Clinical Investigation and Reports

Elevated Plasma Levels of the Atherogenic Mediator Soluble CD40 Ligand in Diabetic Patients
A Novel Target of Thiazolidinediones

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Background—Considerable evidence implicates the proinflammatory cytokine CD40 ligand (CD40L) in atherosclerosis and accumulating data link type 1 and 2 diabetes, conditions associated with accelerated atherosclerosis, to inflammation. This study therefore evaluated the hypothesis that diabetic patients have elevated plasma levels of soluble CD40L (sCD40L) and that treatment with the insulin-sensitizing thiazolidinediones lowers this index of inflammation.

Methods and Results—Subjects with type 1 (n=49) or type 2 diabetes (n=48) had higher (P<0.001) sCD40L plasma levels (6.56±3.27 and 6.67±2.90 ng/mL, respectively) compared with age-matched control groups (1.40±2.21 and 1.32±2.68 ng/mL, respectively). Multiple regression analysis demonstrated a significant (P<0.001) association between plasma sCD40L and type 1 as well as type 2 diabetes, independent of total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, blood pressure, body mass index, gender, C-reactive protein, and soluble intracellular adhesion molecule-1. Furthermore, in a pilot study, administration of troglitazone (12 weeks, 600 mg/day), but not placebo, to type 2 diabetics (n=68) significantly (P<0.001) diminished sCD40L plasma levels by 29%. The thiazolidinedione lowered plasma sCD40L in type 2 diabetic patients with long-standing disease (>3 years) with or without macrovascular complications (−34% and −29%, respectively) as well as in type 2 diabetic patients with more recent (<3 years) onset of the disease (−27%; all P<0.05).

Conclusions—This study provides new evidence that individuals with type 1 or 2 diabetes have a proinflammatory state as indicated by elevated levels of plasma sCD40L. Troglitazone treatment of type 2 diabetic patients diminishes sCD40L levels, suggesting a novel antiinflammatory mechanism for limiting diabetes-associated arterial disease. (Circulation. 2003;107:2664-2669.)

Key Words: atherosclerosis ■ diabetes mellitus ■ inflammation ■ immunology

Recent evidence implicated CD40 signaling in the pathogenesis of the chronic inflammatory disease atherosclerosis. Indeed, ligation of CD40 on human vascular endothelial and smooth muscle cells, as well as on mononuclear phagocytes, mediates a broad gamut of proatherogenic functions in vitro.1–3 Furthermore, disruption of CD40 signaling in hypercholesterolemic mice diminishes the formation and progression of atherosclerotic plaques.4–5 Of even greater clinical relevance, interference with CD40 ligation promotes changes in plaque composition associated in humans with less rupture-prone lesions, namely augmented content of smooth muscle cells and fibrillar collagen as well as diminished lipid and macrophage accumulation.6,7

In addition to the 39-kDa cell membrane–associated form, CD40 ligand (CD40L) can occur in soluble form in plasma (sCD40L).1–2 Patients with unstable angina have higher concentrations of sCD40L than those with stable angina or healthy volunteers, perhaps due to release from activated platelets or T lymphocytes.8 Moreover, we previously demonstrated a correlation between elevated levels of sCD40L with future cardiovascular events in apparently healthy, middle-aged women.9 Of note, statins diminish plasma sCD40L, suggesting that reduced inflammation can contribute to the reduction of cardiovascular events by these drugs.10,11

Diabetes mellitus affects nearly 16 million adults in the United States and markedly augments cardiovascular risk.12...
Many factors contribute to accelerated macrovascular disease in diabetic patients. Beyond impaired insulin secretion and resistance, inflammation has recently attracted much attention as a contributor to diabetes. In this regard, experimental evidence and cross-sectional data suggest that interleukin 6 (IL-6) and C-reactive protein (CRP), both markers of inflammation, correlate with hyperglycemia, insulin resistance, and incident type 2 diabetes. Diabetes also elevates plasma levels of tumor necrosis factor-α and plasminogen activator inhibitor-1 expression. However, little is known regarding modulation of the proinflammatory and atherogenic cytokine CD40L in diabetes.

Thiazolidinediones (TZDs), a class of insulin-sensitizing drugs, act by binding to peroxisome proliferator-activated receptor γ (PPAR-γ). TZDs decrease insulin resistance and improve insulin action, thus ameliorating glycemic control and reducing circulating insulin concentrations. Inflammatory cells in human atheroma express PPAR-γ, and PPAR-γ agonists exert antiinflammatory functions in vitro. Thiazolidinediones can reduce inflammation, as indicated by diminished serum levels of CRP in patients with type 2 diabetes. However, possible effects of TZDs on CD40L remain undetermined.

Methods

Subjects
Plasma samples were collected from type 1 (n = 49) or 2 diabetic patients (n = 48) participating in the Diabetes Nutrition and Complication Trial, a multicenter, observational study initiated in 1993 and designed to correlate nutrition with complications in a general diabetic population in Spain. Blood samples and clinical information were collected on average 7.5 years after diabetes was diagnosed (according to the recommendations of the American Diabetes Association). The mean age for type 1 diabetes was 30 ± 10 years and for type 2 diabetes 70 ± 11 years. Twenty-two percent of the patients with type 2 diabetes received hypoglycemic treatment other than metformin (sulfonylureas, glitazones, dipeptidyl peptidase-4 inhibitors, or glucagon-like peptide 1 receptor agonists). Thirty-two percent of the patients with type 2 diabetes received antihypertensive treatment. Moreover, 9% and 47% of the patients with type 1 and 2 diabetes, respectively, received hypolipidemic agents including statins. Thirty-two percent of the patients with type 2 diabetes received hypoglycemic treatment other than metformin (sulfonylureas, α-glucosidase inhibitors, or biguanides). No patient received thiazolidinediones. Age-matched healthy volunteers were recruited as controls.

For hypothesis-generating purposes, a pilot study of the effect of thiazolidinediones on sCD40L plasma levels was conducted in 68 type 2 diabetic patients followed at the Joslin Diabetes Center (Boston, Mass). Exclusion criteria for study participants were as follows: smoking during the previous 6 months, cardiac arrhythmia, congestive heart failure, recent stroke, chronic renal disease, macroalbuminuria (expressed as albumin/creatinine ratio > 300 μg/mg), severe dyslipidemia (triglycerides > 600 mg/dL or total cholesterol > 300 mg/dL), or other comorbidity requiring active treatment, as described elsewhere. Exclusion criteria included treatment with glucocorticoids, antineoplastic agents, psychoactive agents, bronchodilators, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitors (statins), thiazolidinediones, antihypertensive drugs, or insulin. Three groups were studied. The first consisted of 28 subjects with type 2 diabetes (diagnosed < 3 years) without evidence of macrovascular disease. The second and third groups consisted of subjects with long-term type 2 diabetes (> 3 years) with (n = 21) or without (n = 19) evidence of macrovascular disease, respectively. Macrovascular disease was defined as the presence of coronary artery disease, determined by positive exercise stress tests, unequivocal ECG abnormalities consistent with ischemia, prior coronary revascularization, a history of carotid artery disease requiring endarterectomy, or peripheral vascular disease requiring lower extremity revascularization. Participants were allocated randomly to 12 weeks of treatment with either troglitazone (600 mg/day) or placebo in a double-blind design, as described elsewhere. Patients in the group of type 2 diabetics with macrovascular complications were older than those without complications or subjects with a more recent onset of disease (63.1 ± 5.4 versus 56.4 ± 10 or 55.7 ± 8.9 years, respectively; P < 0.001). Patients with macrovascular complications had a higher fasting insulin level than did those free of macrovascular disease (21 ± 5.4 versus 16 ± 8 mU/mL, respectively; P < 0.01). In contrast, total cholesterol and LDL were lower in the group with macrovascular complications compared to those without or those with a more recent onset of diabetes (P < 0.01). There were no statistically significant differences in the baseline characteristics between those individuals assigned to the active treatment and those receiving placebo in any of the 3 groups. Because of the potential hepatotoxicity of the study drug, only individuals without history of liver disease and with continued normal liver function participated in and completed the study, which was finished before the withdrawal of troglitazone.

In addition to sCD40L, the following variables were determined: body mass index, blood pressure, waist, hip, and brachial circumferences, HbA1c, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, apolipoprotein B, apolipoprotein A1, lipoprotein (a), CRP, and soluble intracellular adhesion molecule-1 (sICAM-1).

Citrated blood was collected at enrolment and for the thiazolidinedione study again after 12 weeks of treatment. Blood specimens were centrifuged (2000g), and aliquots of platelet-free plasma stored at −80°C until laboratory analysis. Study protocols were approved by the ethics committee or institutional review board at each center, and all participants gave written informed consent.

Laboratory Methods
Plasma sCD40L concentrations were determined blinded by ELISA (BenderMed Systems), as described previously. The intra-assay variation among the triplicates for all samples was less than 10%. The detection limit was 10 pg/mL sCD40L. To verify findings, the samples were also analyzed with a second ELISA (R&D Systems), yielding comparable results. Plasma sICAM-1 (R&D Systems) and hs-CRP (ICN Pharmaceuticals) were also measured by ELISA.

Data Analysis
Statistical analysis utilized The Statistical Package for Social Sciences. Results are presented as mean ± SD. Means for baseline clinical characteristics of the study participants were compared using the Student’s t test. Correlation between variables was tested using Pearson correlation analysis. Multiple regression models were used to correct for confounding factors to assess the association between plasma sCD40L and diabetes.

Analysis of the troglitazone treatment study utilized a paired t test to compare baseline data and changes in all plasma sCD40L levels at each study point within each group. The t test was used to compare the baseline characteristics between those receiving active treatment and those receiving placebo in all groups, whereas the ANOVA test was used to compare differences among the 3 groups of diabetics. All probability values are two-tailed, and all confidence intervals computed at the 95% level. The cross-sectional and longitudinal study had 99% and 70% power, respectively, to detect differences in sCD40L plasma levels between the respective subgroups.

Results
This study tested the hypotheses that diabetic subjects have elevated sCD40L plasma levels and that administration of thiazolidinediones lowers circulating levels of this proatherogenic mediator in a two-tiered approach: (1) a cross-sectional analysis evaluated average sCD40L plasma levels in a gen-
eral diabetes population, and (2) a longitudinal study in type 2 diabetics explored modulation of sCD40L levels by TZDs.

Elevated Plasma Concentrations of sCD40L in Diabetes

Baseline characteristics of participants in the cross-sectional study are shown in Table 1 and Table 2. Controls were age-matched to type 1 or type 2 diabetic patients, respectively. There were no significant differences in plasma concentrations of total cholesterol, LDL cholesterol, or HDL cholesterol in either diabetic group compared with the respective control group. Triglycerides levels were lower in type 1 diabetic subjects compared with the control group or type 2 diabetic patients ($P<0.05$). Systolic and diastolic blood pressure did not differ significantly between type 1 diabetic patients and the controls (123±18 versus 124±2 mm Hg and 75±2 versus 74±11 mm Hg, respectively). However, type 2 diabetic patients had higher systolic and diastolic blood pressure than controls or type 1 diabetic patients (146±16 and 81±9 mm Hg, respectively; $P<0.001$).

Subjects with type 1 or type 2 diabetes had significantly higher sCD40L plasma levels ($P<0.001$) than age-matched controls (6.56±3.27 or 6.67±2.90 ng/mL versus 1.40±2.21 or 1.32±2.68 ng/mL, respectively; Figures 1A and 1B).

![Figure 1](http://circ.ahajournals.org)
Plasma concentrations of sCD40L did not differ significantly between males and females comparing type 1 diabetic patients with the control group (Figure 1C). However, females with type 2 diabetes had higher plasma sCD40L (P<0.05) than males. Multiple regression analysis showed a significant (P<0.001) association between plasma sCD40L and type 1 as well as type 2 diabetes, independent of total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, blood pressure, waist, hip, and brachial circumference, body mass index, and sex.

In the control group, plasma sCD40L did not correlate significantly with other variables. However, plasma levels of sCD40L correlated with apolipoprotein B in type 1 (r=0.20, respectively; P<0.05) as well as total cholesterol and albuminuria/urine creatinine plasma levels in type 2 diabetic patients (r=0.25 and 0.28, respectively; P<0.05). No significant association was found between the degree of diabetic control (HbA1c) and plasma sCD40L levels.

Soluble CD40L plasma levels in type 2 diabetic patients with known coronary heart disease tended to be higher compared with patients without (6.80±2.75 [n=28] versus 6.25±3.35 ng/mL [n=61]). However, this difference did not reach statistical difference, probably due to the relatively small number of cases. Similarly, type 2 diabetes patients with intermittent claudication or patients with type 1 or type 2 diabetes and micro- or macroalbuminuria showed higher levels of plasma sCD40L compared with those without; however, none of these trends achieved statistical significance.

In addition to sCD40L, we measured plasma levels of the established markers of inflammation, CRP and sICAM-1. Type 1 and type 2 diabetic patients showed a trend toward higher values of CRP (3.8±2.8 and 3.7±3.0 mg/L, respectively) than the respective age-matched control groups (3.0±2.4 and 3.1±2.6 mg/L; respectively), although this difference did not achieve statistical significance. CRP levels correlated with those of total cholesterol, triglycerides, and LDL cholesterol (r=0.30 [P<0.01], r=0.29 [P<0.05], and r=0.23 [P<0.05], respectively). Type 1 and 2 diabetic subjects had significantly higher levels of plasma sICAM-1 (440±17 and 496±16 ng/mL, respectively) compared with the age-matched controls (398±14 and 399±13 ng/mL; P<0.05 and P<0.001, respectively). Notably, type 2 diabetic patients had significantly higher plasma sICAM-1 levels than did type 1 diabetic patients (496±16 versus 440±17 ng/mL; P<0.05). sICAM-1 levels correlated with HbA1c, HDL cholesterol, sCD40L, and CRP (r=0.21, r=0.20, r=0.15, and r=0.27; all P<0.05, respectively). However, after adjusting for other risk factors, only HbA1C was significantly associated with sICAM-1.

**Troglitazone Diminishes sCD40L Plasma Levels**

To test whether treatment with a thiazolidinedione affects plasma sCD40L, 68 type 2 diabetic patients, characterized by either the recent onset of the disease (<3 years) (n=28) or longstanding diabetes (>3 years) with (n=21) or without (n=19) macrovascular complications, were administered troglitazone or placebo.

Plasma levels of sCD40L in this separate study group at baseline were significantly (P<0.05) elevated (2.93 ng/mL) compared with nondiabetic controls (1.32 ng/mL), but did not differ significantly between the subjects of the treatment groups assigned to troglitazone or placebo across all 3 groups. Within the entire study population (n=37), troglitazone treatment for 12 weeks (600 mg/day) significantly lowered sCD40L plasma levels by 29% (P<0.001, compared with baseline), a finding not observed in the placebo (n=31) group (Figure 2). All 3 subgroups had reduced sCD40L levels, including patients with a more recent onset of diabetes (~27%, n=15; P<0.05) and those with long-standing (>3 years) type 2 diabetes with or without macrovascular complications (~34% and ~29%, respectively, both n=11; P<0.05), but did not differ significantly between the 3 subgroups.
TABLE 2. Characteristics of Diabetic Patients

<table>
<thead>
<tr>
<th></th>
<th>Type 1 DM</th>
<th>Type 2 DM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c</td>
<td>7.8 ± 1.6</td>
<td>7.5 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Apolipoprotein A1</td>
<td>159 ± 90</td>
<td>160 ± 100</td>
<td>NS</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td>78 ± 25</td>
<td>101 ± 29</td>
<td>*</td>
</tr>
<tr>
<td>Lipoprotein (a)</td>
<td>22 ± 25</td>
<td>39 ± 37</td>
<td>*</td>
</tr>
<tr>
<td>Body mass index</td>
<td>24 ± 3</td>
<td>29 ± 5</td>
<td>*</td>
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<tr>
<td>Waist circumference</td>
<td>81 ± 11</td>
<td>100 ± 13</td>
<td>*</td>
</tr>
<tr>
<td>Brachial circumference</td>
<td>28 ± 3</td>
<td>32 ± 10</td>
<td>*</td>
</tr>
<tr>
<td>Hip circumference</td>
<td>96 ± 8</td>
<td>108 ± 12</td>
<td>*</td>
</tr>
<tr>
<td>Diabetic retinopathy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes, nonproliferative</td>
<td>24</td>
<td>23</td>
<td>NS</td>
</tr>
<tr>
<td>Yes, proliferative</td>
<td>9</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>No</td>
<td>55</td>
<td>57</td>
<td>NS</td>
</tr>
<tr>
<td>Intermittent claudication</td>
<td>1</td>
<td>8</td>
<td>†</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>1</td>
<td>28</td>
<td>*</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>15</td>
<td>36</td>
<td>†</td>
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<tr>
<td>Macroalbuminuria</td>
<td>5</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>Renal insufficiency</td>
<td>1</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>No</td>
<td>66</td>
<td>50</td>
<td>†</td>
</tr>
</tbody>
</table>

NS indicates nonsignificant. *P<0.001 type 1 vs type 2; †P<0.05 type 1 vs type 2.

Discussion

This study demonstrates elevated levels of plasma sCD40L in patients with type 1 or type 2 diabetes, independent of all other variables studied. Although the function of the soluble form of CD40L in inflammation remains somewhat controversial, sCD40L can induce chemokine production in peripheral blood mononuclear cells, PGE2 production in lung fibroblasts, and adhesion molecule expression in endothelial cells, as reviewed elsewhere.2 Moreover, platelet-rich plasma from CD40L-deficient mice clots more slowly and forms less stable thrombi than preparations from wild-type mice.10,29 Apparently healthy women with elevated baseline plasma concentrations of sCD40L (in the range found here in type 1 and 2 diabetic patients) had increased risk for future cardiovascular events.9 Indeed, the present study discloses a trend for elevated sCD40L levels and cardiovascular complications; however, the cross-sectional approach lacked sufficient power to show such differences. To address the variation in elevations observed in the present study between different populations, further study is needed to extend these results and evaluate whether ethnicity, diet, or lifestyle affect plasma levels of sCD40L.

Several cell types may release sCD40L and thus contribute to blood levels of this soluble cytokine. Platelets release sCD40L on activation by thrombin in vitro and during thrombus formation in vivo.30 In this regard, diabetic subjects have increased platelet activation and decreased endogenous inhibitors of platelet activity.31 Accordingly, heightened platelet activation could augment plasma sCD40L concentrations. However, other cell types, including endothelial cells, macrophages, or T lymphocytes, might also release sCD40L.2,32 Definitive determination of the source(s) of elevated sCD40L in diabetic patients will require further investigation.

In addition, we show that a thiazolidinedione reduces sCD40L plasma levels in type 2 diabetic patients. Of note, these subjects did not receive other medical treatment affecting sCD40L levels, such as 3-hydroxyl-3-methyl-glutaryl coenzyme A reductase inhibitors.28 The PPARγ agonist troglitazone improves the glycemic control of type 2 diabetic patients.33 Recently, however, other actions of thiazolidinediones have attracted attention. In the context of inflammation, rosiglitazone diminished serum levels of markers of inflammation such as CRP and matrix metalloproteinase-9 (MMP-9) in patients with type 2 diabetes.25 In addition, troglitazone may benefit atherosclerosis by attenuating the inflammatory response via the diminished expression of vascular cellular adhesion molecule-1 and ICAM-1 in activated endothelial cells and the significantly reduced monocyte/macrophage accumulation in atherosclerotic plaques.34 The decrease in circulating levels of sCD40L in diabetic patients after troglitazone treatment in this study suggests that this thiazolidinedione might attenuate inflammatory responses in these patients. Interpretation of the results of this study, however, require care due to the statistical power of 70% to detect differences in sCD40L plasma levels before and after treatment, due to the relatively small number of patients in some groups. Of note, a recent study verified the data presented herein using a different TZD,35 suggesting that thiazolidinediones as a class affect plasma sCD40L levels.

In conclusion, this study demonstrates increased sCD40L plasma levels in diabetic patients that decrease after treatment with troglitazone. Our results underscore the inflammatory nature of diabetes and suggest a novel antiinflammatory mechanism, which may mitigate diabetes-associated arterial disease, a hypothesis to be tested in future mechanistic studies.

Appendix


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References


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