The strong correlation between type 2 diabetes mellitus (T2DM) and atherosclerosis suggests that both conditions may share a common background (1). A clustering of type 2 diabetes mellitus (T2DM) and atherosclerosis suggests that both conditions related directly with metabolic control (3,4). Inflammatory and pro-thrombotic responses, and platelet activation are involved in the pathogenesis of type 2 diabetes (T2DM) and its complications. The CD40-CD40L interactions result in inflammatory and pro-thrombotic responses.

**METHODS**

Urinary 8-iso-prostaglandin (PG)F$_2$$\alpha$ and 11-dehydro-thromboxane (TX)B$_2$, in vivo markers of oxidative stress and platelet activation, respectively, plasma CD40L, and C-reactive protein (CRP) were measured in 114 T2DM patients and 114 control patients. A randomized, parallel group, 17-day study of aspirin (30, 100, or 325 mg/day) was performed in 18 T2DM patients. A similar study was performed in six healthy volunteers (aspirin, 100 mg/day). Twenty poorly controlled T2DM patients were studied before and after improved metabolic control.

**RESULTS**

Compared with control patients, diabetic patients showed significantly higher levels of 8-iso-PGF$_2$$\alpha$, 11-dehydro-TXB$_2$, sCD40L, and CRP. On multiple regression analysis, 11-dehydro-TXB$_2$ and 8-iso-PGF$_2$$\alpha$ excretion rates predicted sCD40L levels. Soluble CD40L linearly correlated with 11-dehydro-TXB$_2$ (rho = 0.67, p < 0.0001), and both were reduced after one week of aspirin (p < 0.0026), with slow recovery over 10 days after aspirin withdrawal. Improved metabolic control was associated with a reduction in sCD40L, 8-iso-PGF$_2$$\alpha$, and 11-dehydro-TXB$_2$.

**CONCLUSIONS**

This study provides several lines of evidence for the dependence of sCD40L release on TXA$_2$-dependent platelet activation in T2DM and provides novel mechanistic insight into the amplification loops of persistent platelet activation in this setting. (J Am Coll Cardiol 2006;47:391–7) © 2006 by the American College of Cardiology Foundation
TXA2-dependent platelet activation. Therefore, the goals of this study were to characterize the platelet contribution to sCD40L in T2DM, to correlate its formation with the extent of oxidative stress, and to investigate the effects of improved metabolic control and low-dose aspirin on these processes.

**METHODS**

**Patients.** One-hundred and fourteen patients with T2DM (61 female, 53 male; mean age, 64.7 ± 9.2 years), as defined in accordance with the criteria of the American Diabetes Association (20), and 114 age- and gender-matched healthy subjects were enrolled in the study. The baseline characteristics of patients and control subjects are detailed in Table 1. Exclusion criteria were represented by a recent history (<6 months) of thrombotic event, pregnancy or delivery in the previous six months, current medication for birth control or over-the-counter hormone replacement therapy, regular use of antioxidants (vitamins C and E), non-steroidal anti-inflammatory drugs, antiplatelet agents, and iron treatment. Patients with cancer, recent infectious diseases, autoimmune disorders, renal insufficiency or proteinuria (by serum creatinine levels and urinalysis), altered hepatic function (by liver enzymes), or alcohol abuse were also excluded.

Diabetic patients were examined for the presence of microvascular and macrovascular complications. Of the 114 patients, 13 (11%) had microvascular complications (10 subjects had retinopathy, 3 had microalbuminuria). Moreover, 26 had a history (>6 months) or physical examination positive for evidence of macrovascular complications: 15 patients had stable angina pectoris, 5 had cerebrovascular disease (transient ischemic attack or previous stroke), and 6 had peripheral vascular disease. Patients with coronary heart disease were in a stable phase. Patients with peripheral vascular disease were in Fontaine stage II (intermittent claudication, ankle-arm pressure index of <0.85, and no resting pain). In none of the patients had vascular disease undergone detectable progression during the previous six months, as judged by clinical examination during outpatient visits. At the time of the study, diabetic patients were being treated by diet alone (8 patients), insulin alone (3 patients), or by diet plus oral hypoglycemic agents (metformin and/or sulfonlurea) (96 patients); in 7 patients, insulin was added to the oral hypoglycemic agents. Fifty-three patients had arterial hypertension, defined as current systolic/diastolic blood pressure >130/85 mm Hg. Fifty-four patients were hypercholesterolemic (blood cholesterol level >200 mg/dl). Informed consent was obtained from each subject participating in the study. The local ethics committee approved the protocol.

**Design of the studies.** In the first study, a cross-sectional comparison of circulating sCD40L, urinary 8-iso-PGF2α, and 11-dehydro-TXB2 was performed among all patients and controls. All subjects were studied as out-patients after a 12-h fast and performed an overnight urine collection immediately before blood sampling. Urine samples were added with the antioxidant 4-hydroxy-tempro (1 mmol/l) (Sigma Chemical Co., St. Louis, Missouri) and stored at −20°C until extraction.

To assess the potential influence of improved metabolic control on sCD40L plasma levels, F2α-isoprostane formation, and platelet activation, a second study was performed in 20 of the 114 T2DM patients in whom inadequate metabolic control had been achieved (fasting blood glucose >200 mg/dl, hemoglobin A1c [HbA1c] >8%) at the time of the cross-sectional study, despite taking oral antidiabetic agents for months. These patients were examined over a four-week period with blood glucose monitoring, and oral antidiabetic therapy was adjusted accordingly and/or insulin therapy was instituted to achieve improved metabolic control. Throughout the study, patients followed an isocaloric diet that provided 50% of calories as carbohydrates, 30% as fat, and 20% as protein. The level of dietary cholesterol was about 0.3 g/day. Physical activity was encouraged, and patients were instructed to walk at least 30 min after each meal. No patient experienced a hypoglycemic reaction during the study period. Blood and overnight urine samples were obtained before and after this intensive metabolic monitoring and treatment program for determination of fasting glucose and HbA1c levels, sCD40L, and urinary 8-iso-PGF2α and 11-dehydro-TXB2.

To test the hypothesis of a platelet origin of sCD40L, aspirin (100 mg/day) was administered for one week to 6 of the 114 T2DM patients. Overnight urine and fasting blood samples were obtained before dosing and on the last day of

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**Abbreviations and Acronyms**

- CRP: C-reactive protein
- 11-dehydro-TXB2: 11-dehydro-thromboxane B2
- HbA1c: Hemoglobin A1c
- IQR: Interquartile range
- 8-iso-PGF2α: 8-iso-prostaglandin F2α-alpha
- LDL-C: Low-density lipoprotein cholesterol
- sCD40L: Soluble CD40 ligand
- T2DM: Type 2 diabetes mellitus
- TC: Total cholesterol

**Table 1.** Clinical Characteristics of Type 2 Diabetic Patients and Healthy Subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>Type 2 Diabetes Mellitus (n = 114)</th>
<th>Healthy (n = 114)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male 61/53</td>
<td>Female 60/54</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>64.7 ± 9.2</td>
<td>65.2 ± 3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.4</td>
<td>24</td>
</tr>
<tr>
<td>Diabetes duration (yrs)</td>
<td>11.85</td>
<td>—</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>205.7 ± 83.7</td>
<td>78.4 ± 9.4</td>
</tr>
<tr>
<td>Hemoglobin A1c (%)</td>
<td>8.2 ± 1.6</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td>Hypertension, %</td>
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<td>0</td>
</tr>
<tr>
<td>Hypercholesterolemia, %</td>
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<td>0</td>
</tr>
<tr>
<td>Microvascular complications, %</td>
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<td>0</td>
</tr>
<tr>
<td>Macrovascular complications, %</td>
<td>23</td>
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</tr>
</tbody>
</table>
aspirin treatment for measurement of circulating sCD40L, urinary 8-iso-PGF2α and 11-dehydro-TXB2.

Based on the results obtained in this preliminary study, we performed a randomized, parallel group study of three different doses of aspirin in 18 T2DM patients (10 female and 8 male; mean age, 60.7 ± 6.1 years; body mass index, 29.4 ± 4.4; diabetes duration, 14.7 ± 7.1 years; fasting blood glucose level, 148.9 ± 39; HbA1c level, 7.8 ± 1.1; hypercholesterolemia, 66%; hypertension, 44%; microvascular complications, 22%; macrovascular complications, 5.5%). This study included a one-week treatment period and a 10-day wash-out period. Patients were randomly assigned to receive the following: aspirin, 30, 100, or 325 mg/day (Bayer, Milan, Italy). During the treatment period, each patient took aspirin once daily at 9:00 AM. Moreover, during this period, blood samples were collected at baseline, after 2 h, after 24 h, and on the 7th day. During the wash-out period, blood samples (9:00 AM, after 12 h of fasting) were collected on the 3rd, 7th, and 10th day to characterize the time course of recovery of aspirin-induced biochemical changes. At each visit, blood samples were drawn for the following measurements: serum TXB2 obtained by incubating 1-ml whole blood samples for 1 h at 37°C (21) and plasma sCD40L levels.

Finally, the same study was performed in six healthy volunteers (4 female and 2 male; age 29.3 ± 1.9 years) who received aspirin 100 mg/day for one week. During the treatment and wash-out periods, blood samples were collected at the same times and with the same modalities used for the 18 diabetic patients.

Biochemical measurements. Fasting plasma glucose was measured by the glucose oxidase method. The HbA1c level was determined by automated high-performance liquid chromatography (22). Total cholesterol (TC) and triglyceride levels were determined by an enzymatic method; high-density lipoprotein cholesterol was measured after phosphotungstic acid/MgCl2 precipitation on fresh plasma; low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula.

Enzyme immunoassays. The sCD40L was determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota). Plasma C-reactive protein (CRP) levels were measured with a highly sensitive immunoassay (23).

Urinary eicosanoid assays. Urinary 8-iso-PGF2α and 11-dehydro-TXB2 were measured by previously described radioimmunoassay methods (24,25). Measurements of urinary 8-iso-PGF2α and 11-dehydro-TXB2 by these radioimmunoassays have been validated using different antisera and by comparison with gas chromatography/mass spectrometry, as detailed elsewhere (24,25).

Statistical analysis. With 114 subjects recruited in the cross-sectional comparison, the study had 95% power to detect a 40% difference in sCD40L serum levels between the T2DM and control groups with a two-tailed α of 0.05.

The data were analyzed by non-parametric methods to avoid assumptions about the distribution of the measured variables. Comparisons between groups were made with the Mann-Whitney U test. Correlations were analyzed by the Spearman rank correlation test. A multiple linear regression analysis was performed to further quantify the relationship between sCD40L and the variables in study.

The intensive metabolic control and low-dose aspirin studies had an 80% power to detect a 20% and a 40% change, respectively, in plasma sCD40L levels. The differences between pre-intensive and post-intensive metabolic control were assessed by the Wilcoxon test. Data are presented as mean (1 standard deviation) or as median and interquartile range (IQR) (25th, 75th percentile). Only p values <0.05 were regarded as statistically significant. All tests were two-tailed, and analyses were performed using a computer software package (Statistica 1999 edition, StatSoft Inc., Tulsa, Oklahoma, or Statistical Package for the Social Sciences, version 11.0, SPSS Inc., Chicago, Illinois).

RESULTS

Plasma CD40L levels were significantly higher in patients with T2DM than in age- and gender-matched healthy patients (median [IQR]: 4.5 [2.3 to 7.9] ng/ml vs. 2.0 [1.4 to 3.1] ng/ml, p < 0.0001). Plasma CRP levels were also significantly increased in diabetic versus control patients (0.50 [0.30 to 1.0] mg/l vs. 0.36 [0.26 to 0.50] mg/l, p = 0.0018).

The T2DM patients had a significantly higher urinary 8-iso-PGF2α excretion rate in comparison with control patients (median [IQR]: 316 [199 to 435] pg/mg vs. 190 [150 to 269] pg/mg creatinine, p < 0.0001) (Fig. 1). Urinary 11-dehydro-TXB2 excretion was also significantly higher in diabetic patients compared with control patients (745 [477 to 1,531] pg/mg vs. 290 [206 to 467] pg/mg creatinine, p < 0.0001) (Fig. 1).

A significant direct correlation was found between plasma CD40L and 8-iso-PGF2α and 11-dehydro-TXB2 excretion rates in diabetic patients (rho = 0.55, p < 0.0001, and rho = 0.67, p < 0.0001, respectively) (Fig. 2). Moreover, a significant linear correlation was found between plasma CD40L and CRP levels (rho = 0.43, p < 0.0001).

The CRP was significantly related to 8-iso-PGF2α and 11-dehydro-TXB2 excretion rates in diabetic patients (rho = 0.32, p = 0.0004, and rho = 0.455, p < 0.0001, respectively). Furthermore, a statistically significant correlation was observed between urinary excretion of 8-iso-PGF2α and 11-dehydro-TXB2 (rho = 0.62, p < 0.0001).

To further define the relationship between sCD40L, metabolic variables, additional atherosclerotic risk factors, urinary prostaglandin metabolites, and CRP, a multiple regression analysis was performed at baseline with sCD40L as the dependent variable. Stepwise linear regression yielded a model in which only 11-dehydro-TXB2 (regression coefficient = 0.45, standard error of the mean = 0.09, p <
0.0001) and 8-iso-PGF$_{2\alpha}$ (regression coefficient = 0.25, standard error of the mean = 0.09, p = 0.007) excretion rates predicted sCD40L levels, independently of fasting blood glucose, hypercholesterolemia, hypertension, presence of macrovascular complications, and CRP. Thus, as shown in Figure 2, among 43 diabetic patients with 11-dehydro-TXB$_2$ and 8-iso-PGF$_{2\alpha}$ excretion in the third and fourth quartiles, 37 (86%) had increased levels of CD40L. In contrast, only 19% of T2DM patients with 11-dehydro-TXB$_2$ and 8-iso-PGF$_{2\alpha}$ urinary excretion in the first and second quartiles had increased levels of CD40L (p < 0.0001).

**Influence of metabolic control on plasma CD40L levels.** Twenty patients with T2DM in whom adequate metabolic control had not been achieved (HbA$_{1c}$ >8%) were subjected to intensive monitoring and treatment, and blood samples were obtained before and after metabolic control over four weeks. At the end of this period, the median HbA$_{1c}$ level decreased from 9.5% (range, 9.0% to 10.3%) to 7.0% (range, 7.0% to 8.0%) (p < 0.0001). Improvement in metabolic control was associated with a significant reduction in CD40L levels (from 6.2 [range, 2.7 to 10.1] ng/ml to 4.2 [range, 2.5 to 5.8] ng/ml, p < 0.003) (Fig. 3) and in both 8-iso-PGF$_{2\alpha}$ (from 516 [range, 294 to 790] pg/mg to 318 [range, 143 to 609] pg/mg creatinine; p < 0.0002) and 11-dehydro-TXB$_2$ (from 1,360 [range, 930 to 2,172] pg/mg to 764 [range, 572 to 1,015] pg/mg creatinine; p < 0.0007) (Fig. 3) excretion. The CRP levels were not significantly affected by improved metabolic control (from 0.95 [range, 0.60 to 1.35] mg/l to 0.95 [range, 0.50 to 1.20] mg/l, p = 0.2959).

**Effects of low-dose aspirin.** Aspirin (100 mg/day) was administered for one week to 6 of the 114 T2DM patients. At the end of this period, plasma CD40L decreased from 7.1 ± 1.1% to 4.7 ± 1.3% (p < 0.0026), with a concomitant reduction in urinary 11-dehydro-TXB$_2$ excretion (1,367 pg/mg to 420 ± 132 pg/mg creatinine; p < 0.0001). The CRP levels did not show any significant change after aspirin therapy (from 1 ± 0.3 mg/l to 0.9 ± 0.3 mg/l, p = 0.69).

Based on the results obtained in this preliminary study, we performed a randomized parallel group study of three different doses of aspirin in 18 T2DM patients in a 17-day study. Plasma CD40L was significantly reduced after 2 h, 24 h, and 7 days of treatment with 30 mg, 100 mg, and 325 mg of aspirin (Fig. 4) with no apparent dose effect. The reduction in plasma CD40L at seven days averaged 53 ± 14%, 39 ± 8%, and 52 ± 11% after 30, 100, and 325 mg, respectively. Whole-blood TXB$_2$ production, a measure of the maximum cyclooxygenase-dependent biosynthetic capacity of blood platelets, was inhibited by 93 ± 4%, 98 ± 2%, and 99 ± 1% seven days after 30, 100, and 325 mg of aspirin, respectively. The recovery of plasma CD40L levels

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![Figure 1](image1.png)

**Figure 1.** Box and whisker plots of urinary excretion of 11-dehydro-thromboxane (TX)B$_2$ (A), 8-iso-prostaglandin F$_{2\alpha}$, (B), and plasma levels of CD40L (C) in healthy patients and in type 2 diabetic patients. PG = prostaglandin.

![Figure 2](image2.png)

**Figure 2.** Correlation between 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ excretion rates in type 2 diabetic patients according to quartiles of plasma CD40L. Vertical and horizontal lines mark the boundaries of median values of both urinary metabolites. Open and closed circles represent individual measurements, according to plasma CD40L: open circles = first and second quartile; solid circles = third and fourth quartile. PG = prostaglandin; TX = thromboxane.
occurred slowly over the 10 days after aspirin withdrawal, regardless of the aspirin dose.

Plasma CD40L was significantly reduced by 60 ± 39% after 7 days of treatment with 100 mg aspirin in six healthy volunteers (p < 0.04), with a slow pattern of recovery over the 10-day wash-out period (Fig. 5).

**DISCUSSION**

The CD40 ligand, a transmembrane protein structurally related to tumor necrosis factor-alpha, was originally identified on CD4+ T cells, but it was also found on activated platelets (15). Both membrane-bound and soluble forms of this ligand may interact with CD40 expressed on vascular cells, resulting in inflammatory responses (9). Platelets are major contributors to enhanced sCD40L in patients with acute coronary syndromes (17) and hypercholesterolemia (18).

Evidence of in vivo platelet activation has been previously reported in diabetics (3,4), and increased plasma levels of CD40L have been described recently in both type 1 and type 2 diabetes mellitus (12,13). Moreover, significantly increased co-expression of CD40 and CD40L on platelets of diabetic patients compared with non-diabetic control patients has been reported, with a significant correlation of sCD40L with CD40L expression on platelets (26). How-
ever, the contribution of persistent platelet activation to increased shedding of CD40L has not been previously investigated.

In the present study, the highly significant correlation between plasma CD40L levels and the urinary excretion rate of 11-dehydro-TXB₂, a non-invasive index of in vivo platelet activation (4) (Fig. 2), supports the likelihood of CD40L release during TXA₂-dependent platelet activation in T2DM. This relationship is in keeping with previously reported evidence showing that CD40L is rapidly up-regulated during platelet activation (15) and that platelet CD40 itself provides a mechanism for platelet activation (16). The contribution of platelets to enhanced CD40L release in T2DM is strengthened by the observation that both improved metabolic control and low-dose aspirin, two independent interventions down-regulating platelet activation in this setting, significantly reduced plasma CD40L levels without any measurable impact on systemic inflammation, as reflected by the non-significant change in CRP levels.

In addition, we found a positive correlation between enhanced sCD40L and 8-isoPGF₂α, in line with the recent report of increased production of endothelial reactive oxygen species by CD40L (27), suggesting that in T2DM the release of sCD40L from activated platelets may contribute to increased oxidant stress. Increased lipid peroxidation and persistent platelet activation have previously been reported in patients with T2DM (3,4). Thus, our findings suggest a possible vicious cycle in which inflammatory stimuli induce increased lipid peroxidation with consequent platelet activation, resulting in further oxidant stress (15).

To further characterize the platelet origin of plasma CD40L, we investigated the dose- and time-dependence of the effects of aspirin in T2DM. Low-dose aspirin is able to reduce TXA₂-dependent platelet activation in several clinical settings (28), and a recent report indicates that platelet-derived interleukin-1-beta is down-regulated by low-dose aspirin in hypercholesterolemia (29). Thus, we performed an open pilot study and then a randomized dose-response study with aspirin (30, 100, and 325 mg) in T2DM patients. Patients’ compliance was verified by the profound suppression of TXB₂ production during whole-blood clotting. Plasma CD40L levels were significantly reduced by 40% to 50% at any aspirin dose, indicating that TXA₂-dependent mechanisms are responsible, at least in part, for CD40L release by platelets: in fact, the r² value for the linear relationship between TXA₂-dependent platelet activation and CD40L levels was 0.45 in the present study. Both the satura-tability of the effect of aspirin at low doses and the time-dependent pattern of recovery of plasma CD40L levels on aspirin withdrawal are consistent with an effect dependent on platelet cyclooxygenase-1 inactivation (30). Moreover, the lack of a dose-effect in the 30 to 325 mg range argues against an anti-inflammatory effect of the drug contributing to the reduction in plasma CD40L.

The aspirin study in T2DM raises the question of whether only 40% to 50% of plasma CD40L is TXA₂-dependent, or the enhanced release of CD40L observed in T2DM is largely TXA₂-dependent but cannot be further reduced below normal levels. To address this question, we performed an additional study in six healthy volunteers, who were administered aspirin (100 mg/day) for 7 days, and their time course of plasma CD40L levels over 17 days was characterized. The similar reduction in plasma sCD40L levels within the normal range, as well as the similar time-course of recovery, strongly supports the hypothesis that in the setting of T2DM, both TXA₂-dependent and independent mechanisms of platelet activation contribute to enhanced release of sCD40L.

Previous results obtained from volunteer donors before and after seven days of aspirin (325 mg/day) showed a 50% decrease in sCD40L release measured ex vivo from platelet aggregates stimulated with collagen, a platelet agonist dependent on TXA₂ production (31). However, the latter study presents two main limitations: first, the choice of a 325 mg/day dose, together with the lack of any information about the time course of recovery of plasma CD40L levels on aspirin withdrawal, does not allow exclusion of an impact of aspirin on systemic inflammation, which could influence per se sCD40L levels; second, this experiment was performed ex vivo in a quite artificial milieu reproducing collagen stimulation. Conversely, our results clearly show that low-dose aspirin is able to down-regulate sCD40L production in vivo both in diabetic and in healthy subjects.

These findings may have therapeutic implications for atherothrombotic disease. Previously, the studies by Lindemann et al. (32) and by May et al. (33) showed that inhibition of glycoprotein IIb/IIIa engagement markedly attenuated the synthesis of platelet interleukin-1-beta and CD40L, respectively. Because platelet inflammatory mediators, such as interleukin-1-beta and CD40L, might trigger
a cascade of activation pathways substantially contributing to atherogenesis, matrix degradation, and plaque rupture (8), prevention by antiplatelet agents of platelet cytokine release may contribute to their cardioprotective effects.

We conclude that persistent platelet activation is responsible for increased levels of sCD40L in T2DM. Because low-dose aspirin can only incompletely down-regulate this phenomenon, we suggest that additional antiplatelet strategies should be investigated in an attempt to interrupt the vicious circle triggered by sCD40L-mediated events in this setting (Fig. 6).

References: