Influence of pre-analytical and analytical factors on soluble CD40L measurements

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ABSTRACT

The soluble form of CD40L (CD40 ligand), a pro-atherogenic mediator, has emerged as a diagnostic and prognostic marker for cardiovascular events. However, as platelets can shed CD40L upon activation, accurate measurement has proved challenging. The present study addresses the controversy regarding the appropriate specimen and preparation for laboratory evaluation of blood sCD40L (soluble CD40L). Serum and plasma (collected in EDTA, citrate or heparin) were collected from healthy volunteers (n = 20), and sCD40L was analysed by ELISA immediately or after one to three freeze–thaw cycles and at different centrifugation speeds. Urine sCD40L levels were measured in subjects with low- and high-plasma sCD40L levels. Serum sCD40L levels (5.45 ± 4.55 ng/ml; P < 0.001) were higher than in citrate, EDTA or heparin plasma (1.03 ± 1.07, 1.43 ± 1.03 or 1.80 ± 1.25 ng/ml respectively), with no significant differences between plasma preparations. Increasing g values (200–13 000 g), which gradually deplete plasma of platelets, yielded lower sCD40L levels. Repeated freeze–thaw cycles significantly (P < 0.05) increased sCD40L concentrations in platelet-rich, but not platelet-depleted, plasma (up to 2.4-fold). Bilirubin and haemoglobin interfered positively, and triacylglycerols (triglycerides) and cholesterol quenched CD40L signalling. No sCD40L was detected in urine samples. In conclusion, serum yields higher sCD40L concentrations than plasma; accurate measurements of sCD40L require exclusion of platelets and avoiding their post-hoc activation. Samples with high concentrations of bilirubin, haemoglobin and/or triacylglycerols should be excluded, as these substances interfere with the assay.

INTRODUCTION

The soluble form of the inflammatory and pro-atherogenic mediator CD40L (CD40 ligand), termed sCD40L, can be measured in serum or plasma by immunoassay [1]. sCD40L has emerged as a diagnostic and prognostic marker. Patients with unstable angina have higher sCD40L plasma concentrations than healthy volunteers or those with stable angina [2]. Furthermore, elevated plasma levels of sCD40L precede thrombotic complications and identify healthy individuals at heightened risk of cardiovascular complications [3]. Also, sCD40L levels predict recurrent cardiovascular events in patients with acute coronary syndromes and correlate with features of plaque composition, such as the presence of a lesional lipid core [4–6]. Finally, elevated levels of sCD40L associate with traditional cardiovascular risk factors, such as diabetes and hypercholesterolaemia, and can be modulated by treatment with thiazolidinediones and statins [7–10].

However, interest in this new clinical application of sCD40L has engendered controversy regarding the

Key words: assay interference, cardiovascular risk, CD40 ligand (CD40L), cytokine, soluble CD40L.

Abbreviations: CD40L, CD40 ligand; LDL, low-density lipoprotein; PRP, platelet-rich plasma; rCD40L, recombinant CD40L; sCD40L, soluble CD40L.

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preferable specimen, appropriate preparation and method of measurement [11,12]. Also, studies agree only poorly regarding sCD40L concentrations in apparently healthy individuals, possibly explained by the type of sample employed [2,4,5]. However, analytical performance of the different commercial assays could influence the results as well [12]. Platelets contain preformed CD40L, and activation of platelets causes shedding of this mediator. Platelets may represent the predominant source of sCD40L in the circulation, and platelet activation may affect blood levels of sCD40L [13]. Hence activation of platelets post-phlebotomy presumably would artificially increase the levels of non-cell-associated blood sCD40L, explaining the different sCD40L levels obtained in serum and plasma. However, no currently available studies compare serum and plasma obtained from the same patient with different anticoagulants or explore the effect of the centrifugation speed on sCD40L levels.

Moreover, analytical interference in immunoassays can lead to erroneous results. To date, little information exists regarding the effect of substances that interfere with the assay of sCD40L. The present study aims to provide a direct comparison of serum and plasma, explores optimum methods for plasma preparation, studies sCD40L thermostability, determines the effects of different anticoagulants, and evaluates analytical interferences affecting sCD40L quantification. The results should aid accurate measurements of this emerging marker of cardiovascular risk and prognosis in primary and secondary prevention.

**METHODS**

**Blood and urine samples**

Using Vacutainer® tubes, serum and plasma (collected in citrate, EDTA or heparin) were collected from healthy volunteers (n = 20; age, 35 ± 11 years) not taking any medication. The Institutional Review Board at Brigham and Women’s Hospital approved study protocols, and all participants gave written informed consent.

If not stated otherwise, samples were centrifuged at 2000 g for 10 min before measuring sCD40L concentrations. In addition, citrated blood was centrifuged at increasing g values (200, 400, 1000, 2000 and 13,000 g). Supernatants were collected and platelets and other cell types were counted (Beckman Coulter). Subsequently, sCD40L concentrations were measured in the supernatants immediately or following one, two, or three freeze–thaw cycles. To evaluate how the time from blood sampling to coagulation influenced serum sCD40L levels, serum samples were centrifuged following 30 min, 2 h or 6 h of storage at room temperature and sCD40L concentrations were measured.

Healthy volunteers and diabetic patients (both n = 10) provided plasma and urine samples after providing informed written consent.

In addition, urine samples were supplemented with 10 ng/ml of rCD40L (recombinant CD40L; Leinco) to quantify recovery from these specimens. Finally, urine solutions containing 10 ng/ml rCD40L were prepared at different pHs (2–9). sCD40L was measured by ELISA (BenderMedSystems) in all urine samples.

**Platelet intracellular CD40L**

To quantify intracellular CD40L content, platelets were isolated from PRP (platelet-rich plasma) by a metrizamide gradient and resuspended in platelet buffer [140 mmol/l NaCl, 3 mmol/l KCl, 0.5 mmol/l MgCl2, 5 mmol/l NaHCO3, 10 mmol/l glucose and 10 mmol/l Heps (pH 7.4)], as described previously [14]. Platelet lysates were obtained by centrifuging the platelet suspension for 15 min at 1000 g and resuspending the pellets in lysis buffer [1 % (w/v) Triton X-100, 20 mmol/l Tris/ HCl (pH 7.4), 150 mmol/l NaCl and 1 mmol/l EDTA (pH 8.0)]. Total protein concentrations were determined by the bicinchoninic acid method, and CD40L was measured by ELISA. Results are expressed as ng of CD40L/total protein.

**sCD40L measurements**

sCD40L concentrations in serum, plasma and urine were determined by ELISA (BenderMedSystems), as described previously [7]. Each sample was measured in duplicate; the intra-assay variation among the duplicates for all samples was < 10 %. The detection limit was 10 pg/ml sCD40L.

Recovery of exogenous analyte was evaluated by adding rCD40L (10 and 50 ng/ml) to normal human citrated plasma and urine (n = 10). The amount of endogenous sCD40L in the plasma or urine was subtracted from the spiked values. Recoveries of exogenous rCD40L ranged from 80–110 % in plasma and 72–116 % in urine, with an average recovery of 90 ± 16 and 76 ± 18 % respectively.

**Western blotting**

T-cells incubated in the presence or absence of PMA (50 ng/ml for 12 h) were lysed in lysis buffer, and total protein concentrations were determined by the bicinchoninic acid method. Lysates were separated (50 µg of total protein/lane) by SDS/PAGE under reducing conditions and proteins were then blotted on to PVDF membranes (Millipore). Blots were blocked for 1 h in 5 % (w/v) non-fat milk/PBS/0.1 % Tween 20, and primary antibody was added overnight (mouse anti-human CD40L, 1:200 dilution; Bender Medsystems). Immunoreactive proteins were visualized after incubation with a secondary antibody (goat anti-mouse) employing the Western Lighting Chemiluminiscence system (PerkinElmer).
Stability of rCD40L and human endogenous sCD40L

To evaluate the stability of human whole-blood sCD40L, aliquots of rCD40L (10 ng/ml), human serum and human plasma were frozen immediately, or kept for 6, 24 or 48 h at either room temperature or 4 °C before ELISA.

Analytical interference

Analytical interference of the immunoassay with molecules frequently found at high levels in human plasma specimens was analysed in experiments that employed serum and citrated plasma supplemented with exogenous bilirubin (50, 100 and 200 mg/ml), haemoglobin (12, 24 and 36 mg/ml), triacylglycerols (triglycerides; 50, 100, 200 and 400 mg/dl), cholesterol (150, 200, 250 and 300 mg/dl) or LDL (low-density lipoprotein)-cholesterol (1.5, 2 and 2.5 mg/ml) (all from Sigma). Subsequently, sCD40L levels were measured by ELISA.

To ascertain the validity of the assay for cell culture specimen, interference by PBS, Hepes, RPMI, ethanol, DMSO, assay buffer (PBS/0.2 % BSA) or lysis buffer was determined.

Statistical analysis

Statistical analysis utilized the Statistical Package for Social Sciences (SPSS 11.0). Results are means ± S.D. Normal distribution of samples was assessed by the Shapiro–Wilks test. ANOVA, followed by a Tukey test, was employed to detect differences in sCD40L levels between serum and the different plasma samples. Means for continuous variables were compared using the Student’s t test. Correlation between variables was tested using Pearson’s correlation analysis. All P values are two-tailed, and all confidence intervals were computed at the 95 % level.

RESULTS

Higher levels of sCD40L in serum compared with heparin, EDTA or citrated plasma

Comparison of serum and plasma (platelet-free) samples from 20 donors demonstrated significantly (*P = 0.001) elevated sCD40L levels in serum samples, yielding 0.65–12.93 ng/ml sCD40L (mean, 5.45 ± 4.55 ng/ml sCD40L) compared with citrated, EDTA or heparinized plasma (1.03 ± 1.07, 1.43 ± 1.03 or 1.80 ± 1.25 ng/ml sCD40L respectively) (Figure 1A). sCD40L levels in plasma collected in EDTA, citrate or heparin did not differ significantly. Furthermore, serum sCD40L concentrations did not correlate with sCD40L levels in plasma collected using any of the anticoagulants tested. Plasma sCD40L levels measured in EDTA and heparin correlated significantly (*r = 0.65, *P = 0.009). Increasing the time from blood sampling to sample processing (2 and 6 h) resulted in a significant (*P < 0.05) increase in serum sCD40L levels (15 % and 22 % respectively) compared with samples processed after clotting for 30 min.

Interestingly, we found a significant correlation between the intracellular CD40L content in non-activated platelets (in ng of CD40L/µg of protein) and sCD40L serum (*r = 0.52, *P = 0.04; Figure 1B), but not plasma levels.

Influence of platelet content and freeze–thaw cycles on sCD40L concentrations

Whole blood collected in sodium citrate (0.129 mol/l) and centrifuged at 200 g for 10 min yielded PRP and high concentrations of detectable sCD40L (Figure 2A). Gradual increases in the g value (from 200 to 13 000 g), which achieved graded depletion of platelets and leucocytes from plasma, reduced measured sCD40L levels. In plasma with high sCD40L concentrations, centrifugation at 200 g yielded higher concentrations (10.25 ± 4.23 ng/ml) than plasma centrifuged at 2000 or 13 000 g (6.18 ± 0.81 and 6.31 ± 0.80 ng/ml respectively). Centrifugation at 2000 or 13 000 g did not yield significant differences in sCD40L concentrations between the samples.
Presence of platelets in plasma yields higher concentrations of sCD40L than platelet-free plasma

Citrated blood from healthy volunteers (n = 3) was centrifuged at increasing g values (200, 400, 1000 and 2000 g respectively). Supernatants were collected and platelets were counted microscopically (A). Subsequently, supernatants underwent one, two, three or no freeze–thaw cycles (B). Values are mean sCD40L concentrations measured by ELISA. PRP, plasma centrifuged at 200 g.

Repeated freeze–thaw cycles (up to three cycles) yielded similar sCD40L concentrations in samples with low or absent platelet count (coefficient of variation was < 5%). However, repeated freeze–thaw cycles on PRP resulted in sCD40L release that increased along with the number of cycles, probably due to sCD40L shedding from platelets (Figure 2B).

To test whether the ELISA employed could distinguish full-length CD40L and sCD40L, the anti-CD40L antibody provided by the manufacturer of the ELISA kit was applied to Western blot analysis of lysates obtained from T-cells, which express full-length CD40L on the cell surface. Cells were cultured in the presence or absence of PMA/ionomycin (50 ng/ml for 12 h), lysed in 1 % Triton X-100 and separated by SDS/PAGE. An immunoreactive band at 39 kDa was observed, proving that the antibody recognizes full-length CD40L.

Thermostability of rCD40L and sCD40L

The thermostability of rCD40L and sCD40L was evaluated by measuring sCD40L concentrations in aliquots of serum and citrated plasma (centrifuged at 2000 g), as well as solutions of rCD40L, immediately or after storage for 6, 24, or 48 h at room temperature or 4 °C. No significant changes in serum or plasma sCD40L concentrations were detected after storage at 4 °C for up to 48 h (Table 1). However, significant (P < 0.05) loss of sCD40L immunoreactivity occurred in serum and plasma, and also in rCD40L, during long-term storage at room temperature.

Interference in the sCD40L assay by bilirubin, haemoglobin, triacylglycerols or cholesterol

We evaluated further the analytical interference of the immunoassay with different molecules frequently found at high levels in human plasma by adding various concentrations of bilirubin, haemoglobin, triacylglycerols, cholesterol and LDL-cholesterol to citrated plasma, serum or citrated plasma supplemented with 10 ng/ml
Analytical interference of sCD40L measurements

Analytical interference of different solvents in pH of the sample influences the measured sCD40L.

LDL-cholesterol

Cholesterol

Triacylglycerols

Haemoglobin

rCD40L. A positive concentration-dependent interference of bilirubin and haemoglobin occurred in all of the samples analysed (Table 2). Moreover, addition of exogenous lipids at physiological concentrations had minimal analytical interference; however, detectable sCD40L in pathological lipid concentrations (triacylglycerols > 200 mg/dl, total cholesterol > 250 mg/dl and LDL-cholesterol > 2 mg/dl) diminished significantly.

Finally, we dissolved defined concentrations of rCD40L (5 and 10 ng/ml) in different solvents and buffers commonly employed in cell culture studies. Recoveries, as determined by ELISA, ranged from 78–109 % in most of the buffers and solvents analysed (Table 3). Dissolving rCD40L in 100 % ethanol or 100 % DMSO resulted in a loss of detectable sCD40L; however, recovery of the analyte at the solvent concentrations (< 10 %) commonly used in experimentation was > 65 % (Table 3).

Urine samples

sCD40L concentrations were measured in urine and in plasma from healthy controls and diabetic patients (both n = 10) who previously had elevated levels of circulating sCD40L. Plasma concentrations ranged from 0.01–1.5 ng/ml (mean, 0.97 ± 0.4 ng/ml) in controls and from 1.17–3.20 ng/ml (mean, 2.1 ± 0.8 ng/ml) in diabetic patients. However, we detected no sCD40L in urine from healthy volunteers or diabetic patients. The addition of rCD40L (10 ng/ml) to these samples yielded a mean recovery of 76 ± 18 %, suggesting the absence of antigen, rather than assay interference, as the cause of the lack of signal.

Furthermore, to clarify whether pH variations in urine could affect sCD40L measurements, urine solutions with 10 ng/ml rCD40L were prepared at different pHs. Average recovery was 106 % at pHs between 5 and 8 (Figure 4).

Table 2 Analytical interference of sCD40L measurements

Values are means ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Interference (%)</th>
<th>Serum</th>
<th>Plasma</th>
<th>Plasma + rCD40L (10 ng/ml)</th>
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<tbody>
<tr>
<td>Bilirubin</td>
<td></td>
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<tr>
<td>50 mg/ml</td>
<td>105 ± 159</td>
<td>1729 ± 907</td>
<td>120 ± 215</td>
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<tr>
<td>100 mg/ml</td>
<td>98 ± 108</td>
<td>2928 ± 2792</td>
<td>155 ± 199</td>
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<tr>
<td>200 mg/ml</td>
<td>85 ± 58</td>
<td>3634 ± 3671</td>
<td>187 ± 256</td>
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<tr>
<td>Haemoglobin</td>
<td></td>
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<td></td>
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<tr>
<td>12 mg/ml</td>
<td>332 ± 126</td>
<td>2403 ± 2516</td>
<td>201 ± 364</td>
</tr>
<tr>
<td>24 mg/ml</td>
<td>808 ± 152</td>
<td>2906 ± 1382</td>
<td>260 ± 1526</td>
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<tr>
<td>36 mg/ml</td>
<td>640 ± 393</td>
<td>4328 ± 1526</td>
<td>610 ± 887</td>
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<tr>
<td>Triacylglycerols</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>−4 ± 3</td>
<td>−4 ± 20</td>
<td>−3 ± 23</td>
</tr>
<tr>
<td>100 mg/ml</td>
<td>−13 ± 20</td>
<td>−15 ± 16</td>
<td>−17 ± 37</td>
</tr>
<tr>
<td>200 mg/ml</td>
<td>−51 ± 40</td>
<td>−53 ± 27</td>
<td>−29 ± 19</td>
</tr>
<tr>
<td>400 mg/ml</td>
<td>−83 ± 40</td>
<td>−62 ± 17</td>
<td>−48 ± 25</td>
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<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>150 mg/dl</td>
<td>−8 ± 5</td>
<td>−8 ± 19</td>
<td>−1 ± 29</td>
</tr>
<tr>
<td>200 mg/dl</td>
<td>25 ± 1</td>
<td>−29 ± 1</td>
<td>−8 ± 13</td>
</tr>
<tr>
<td>250 mg/dl</td>
<td>−81 ± 2</td>
<td>−92 ± 16</td>
<td>−6 ± 4</td>
</tr>
<tr>
<td>300 mg/dl</td>
<td>−96 ± 13</td>
<td>−62 ± 20</td>
<td>−14 ± 32</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1.5 mg/ml</td>
<td>−25 ± 14</td>
<td>−4 ± 30</td>
<td>−3 ± 20</td>
</tr>
<tr>
<td>2 mg/ml</td>
<td>−39 ± 5</td>
<td>−31 ± 18</td>
<td>−8 ± 9</td>
</tr>
<tr>
<td>2.5 mg/ml</td>
<td>−52 ± 55</td>
<td>−61 ± 37</td>
<td>−8 ± 4</td>
</tr>
</tbody>
</table>

Figure 4 pH of the sample influences the measured sCD40L concentrations

Urine solutions with 10 ng/ml rCD40L were prepared at different pHs (2–9). Values are mean sCD40L concentrations measured by ELISA.
DISCUSSION

sCD40L has emerged as a diagnostic and prognostic marker for cardiovascular endpoints [3–5]. The development of commercial assays for the determination of this marker makes sCD40L measurements available to the research community, and might contribute to unravelling the roles of inflammation in atherosclerosis and its complications. However, controversy has arisen regarding technical aspects of sCD40L measurement as well as optimum sample preparation [1,11,12,15]. In addition, little information is currently available regarding factors that may lead to false-positive or low values and pre-analytical and analytical interferences that may confound interpretation of this measurement.

The observation that serum contains significantly higher levels of sCD40L than plasma agrees with earlier reports that employed serum with average sCD40L levels higher than those previously described in plasma [3,9,10], probably due to release from activated platelets. Presumably, post-harvesting activation of platelets when obtaining serum artificially increases free sCD40L levels compared with plasma. In accordance with this hypothesis, we observed a significant positive correlation between serum sCD40L levels and platelet content, suggesting that serum sCD40L concentrations reflect the CD40L content of platelets and other blood cells. Indeed, the observed correlation of serum sCD40L and intra-platelet CD40L content suggests that serum sCD40L reflects platelet CD40L content. Previous reports showing that serum sCD40L correlates with platelet count also support this hypothesis [1]. Future studies will be required to determine the use of this marker in different disease states.

We found no significant differences in sCD40L concentrations between plasma obtained with the different anticoagulants tested, suggesting the suitability of plasma samples prepared in these conditions for sCD40L measurements. Previous studies [16–18] reported interference of samples anticoagulated with EDTA with immunoassays that use alkaline phosphatase for detection, probably because EDTA can chelate the zinc necessary for the activity of this enzyme. However, the recovery tests performed in the present study and the correlation of sCD40L concentrations in EDTA with sCD40L concentrations in heparinized plasma suggest that EDTA does not interfere with the analysis, probably because the commercial assay used in the present study employs horseradish peroxidase for amplification.

The present study indicates that avoiding the presence of platelets while preparing plasma samples to determine free sCD40L limits interference by post-hoc release of the analyte provoked by freeze–thaw cycles. Our observations support centrifugation of blood preparations at ≥ 2000 g as optimum for platelet depletion without activation for sample preparation for the sCD40L assay. Previous studies have shown that most of the CD40L binds to microparticles in serum; in contrast, all CD40L in plasma is soluble [19]. Thus unremoved platelet microparticles might be measured as soluble molecules. In view of the poor standardization of measurements, we employed plasma for the centrifugation experiments, as most of the CD40L is expected to be in the soluble form.

A previous report assessed the effect of time and temperature on measured sCD40L concentrations and found an increase in serum sCD40L levels after 3 h of storage at room temperature and no changes in plasma sCD40L [12]. On the contrary, we report in the present study a significant loss of sCD40L both in serum and plasma after longer storage at room temperature (6–24 h) and no change in sCD40L concentrations after storage at 4 °C for up to 48 h.

Increasing concentrations of exogenous haemoglobin spuriously elevated concentrations of sCD40L, showing that haemolysis interferes with the assay. Thus haemolysed samples should be avoided, as recommended by the assay’s manufacturer. Bilirubin also causes false-positive results. In contrast, high concentrations of lipids, another marker of cardiovascular risk, appear to mask sCD40L concentrations, at least partially. Consequently, data obtained from highly icteric or lipaemic samples require careful interpretation and assay of dilutions.

Finally, we did not detect sCD40L in the urine of subjects with low or high sCD40L plasma levels. The good recovery obtained with the addition of exogenous rCD40L favours the hypothesis of absence of antigen, rather than analytical interference. Also, we detected no rCD40L at a pH < 4 or > 9; however, the pH values of all urine samples employed for these experiments were between 5 and 7, thus excluding the possibility that pH variations obliterated sCD40L.

The use of sCD40L in clinical studies requires reliable methods. Our present results underscore the importance of careful removal of platelets to determine free sCD40L and to avoid interference with the ligand derived from cell-associated pools. Plasma prepared in this manner can undergo at least three freeze–thaw cycles without perturbing the analysis. Serum, on the other hand, may provide a useful biomarker for platelet sCD40L content. For the correct interpretation of results, clinical and research laboratories and clinicians must be aware of the limitations of immunoassays for this pro-atherogenic and pro-inflammatory cytokine increasingly linked to cardiovascular outcomes.

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