy controls, an observation consistent with previous reports [13, 14, 20]. Because there were differences in the baseline characteristics between controls and type 1 diabetes (especially in terms of sex, BMI and serum triglyceride), we performed a stepwise multivariate regression analysis that included type 1 diabetes, sex, BMI and serum triglyceride as independent variables, and found that the presence of type 1 diabetes was an independent determinant of a high sCD40L value. Although there was no significant association between sCD40L and HbA_{1c} in the present study, controversy remains as to whether there is a relationship between sCD40L and HbA_{1c} levels [13, 14, 20, 21]. The current study also showed that serum levels of sCD40L were correlated with those of hs-CRP and that hs-CRP is an independent determinant of a high sCD40L value. Although our data do not permit any conclusions to be drawn as to whether the elevated sCD40L is a cause or a consequence of elevated hs-CRP, these results might reflect the pathophysiological relationship between inflammation and platelet hyperactivation in atherothrombosis [22].

Carotid IMT is associated with serum level of sCD40L It has been reported that increased levels of sCD40L are associated with atherosclerotic diseases [8–12]. Previous studies have also established carotid arterial wall thickness, IMT, as one of the most reliable markers of systemic atherosclerosis, both in subjects with and without diabetes [23]. However, the association between sCD40L levels

and carotid IMT in subjects with type 1 diabetes has not been reported. To the best of our knowledge, this is the first report showing the relationship between subclinical, relatively early stage atherosclerosis and a marker of platelet hyperactivity (sCD40L) in subjects with type 1 diabetes. The present study has shown that carotid IMT is significantly greater in patients with type 1 diabetes than in control subjects and that there is a statistically significant association between sCD40L levels and carotid IMT. There was a significant association between hs-CRP and carotid IMT, linking inflammation to the development of atherosclerosis. Furthermore, hs-CRP was also linked to sCD40L, indicating an association between inflammation and thrombocyte activation. However, on multivariate analysis, sCD40 L remained an independent predictor of carotid IMT. Since type 1 diabetes is a major cause of cardiovascular morbidity and mortality, the results of the current study provide important information, suggesting that routine screening for carotid IMT could be important as a target for therapeutic interventions and monitoring to improve the clinical outcome in this high-risk population. However, this is a cross-sectional survey with a rather small number of subjects, and the association demonstrated between sCD40L levels and carotid IMT was statistically significant but not strong. Therefore, further study would be necessary to examine whether sCD40L actually accelerates the progression of atherosclerosis. Although sources of elevated sCD40L in diabetic patients will require further investigation, sCD40L may activate circulating leucocytes to enhance the release of proinflammatory cytokines, enhance the release of chemokines by mononuclear cells, and increase the expression of adhesion molecules at the endothelium [8, 19, 24]. These inflammatory responses may further promote the infiltration of activated leucocytes into the atherosclerotic lesion, which, in turn, may directly activate small mononuclear cells, macrophages and T cells inside the vessel wall, leading to thickening of the arterial wall [25]. Taking into account the pathological role of sCD40L reported in the previous studies, it would not merely be a marker but one of the key players in the progression of atherosclerosis [6–12, 20, 24, 25].

In contrast to the significant association observed between sCD40L levels and carotid IMT, no significant difference in serum sCD40L levels was seen between individuals with and without diabetic microangiopathy (retinopathy and nephropathy). These results suggest that sCD40L levels are not closely associated with the progression of microangiopathy, although, because of the limited number of subjects with microangiopathy in the present study, we cannot exclude the possibility that sCD40L levels somehow influence the progression of microangiopathy. Further studies with larger study populations would be necessary to clarify this association.

In summary, serum levels of sCD40L and hs-CRP are elevated in young patients with type 1 diabetes, suggesting that platelet activity and inflammation are simultaneously accelerated in type 1 diabetes, even in younger generations. Furthermore, it is suggested that increased levels of

serum sCD40L are associated with accelerated atherosclerotic changes observed in young patients with type 1 diabetes.

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