Original Article: Metabolism

Relationship between soluble CD40 ligand and γ-glutamyltransferase concentrations in non-drinking, young Type 1 diabetic individuals

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Abstract

Aims   To assess the association between circulating levels of soluble CD40 ligand (sCD40L), an emerging cardiovascular risk factor, and γ-glutamyltransferase (GGT) activity concentrations in Type 1 diabetic subjects.

Methods   Plasma concentrations of sCD40L and GGT activity, a marker of liver dysfunction, were measured in 54 non-smoking, non-drinking, young Type 1 diabetic patients, who were free of diagnosed cardiovascular disease.

Results   When participants were grouped according to tertiles of GGT, plasma sCD40L concentrations steadily increased across GGT tertiles ($P = 0.007$ for trend). Similarly, plasma sCD40L concentrations were positively correlated with plasma GGT levels in the whole group of participants ($r = 0.532$; $P < 0.0001$). In multivariate linear regression analysis, plasma GGT activity levels were positively associated with sCD40L (standardized beta coefficient = 0.342; $P = 0.027$) independently of age, gender, diabetes duration, glycated haemoglobin, total cholesterol and systolic blood pressure. Further adjustment for the presence of diabetic retinopathy and microalbuminuria did not appreciably attenuate this association.

Conclusions   Our findings suggest that there is a strong, graded, relationship between plasma GGT activity and sCD40L concentrations in non-smoking, non-drinking, young Type 1 diabetic individuals. This association appears to be independent of numerous confounding factors. Further studies are required to confirm the reproducibility of these results.

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Keywords   cardiovascular risk factors, γ-glutamyltransferase, liver fat, sCD40 ligand, Type 1 diabetes

Abbreviations   ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular disease; GGT, γ-glutamyltransferase activity; GSH, glutathione; HbA_{1c}, glycated haemoglobin; NAFLD, non-alcoholic fatty liver disease; sCD40L, soluble CD40 ligand; TNF, tumour necrosis factor

Introduction

The past two decades have been characterized by growing interest in the idea that atherosclerosis is an inflammatory disease [1]. Interaction of the multi-potent immuno-modulator CD40 ligand (CD40L) with its receptor CD40 has emerged as an important contributor to the inflammatory processes that lead to atherosclerosis and thrombosis. CD40 and CD40L, a member of the tumour necrosis factor (TNF) receptor super-family, are widely expressed in human atheroma and on a range of atheroma-associated cells, including endothelial cells, macrophages, T cells, smooth muscle cells and platelets [1–3]. The CD40–CD40L interaction promotes a broad range of pro-thrombotic and pro-inflammatory effects, including the expression of adhesion molecules and the secretion of numerous cytokines and matrix metalloproteinases involved in extracellular matrix degradation [2,4–7]. Accordingly, inhibition of CD40 signalling reduces experimental atherosclerosis development [8] as well as the evolution of established atherosclerosis [9]. In addition to the cell-associated form, CD40L also exists in a

soluble form that is fully active biologically, termed soluble CD40L (sCD40L). Interestingly, elevated plasma sCD40L concentrations are closely associated with an increased risk of future cardiovascular events in healthy women [10], in patients with acute coronary syndromes [11] and in those with end-stage renal disease on haemodialysis [12].

Recent studies suggested that non-alcoholic fatty liver disease (NAFLD), which is the commonest cause of abnormal serum liver enzymes in adults in Western countries [13–15], may be linked to an increased incidence of cardiovascular disease (CVD), especially in diabetic populations [16–20]. Type 2 diabetic patients appear to have a higher risk of NAFLD, especially in its more progressive forms, than non-diabetic subjects [13–16]. Available information on the frequency of NAFLD in people with Type 1 diabetes is quantitatively limited. However, elevated serum alanine aminotransferase (ALT) levels (defined as ALT > 50 U/l) were found in 9.5% of patients with Type 1 diabetes and 12.1% of those with Type 2 diabetes in a cohort of 1353 adult patients (62% of whom had Type 2 diabetes) [21]. In addition, further indirect evidence of a high frequency of NAFLD in Type 1 diabetes might be derived from recent results of large epidemiological studies reporting a higher frequency of the metabolic syndrome (ranging from 20 to 40%) in this patient population than that observed in non-diabetic individuals [22,23].

The exact biological mechanisms by which NAFLD may contribute to the development and progression of CVD have not been completely elucidated. To our knowledge, the relationship between serum liver enzymes, as markers of NAFLD [13,14] and circulating levels of sCD40L in Type 1 diabetes has not yet been investigated.

Thus, the aim of this study was to assess the association between plasma sCD40L and liver enzymes in non-drinking, young Type 1 diabetic individuals without any clinical evidence of CVD.

Methods

After written informed consent was obtained, we enrolled 54 young Type 1 diabetic patients, who were selected from all outpatients with Type 1 diabetes regularly attending our diabetes clinic after excluding (i) subjects who currently smoked or drank alcohol, (ii) those who had a history of recent acute illness or clinical evidence suggestive of CVD, kidney or liver diseases and (iii) those who were taking anti-platelet drugs. Overall, these participants represented approximately 30% of all the Type 1 diabetic patients ($n = 202$) who regularly attended our clinic. A group of these participants ($n = 27$) has been previously included in a pilot study assessing plasma sCD40L concentrations in relation to diabetes status [24]. All patients were treated with insulin; none was prescribed lipid-lowering drugs, whereas six patients were taking ramipril. Information on daily alcohol consumption and other lifestyle characteristics was obtained from all participants by a questionnaire. To exclude the presence of clinically manifest CVD, a resting electrocardiogram, measurement of ankle brachial pressure index and

carotid ultrasonography (to exclude the presence of significant carotid stenoses but to measure carotid intima-media wall thickness) were performed in all participants.

Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Blood pressure was measured in triplicate with a standard mercury manometer. Venous blood was drawn in the morning (08.00–08.30 h) after an overnight fast in all participants. Serum liver enzymes [ALT, aspartate aminotransferase (AST) and γ-glutamyltransferase (GGT)], lipids and other biochemical blood measurements were determined by standard laboratory procedures (DAX 96; Bayer Diagnostics, Milan, Italy). Reference ranges for ALT, AST and GGT levels, in our laboratory, were 5–40 and 5–55 U/l for women and 5–45 and 5–55 U/l for men, respectively. Serology for viral hepatitis B and C was assessed in all participants. Glycated haemoglobin (HbA_{1c}) was measured by an automated high-performance liquid chromatography analyser (Bio-Rad Diamat, Milan, Italy); normal range values in our laboratory were 3.0–5.5%. Blood for coagulation analysis was collected in citrated tubes and immediately centrifuged at 2500 g for 15 min at 4°C. Several aliquots of each plasma sample were then quick-frozen and stored at −80°C, in plastic vials, until determinations were performed. Within 6 months, all stored plasma samples were assayed in a single session by an experienced laboratory technician. Determination of plasma sCD40L was performed in duplicate with a commercially available ELISA-kit (Bender Med-Systems Diagnostics, Vienna, Austria) with intra-assay and interassay coefficients of variation of 6.6 and 9.7%, respectively. Plasma concentrations of fibrinogen (IL-test-PT-fibrinogen HS; Instrumentation Laboratory, Lexington, MA, USA), adiponectin (by an ELISA-method; B-Bridge International, San Jose, CA, USA), soluble intercellular adhesion molecule-1 (ICAM-1) (by an ELISA-method; Bender Med-Systems Diagnostics, Vienna, Austria) and soluble P-selectin (by an ELISA-method; Bender Med-Systems Diagnostics, Vienna, Austria) were measured in duplicate. Intra- and interassay coefficients of variation were 6.5 and 7.2%, 3.5 and 7.3%, 3.8 and 9%, 4 and 8.9% for fibrinogen, adiponectin, sICAM-1 and P-selectin, respectively.

In all patients, urinary albumin excretion was measured in an early morning urine sample as the albumin-to-creatinine ratio by an immuno-nephelometric method; micro-albuminuria and macro-albuminuria were present if urinary albumin excretion was 30–299 mg/g creatinine and ≥ 300 mg/g creatinine, respectively [25]. No patients had macro-albuminuria. A single ophthalmologist diagnosed retinopathy by fundoscopy after pupillary dilation, according to a clinical disease severity scale [26]. The presence of sensory neuropathy (by biothesiometer) was not extensively recorded in the study participants.

Statistical analysis

Data are presented as means ± SD or proportions. Skewed variables (sCD40L, adiponectin, sICAM-1, P-selectin, fibrinogen and triglycerides) were logarithmically transformed to improve normality before analysis. The following statistical tests were performed: one-way analysis of variance (for continuous variables), χ^2-test (for categorical variables) and univariate linear correlation. The independent association between sCD40L (logarithmically transformed) and GGT (included as a continuous measure) was assessed by two multivariate linear regression models as

Table 1 Main clinical and biochemical characteristics of young Type 1 diabetic patients grouped according to tertiles of serum GGT activity concentrations

Variables	First tertile (< 10 U/l)	Second tertile (10–15 U/l)	Third tertile (> 15 U/l)	P-values
N	19	17	18	
Age (years)	32 ± 9	28 ± 7	34 ± 10	0.11
Sex (% male, n)	36.8 (n = 7)	58.8 (n = 10)	66.7 (n = 12)	0.17
Body mass index (kg/m^2)	23.8 ± 3	23.9 ± 4	23.7 ± 2	0.97
Diabetes duration (years)	12 ± 8	13 ± 6	19 ± 9	0.020
Systolic BP (mmHg)	126 ± 8	120 ± 13	134 ± 22	0.040
Diastolic BP (mmHg)	81 ± 7	77 ± 8	83 ± 7	0.087
HbA$_{1c}$ (%)	6.6 ± 1.2	6.9 ± 1.0	6.6 ± 1.1	0.61
Total cholesterol (mmol/l)	4.8 ± 0.6	4.5 ± 0.9	4.8 ± 0.8	0.35
Triglycerides (mmol/l)	0.9 ± 0.4	1.0 ± 0.6	1.2 ± 0.9	0.37
sICAM-1 (ng/ml)	264 ± 49	280 ± 53	300 ± 70	0.18
P-selectin (ng/ml)	2.44 ± 2.2	2.80 ± 3.2	3.45 ± 3.6	0.51
Fibrinogen (g/l)	3.35 ± 1.1	3.44 ± 0.9	3.11 ± 0.7	0.68
Adiponectin (μg/ml)	8.85 ± 4.4	7.01 ± 3.8	7.71 ± 5.4	0.31
sCD40L (ng/ml)	1.61 ± 0.3	2.23 ± 0.4	3.14 ± 0.5	0.007
Microalbuminuria (%, n)	10.5 (n = 2)	17.6 (n = 3)	27.8 (n = 5)	0.40
Retinopathy (%, n)	31.6 (n = 6)	23.5 (n = 4)	50.0 (n = 9)	0.25
AST (U/l)	15 ± 2	16 ± 4	17 ± 6	0.16
ALT (U/l)	17 ± 5	18 ± 4	24 ± 8	0.017
GGT (U/l)	7 ± 2	11 ± 2	22 ± 9	ND

Data are means ± SD or proportions.
Differences were tested by the one-way ANOVA (for continuous variables) and by the χ^2-test (for categorical variables).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BP, blood pressure; GGT, γ-glutamyltransferase; HbA$_{1c}$, glycated haemoglobin; ND, not determined; sCD40L, soluble CD40 ligand; SD, standard deviation; sICAM-1, soluble intercellular adhesion molecule-1.

follows: model 1 including age, gender, diabetes duration, HbA$_{1c}$, total cholesterol, GGT and systolic blood pressure as covariates; and model 2 including age, gender, diabetes duration, HbA$_{1c}$, GGT, retinopathy (yes/no) and microalbuminuria (yes/no) as covariates. $P < 0.05$ were considered statistically significant. Statistical analyses were performed using SPSS 14.0 (SAS Institute, Cary, NC, USA).

Results

Patients enrolled in the study were predominantly males (53.7% of total, n = 29), lean (BMI = 23.8 ± 2.8 kg/m^2), normotensive (systolic blood pressure = 126 ± 15 mmHg; diastolic blood pressure = 80 ± 8 mmHg), normolipidaemic (total cholesterol = 4.7 ± 0.7 mmol/l; triglycerides = 1.0 ± 0.6 mmol/l) and had a mean age of 32 ± 9 years, duration of disease of 14.8 ± 8 years (range: 2–40 years) and were in good glycaemic control (HbA$_{1c}$ = 6.7 ± 1.1%). All participants had normal serum liver enzymes (AST = 17 ± 7 U/l, ALT = 19 ± 6 U/l, GGT = 13 ± 8 U/l) and were non-drinkers and non-smokers. Of participants, 18.5% (n = 10) had microalbuminuria and 35.2% (n = 19) had retinopathy (seven of whom had proliferative retinopathy). The prevalence of microvascular complications was similar to that described in other European populations with comparable age, diabetes duration and glycaemic control [27].

Table 1 shows the clinical and biochemical characteristics of participants grouped according to tertiles of serum GGT activity levels. Diabetes duration, ALT levels and systolic blood pressure increased across GGT tertiles, whereas all other measured variables were not significantly different among the groups.

Notably, plasma sCD40L concentrations steadily increased across GGT tertiles ($P = 0.007$ for trend). Similar insignificant trends were observed across tertiles of ALT ($P = 0.083$) or the ALT-to-AST ratio ($P = 0.091$), respectively (data not shown). Consistently, as shown in Fig. 1, plasma sCD40L concentrations were positively correlated with plasma GGT levels in the whole group of participants ($r = 0.532$; $P < 0.0001$). The significant increase in plasma sCD40L concentrations across GGT tertiles remained essentially unchanged after excluding those (n = 6), who were treated with ramipril ($P = 0.019$ for trend).

In multivariate linear regression analysis, GGT was positively associated with log-transformed sCD40L (standardized beta coefficient = 0.342; $P = 0.027$) independently of age, gender, diabetes duration, HbA$_{1c}$, total cholesterol and systolic blood pressure. All other variables included in this model were not independently associated with sCD40L.

Almost identical results were obtained in another multivariate regression model that included age, gender, diabetes duration, HbA$_{1c}$, presence of retinopathy and microalbuminuria as covariates. Also in this case, GGT was the only variable
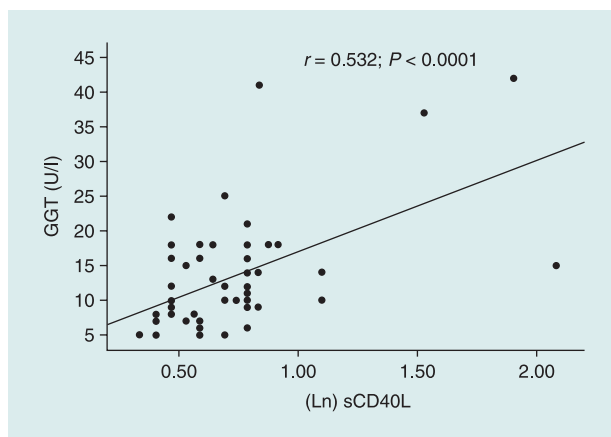
FIGURE 1 Relationship between plasma soluble CD40 ligand (sCD40L) concentrations (logarithmically transformed) and γ-glutamyltransferase (GGT) activity levels in non-smoking, non-drinking, young Type 1 diabetic patients.

independently associated with sCD40L (standardized beta coefficient = 0.324; $P = 0.024$).

Identical results were observed even when the independent association between sCD40L and GGT was assessed by multivariate logistic regression analysis. In that analysis, GGT was positively associated with sCD40L (1st tertile vs. 2nd–3rd tertiles combined) independently of age, gender, total cholesterol, systolic blood pressure, HbA_{1c} and duration of diabetes [multiple-adjusted odds ratio 1.31 (95% confidence intervals 1.06–1.49), $P = 0.011$].

Discussion

NAFLD, in its whole spectrum of disease ranging from pure steatosis to steatohepatitis and cirrhosis, is the commonest chronic liver disease in the developed world and is now regarded as the liver manifestation of the metabolic syndrome [13,14]. NAFLD prevalence has been estimated to range from 15 to 30% in the general population in Western countries [14,15] and is almost certainly increasing. Compared with non-diabetic subjects, people with diabetes appear to be at increased risk of developing NAFLD and certainly have a higher risk of developing fibrosis and cirrhosis [13–16,21].

Mild elevations of serum GGT activity levels and other serum liver enzymes are suggested to have a clinical and epidemiological significance as markers of NAFLD and related liver dysfunction [13,14]. A strong positive association between mildly elevated serum GGT levels and increased incidence of CVD has been shown in several population-based studies [28–32]. For example, in a study of 163 944 middle-aged Austrian men and women, serum GGT levels, even within their reference range, were independently associated with increased CVD mortality in both genders [29].

The major finding of our study is that, in Type 1 diabetic patients, increasing GGT levels are closely associated with elevated plasma sCD40L concentrations independently of a broad range of confounding factors, including age, gender, systolic blood pressure, total cholesterol, HbA_{1c}, diabetes duration and microvascular complications status. Notably, our patients were young, in good glycaemic control and free of diagnosed CVD. Moreover, all our patients were non-smokers, non-drinkers and had serum liver enzymes within the reference ranges. We believe that all of these conditions would possibly enhance the validity of our findings.

As many patients with NAFLD have normal serum liver enzymes [13–17], there is now increasing consensus among hepatologists supporting the notion that the currently used 'normal' reference values for serum liver enzymes for excluding NAFLD need to be revised. Recently, Prati *et al.* have proposed upper limits of normal range for ALT (i.e. 30 U/l for men and 19 U/l for women) that were substantially lower than the levels that laboratories currently consider to be the upper range of normal (for example, 45 U/l for men and 40 U/l for women in our laboratory) [33]. Using these new definitions of normal, these researchers could more accurately identify the patients with NAFLD and other chronic liver diseases than they could using the old ranges [33]. Notably, when we used these more stringent criteria, nearly half of our patients in the highest GGT tertile had serum ALT levels above the new proposed cut-off values, thus possibly having NAFLD.

Overall, therefore, our findings support a potential link between NAFLD, as reflected by mildly elevated serum GGT levels, and the CD40–CD40L pathway in Type 1 diabetic patients, thus suggesting an additional, underlying, mechanism by which NAFLD might contribute to the development and progression of CVD. The possible molecular mediators linking NAFLD and CVD have been extensively reviewed elsewhere [34], but include the release of pro-atherogenic mediators from the liver, including increased reactive oxygen species, C-reactive protein, fibrinogen, plasminogen activator inhibitor-1, TNF-alpha and other pro-inflammatory cytokines. In this context, it has been experimentally shown that the CD40–CD40L pathway is markedly activated in the liver of patients with chronic liver diseases (especially in those with autoimmune hepatitis and primary biliary cirrhosis) [35–37]. Moreover, in *in vitro* studies it has been demonstrated that TNF-alpha stimulates CD40 expression on hepatocytes via activation of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1)/jun N-terminal kinase (JNK), which are two transcription factors that control the expression of several pro-inflammatory cytokines and adhesion molecules and that are implicated in the control of epithelial cell proliferation and apoptosis [36,37].

Another possible explanation for our findings can rely on recent data suggesting that GGT, even within its reference range, is not simply a marker of liver dysfunction or alcohol abuse but may also be a risk factor actively involved in CVD pathogenesis—possibly also through the activation of the CD40–CD40L pathway [38,39]. GGT, which is found on all cell membranes, with the exception of erythrocytes, is the main determinant of extracellular hydrolysis of glutathione (GSH). In this process, GGT releases the dipeptide cysteinyl–glycine,

which is subsequently cleaved to cysteine and glycine by plasma membrane dipeptidase activities [38]. Thus, GGT activity provides cells with a means for the recovery of precursors needed to reconstitute intracellular levels of GSH, the main cellular antioxidant. However, recent studies have shown that the reactive thiol of cysteinyl–glycine originated during GGT-mediated cleavage of GSH may cause the reduction of ferric Fe(III) to ferrous iron Fe(II), thus starting a redox-cycling process resulting in increased production of the reactive oxygen species superoxide anion and hydrogen peroxide, both capable of stimulating pro-oxidant reactions [38,39]. In turn, oxidative stress can induce chronic inflammation with subsequent activation of the CD40–CD40L pathway. GGT-mediated pro-oxidant/inflammatory effects are likely within atherosclerotic coronary, carotid and cerebral plaques, where catalytically active GGT has been identified histochemically and can be sustained by iron storage proteins such as transferrin and ferritin, or even by free iron, shown to be present within the plaque at sufficient concentrations [38,39].

Our study has some important limitations. First, the cross-sectional design of our study precludes the establishment of causal or temporal relationships between plasma sCD40L and GGT concentrations. Prospective studies will be required to clarify the time sequence of events. Second, NAFLD diagnosis was based on serum liver enzymes and exclusion of other common causes of chronic liver disease (i.e. alcohol abuse, viral hepatitis, use of hepato-toxic medications), but was not confirmed by liver biopsy. However, liver biopsy would be unethical to perform in our patients who had serum liver enzymes within the reference range. Finally, whether these observations can also be extended to non-diabetic individuals remains to be determined.

In conclusion, our findings suggest that there is a strong, graded, relationship between plasma GGT activity and sCD40L concentrations in young Type 1 diabetic patients without any clinical evidence of CVD. This association appears to be independent of numerous confounding factors. Further research is needed to elucidate the underlying molecular mechanisms linking GGT activity and the CD40–CD40L pathway before causality can be firmly established.

Competing interests

Nothing to declare.

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