Increased Plasma Concentrations of Soluble CD40 Ligand in Acute Coronary Syndrome Depend on in Vitro Platelet Activation

Boris T. Ivandic,1*† Eberhard Spanuth,2† Detlef Haase,3 Heiko-Gundmar Lestin,3 and Hugo A. Katus1

Background: Soluble CD40 ligand (sCD40L) was suggested as a novel biomarker of cardiovascular risk. We examined the effect of preanalytical variation on the measurement of sCD40L concentration.

Methods: From healthy control individuals (n = 20) and patients with acute coronary syndrome (ACS) (n = 20) or sepsis (n = 20), we obtained blood drawn into 5 tubes containing citrate or a mixture of citrate, theophylline, adenosine, and dipyridamole (CTAD). The tubes were incubated for 30 min at room temperature or 0 °C before a single or double centrifugation (15 min, 2500 g) at room temperature or 4 °C, respectively. sCD40L, β-thromboglobulin (βTG), and platelet factor 4 (PF4) concentrations were measured using immunoassays.

Results: Concentrations of sCD40L were very low in all CTAD and citrated samples maintained at 0 °C (median <0.076 μg/L). Although increased βTG and PF4 confirmed disease-related in vivo platelet activation, sCD40L was not higher in patients than in controls. In contrast, if the samples were processed at room temperature, sCD40L was significantly higher in ACS patients than in controls (P <0.02 in CTAD and citrated plasma at room temperature). Moreover, the βTG:PF4 ratio decreased in patient but not control CTAD samples, suggesting a greater susceptibility of patient platelets to in vitro activation.

Conclusions: Increased sCD40L concentrations resulted from in vitro platelet activation during sample preparation. Disease-related in vivo activation did not contribute to sCD40L concentrations in plasma. Therefore, published studies of sCD40L demand cautious interpretation, because their preanalytical conditions were not standardized.

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CD40 ligand (CD40L; CD154) is a homotrimeric type II transmembrane protein. Its C-terminus contains a tumor necrosis factor homology domain required for binding to its receptor CD40. Intracellular CD40L is expressed on platelet membranes in response to platelet activation (1). Subsequent cleavage by metalloproteases produces soluble CD40L (sCD40L), an 18-kDa soluble fragment (2). Many reports have suggested that sCD40L is a promising clinical biomarker of atherothrombotic risk. Increased concentrations of sCD40L were reported mostly in disorders associated with platelet activation such as acute and stable coronary artery disease (3–5). Coronary artery bypass graft surgery and percutaneous coronary intervention (PCI) were shown to increase sCD40L (3, 6). Conversely, increased sCD40L was also directly associated with a higher cardiovascular risk in patients undergoing PCI (7).

In spite of the great interest in this biomarker, sCD40L still awaits appropriate clinical validation (8). Although preanalytical conditions may influence sCD40L concentrations significantly, they have not been standardized for the measurement of this marker (9–13). This lack of standardization prompted us to examine the effects of...
preanalytical conditions on the measurement of sCD40L in samples collected from healthy control individuals and patients with acute coronary syndrome (ACS) and sepsis—both diseases are associated with in vivo platelet activation. Platelet activation was independently determined by measurement of β-thromboglobulin (βTG) and platelet factor 4 (PF4) concentrations. In response to activation, these proteins are rapidly secreted from platelet granules in a constant molar ratio. Decrease of the βTG:PF4 ratio is a sensitive indicator of in vitro platelet activation (14).

Materials and Methods

PATIENTS

The study was approved by the institutional review board in accordance with the Declaration of Helsinki. Blood samples were obtained from 3 groups: (a) 20 healthy volunteers (controls), who were not taking any antiplatelet medication within 7 days before sampling; (b) 20 patients admitted with ACS; and (c) 20 patients hospitalized with sepsis. All patients were consecutively recruited and gave informed consent.

PREANALYTICAL SAMPLE PROCESSING

Five samples of venous blood were drawn via a butterfly canula (21-gauge) into 2 plastic syringes (S-Monovette, Sarstedt AG) containing 1/10 of the syringe volume of 0.106 mol/L trisodium citrate, and into 3 plastic syringes containing citrate and the inhibitors of platelet activation theophylline (15 mmol/L), adenosine (3.7 mmol/L), and dipyridamole (0.198 mmol/L) (CTAD). One of the CTAD syringes was precooled (ice-water mix) to minimize in vitro platelet activation, degranulation, and formation of microparticles. We used each syringe to examine a different condition of preanalytical processing, i.e., a combination of anticoagulant (citrate or CTAD) and incubation temperature (0 °C or room temperature). After incubation of whole blood at room temperature or 0 °C (ice-water mix) for 30 min, we obtained plasma samples after a single centrifugation (2500 g at 0 °C) for 15 min and stored them at −20 °C until analysis. Whole blood, which was drawn into the precooled CTAD tube, was incubated at 0 °C before centrifugation (2500g) at 4 °C for 15 min. The plasma was removed from the central portion of the supernatant, centrifuged again to obtain platelet-poor plasma (PPP), and analyzed immediately.

Table 1. sCD40L concentrations (µg/L) in plasma.α

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CTAD PPP</th>
<th>CTAD 0 °C</th>
<th>CTAD room temperature</th>
<th>Citrate 0 °C</th>
<th>Citrate room temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19</td>
<td>0.057 (0.018)</td>
<td>0.063 (0.031)</td>
<td>0.061 (0.044)</td>
<td>0.076 (0.035)</td>
<td>0.098 (0.113)</td>
</tr>
<tr>
<td>ACS</td>
<td>20</td>
<td>0.059 (0.017)</td>
<td>0.057 (0.021)</td>
<td>0.113 (0.056)</td>
<td>0.0036</td>
<td>0.072 (0.041)</td>
</tr>
<tr>
<td>Sepsis</td>
<td>20</td>
<td>0.041 (0.012)</td>
<td>0.0005</td>
<td>0.046 (0.009)</td>
<td>0.0003</td>
<td>0.052 (0.016)</td>
</tr>
</tbody>
</table>

α P values compare patients with ACS and sepsis vs controls. Statistical significance was assumed at P < 0.05 (Wilcoxon signed-rank test). IQR, Interquartile range; NS, not significant.

RESULTS

sCD40L was measured by use of an electrochemiluminescence immunoassay using the ElecSys® 2010 automated analyzer (Roche Diagnostics) (13). Within-run imprecision, reflected by CV, in pools of citrated plasma and serum (n = 21) was 1.8% at 0.1 µg/L and 1.2% at 6.9 µg/L; between-run imprecision, over 6 days, was 4.6% and 6.4%, respectively. Interference of CTAD with sCD40L analysis was negligible: sCD40L was comparable (6.81 and 6.96 µg/L) in a serum sample diluted 10:1 with CTAD and NaCl (0.154 mmol/L), respectively. Commercial ELISAs (Diagnostica Stago/Roche Diagnostics) were used to measure βTG and PF4 concentrations.

STUDY PARTICIPANTS

Twenty healthy individuals were studied as controls. A 57-year-old man was excluded from analysis because his sCD40L concentration was unexplainably very low (plasma 0.013 µg/L). The remaining controls included 9 men and 10 women. Their mean (SD) age was 41 (12) years. The ACS group included 15 men and 5 women, age 66 (10) years. In this group, 9, 7, and 4 patients presented with unstable angina, non-ST-segment elevation, and ST-segment elevation myocardial infarction, respectively. Eleven patients had increased cardiac troponin T (>0.03 µg/L), and PCI was performed in 15 ACS patients. The sepsis group included 13 men and 7 women, age 68 (11) years.

sCD40L, βTG, AND PF4 IN THE DIFFERENT SAMPLE PREPARATIONS

All samples processed at 0 °C (and particularly CTAD PPP samples) exhibited very low concentrations of sCD40L (median =0.076 µg/L). There was no difference between controls and ACS patients, but values were lower in septic patients (Table 1). Samples from ACS patients processed at room temperature had increased sCD40L concentrations (Table 1). In contrast, median concentrations of βTG were significantly higher in CTAD samples (processed at 0 °C) from ACS and sepsis patients compared with controls (Table 2; Mann–Whitney U, P = 0.0157 and P = 0.0388 for ACS and sepsis, respectively). Median concentrations of PF4 were also increased in patients with ACS and sepsis compared with controls (Table 2). Sample processing at room temperature de-
creased $\beta$-TG:PF4 ratios (Table 2). Interestingly, CTAD samples obtained from healthy controls maintained a high $\beta$-TG:PF4 ratio in spite of these conditions.

**Discussion**

Accumulated evidence suggests that sCD40L is an important link between inflammation, atherosclerosis, and thrombosis. Numerous studies reported increased sCD40L in various clinical conditions and diseases associated with platelet activation but did not adequately describe the preanalytical conditions of sCD40L analysis (9). sCD40L concentrations are much higher in serum than in plasma, because platelet CD40L is released during clotting (11,13). Moreover, sCD40L increases during preanalytical sample processing, suggesting that in vitro platelet activation may account for the release of sCD40L in ACS patients. Interestingly, CTAD samples from ACS and sepsis patients if the preanalytical processing temperature was significantly increased to room temperature. In comparison, the ratio remained high in control CTAD samples, suggesting that platelets from healthy individuals may be more resistant to temperature-related in vitro activation than platelets from patients. Temperature-dependent formation of microparticles or specific enzymatic processes, or both, may explain this finding.

We conclude that previously reported plasma concentrations of sCD40L demand cautious interpretation, because they may result from in vitro platelet activation. For the time being, the role of sCD40L as a marker of atherosclerotic thrombotic risk should be considered doubtful awaiting further evaluation.

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**References**


**Table 2. Median values of $\beta$-TG, PF4, and $\beta$-TG:PF4 ratio.**

<table>
<thead>
<tr>
<th>Sample processing</th>
<th>$\beta$TG</th>
<th>PF4</th>
<th>Ratio</th>
<th>$P$</th>
<th>$\beta$TG</th>
<th>PF4</th>
<th>Ratio</th>
<th>$P$</th>
<th>$\beta$TG</th>
<th>PF4</th>
<th>Ratio</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAD 0 °C</td>
<td>44.0</td>
<td>9.0</td>
<td>3.70</td>
<td>NS</td>
<td>61.5</td>
<td>16.5</td>
<td>3.60</td>
<td>0.0117</td>
<td>78.5</td>
<td>10.5</td>
<td>3.60</td>
<td>0.0002</td>
</tr>
<tr>
<td>Room temperature</td>
<td>51.0</td>
<td>13.0</td>
<td>3.80</td>
<td>NS</td>
<td>208.0</td>
<td>98.5</td>
<td>2.25</td>
<td>22.0</td>
<td>88.0</td>
<td>3.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate 0 °C</td>
<td>177.0</td>
<td>54.0</td>
<td>3.30</td>
<td>0.0234</td>
<td>205.0</td>
<td>75.0</td>
<td>2.65</td>
<td>NS</td>
<td>303.0</td>
<td>95.0</td>
<td>2.95</td>
<td>NS</td>
</tr>
<tr>
<td>Room temperature</td>
<td>245.0</td>
<td>108.0</td>
<td>2.90</td>
<td>NS</td>
<td>273.0</td>
<td>122.0</td>
<td>2.30</td>
<td>302.0</td>
<td>98.5</td>
<td>2.70</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Ratios were calculated from concentrations of $\beta$-TG and PF4 measured in individual samples, not from the median values given in the preceding 2 columns.

$^b$ $\beta$-TG:PF4 ratios were compared between samples processed at 0 °C and room temperature. Ratios were measured in samples from patients with ACS and sepsis and from controls. Statistical significance was assumed at $P<0.05$ (Wilcoxon signed-rank test); NS, not significant.

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