Cardiopulmonary Bypass Induces Release of Soluble CD40 Ligand

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Background—Cardiopulmonary bypass (CPB) is known to induce platelet activation, thrombosis, thrombocytopenia, and a systemic inflammatory response. It is known that CD40 ligand (CD40L) exists in platelets, that a soluble form of this protein (sCD40L) is released on platelet activation, that platelets are the primary source of sCD40L in blood, and that sCD40L is involved in thrombosis and inflammation. The present study was designed to determine whether sCD40L is released during CPB.

Methods and Results—Blood was obtained from patients undergoing CPB-requiring surgery and analyzed for sCD40L, interleukin-6, and platelet factor 4 and β-thromboglobulin (markers of platelet activation). Platelets were also isolated and analyzed for their levels of CD40L. Plasma levels of sCD40L increased 1.7-fold (from 0.29 to 0.51 ng/mL, \( P = 0.001 \)) within 1 hour on CPB and increased further to 3.7-fold (to 1.08 ng/mL, \( P = 0.03 \)) 2 hours after the procedure. Half of the released sCD40L was cleared in 2 hours, which allowed the sCD40L to return to approximately baseline levels 8 hours after the procedure. The platelet content of CD40L was decreased by 40% (2.675 to 1.64 ng/10^8 platelets, \( P = 0.001 \)) 1 hour after initiation of CPB and was similar to that observed for platelet factor 4 and β-thromboglobulin. Interleukin-6, a marker of inflammation, also increased during CPB.

Conclusions—The present study demonstrates that CPB causes an increase in the concentration of plasma sCD40L. The corresponding decrease in platelet CD40L suggests that this prothrombotic and proinflammatory protein was derived primarily from platelets and may contribute to the thrombotic and inflammatory complications associated with CPB. (Circulation. 2002;105:2849-2854.)

Key Words: platelets ■ cardiopulmonary bypass ■ inflammation ■ thrombosis ■ platelet-derived factors
cellular risk in women.23 sCD40L appears to be involved in both thrombosis and inflammation. The prothrombotic activity was demonstrated in mice harboring a CD40L gene deletion. These mice have a thrombosis defect, but infusion of recombinant sCD40L normalizes this deficiency,17 which demonstrates the prothrombotic activity of this protein. Other studies have appeared that show that sCD40L is proinflammatory, capable of inducing chemokine production in peripheral blood mononuclear cells18,19 and adhesive protein expression in endothelial cells.11 Thus, sCD40L has the potential of mediating several events within the vasculature. Indeed, elevated levels of sCD40L have been documented in several thrombotic and inflammatory conditions, including acute coronary syndromes,20 peripheral arterial occlusive disease,21 and systemic lupus erythematosus.22 Furthermore, elevated levels of sCD40L are associated with an increased cardiovascular risk in women.23 The present study was designed to determine how CPB affects the plasma levels of sCD40L as a first step in elucidating the role of sCD40L in the systemic vascular thrombotic and inflammatory complications of this procedure. We observed a marked, transient rise in plasma levels of sCD40L during CPB that peaked 2 hours after the procedure. A corresponding loss of CD40L from activated platelets also was observed, which implicates platelets as a source of the sCD40L. The close correlation of the rise in sCD40L with IL-6, a marker of inflammation, suggests the possibility that platelet-derived sCD40L may be involved in both the thrombotic and inflammatory events that accompany CPB.

Methods

The Institutional Review Board of Massachusetts General Hospital approved this study, and all subjects gave informed consent.

Detection of sCD40L in Plasma and Serum

Whole blood was collected from healthy volunteer donors into tubes that contained either EDTA anticoagulant or no additive. Blood samples were incubated at room temperature or on ice for various times. To obtain serum from the plain tube and platelet-poor plasma (PPP) from the EDTA tube, the samples were centrifuged at 15 000 g for 10 minutes at 4°C. The levels of sCD40L were quantified by CD40L ELISA (Alexis Biochemicals).

Release of sCD40L From Aggregated Platelets

Platelet-rich plasma (PRP) was prepared from volunteer donors by obtaining blood through a 19-gauge butterfly into PPACK anticoagulant (Phe-Pro-Arg chloromethyl ketone, final concentration of 8 mmol/L, Calbiochem) and centrifugation of the blood at 160 g for 15 minutes at 4°C, and frozen immediately. For PPP preparation, the blood was put on ice, centrifuged at 15 000 g for 15 minutes at 4°C, and frozen immediately. For platelet lysate preparation, another blood sample was centrifuged at 150 for 15 minutes, and the PRP was collected and centrifuged at 800 g for 8 minutes to obtain a platelet pellet. The platelets were washed once in CGS (13.3 mmol/L sodium citrate pH 7.0, 33 mmol/L dextrose, and 123 mmol/L NaCl) and resuspended in CGS to a platelet concentration of 8x10^9/mL. Lysates were immediately prepared by addition of an equal volume of 4°C lysis buffer (2% Triton X-100, 250 mmol/L NaCl, 100 mmol/L Tris, 4 mmol/L EDTA, 20% glycerol, 200 μg/mL leupeptin, 40 μg/mL aprotinin, and 2 mmol/L PMSF). Plasma samples were analyzed by ELISA for sCD40L (Alexis BioChemical), IL-6 (Endogen), β-thromboglobulin (β-TG: Asserchrome), and platelet factor 4 (PF4: Asserchrome). The CD40L, β-TG, and PF4 content of washed platelet lysates was also determined by ELISA. Subsequently, anticoagulated whole blood from an additional 6 patients was obtained before initiation of CPB, after 1 hour on CPB, and 2, 4, 6, 8, and 24 hours after cessation of CPB to define a more precise time course of the clearance of sCD40L from the circulation. Patients receiving platelet transfusions were omitted from the study at the point of transfusion.

Values are reported as mean±SD for all patients. Results were analyzed statistically with a paired 1-sided t test.

Results

The Table lists the characteristics of the CPB patients studied. Of note, no patient was on antiplatelet therapy at the time of cardiac surgery. Aspirin, clopidogrel, and glycoprotein (GP) IIb/IIIa antagonists had been discontinued at least 24 hours before surgery in all cases. None of the patients analyzed received transfusions of platelets. As expected, all patients experienced a decrease in circulating platelet counts at the completion of bypass, although the effect of hemodilution was not adjusted. Also as expected, all of the patients experienced platelet activation during CPB, as demonstrated by significant rises in plasma β-TG and PF4, platelet α-granule proteins released on activation.24 In comparing samples from normal donors, the levels of β-TG and PF4 were approximately half that seen in the patient samples before bypass (data not shown), which indicates that patients had partially activated platelets before the CPB procedure.

We established conditions for the measurement of the sCD40L released during CPB. Previous studies measuring sCD40L in patients with acute coronary syndromes used serum samples derived from clotted blood.19 However, we found a time-dependent release of sCD40L as a consequence of serum preparation in vitro (Figure 1A), which indicated potential artifacts when this procedure was used. In contrast, EDTA anticoagulated plasma appeared to be a preferred matrix for 3 reasons. First, sCD40L was not formed on storage of EDTA-anticoagulated PRP at either room temperature or on ice (Figure 1A). Second, EDTA blocked the time-dependent release of sCD40L induced by the activation of platelets through the thrombin receptor (PAR-1) by TRAP-6 (Figure 1B). Third, quantification of recombinant sCD40L by ELISA was found to be similar in plasma and serum (data not shown).
The effect of CPB on plasma levels of sCD40L is presented in Figure 2. One hour of CPB induced more than a 1.7-fold increase in sCD40L (from 0.29 to 0.51 ng/mL, \(P < 0.001\)). Four hours after CPB, the levels increased further to 2.4-fold (to 0.69 ng/mL, \(P < 0.001\)). Twenty-four hours after CPB, sCD40L levels were markedly reduced, similar to baseline values (to 0.33 ng/mL, \(P < 0.005\)). Figure 2 also contains data from an additional 6 patients who were studied at shorter time intervals after CPB. This was done to further delineate the kinetics of clearance of sCD40L; therefore, the numbers of patient samples were not equal at each time point. Although the half-life of sCD40L in plasma is not known, we did observe that approximately half of the sCD40L induced by CPB at 2 hours was cleared at the 4-hour time point and that the sCD40L levels returned to baseline by the 6-hour time point. On the basis of these observations, it would appear that the sCD40L generated during CPB had a half-life of 2 hours.

Previous studies by Henn and coworkers \(^{16}\) showed that platelets were the primary source of most of the CD40L found in blood and that this protein was shed on platelet stimulation. We therefore investigated whether activated platelets in circulation contributed to the rise in plasma sCD40L. As shown in Figure 3A, 1 hour of CPB caused a 40% loss of platelet CD40L. In addition, as shown in Figures 3B and 3C, CPB also caused an expected loss in platelet-associated \(\beta\)-TG and PF4, proteins released from platelet \(\alpha\)-granules on stimulation.

Plasma IL-6 levels were also measured to determine the temporal relationship between the generation of sCD40L and inflammation. Figure 4 shows that IL-6 increased 6-fold within 1 hour on bypass (\(P = 0.002\), to \(\approx 50\)-fold 4 hours after

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**Figure 1.** Release of sCD40L during serum preparation and platelet aggregation. A, Blood collected in tubes without anticoagulant was allowed to clot for various times at room temperature (WB-rt; \(\bullet\)) or on ice (WB-ice; \(\triangle\)). Blood anticoagulated with EDTA (triangles) was also incubated. Serum and plasma were analyzed for sCD40L by ELISA. B, Release of sCD40L during platelet aggregation. PRP that was unstimulated (\(\bigcirc\)) or stimulated with 5 \(\mu\)mol/L TRAP (\(\bullet\)) or TRAP plus 5 mmol/L EDTA (\(\bigtriangleup\)) was then centrifuged, and resultant PPP was quantified for sCD40L. Each graph is representative of 3 separate experiments.

**Figure 2.** Plasma sCD40L increases in patients undergoing CPB. Blood was collected from patients before initiation of CPB (Pre), after 1 hour on CPB (ON), and 2, 4, 6, 8, and 24 hours after cessation of CPB (2h-p, 4h-p, etc). Plasma was prepared and quantified for sCD40L by ELISA. As discussed in Methods, additional patient samples (n=6) were subsequently collected to analyze kinetics of sCD40L appearance and clearance from blood of CPB patients. Graph represents summary of both data sets, and number of samples is shown for each time point.
bypass (P<0.001), and decreased to the 20-fold level 24 hours after CPB (P=0.004).

**Discussion**

Previous studies have established that sCD40L is a risk factor for cardiovascular disease, that sCD40L is involved in thrombosis and vascular inflammation, that sCD40L is released after platelet activation, and that platelets are the predominant source of this soluble protein in blood. The present study shows that CPB caused a statistically significant rise in the levels of sCD40L. This increase was observed during the first hour of the procedure, peaked at 2 hours after CPB, and returned toward baseline within 6 hours after CPB, with an apparent clearance rate consistent with a half-life of <2 hours. We also observed a loss of 40% of the CD40L content of circulating platelets within the first hour of the procedure, which implicates platelets as the source of the sCD40L found in plasma. The data suggest that sCD40L may not only be a marker of platelet stimulation induced during CPB but also may contribute to the platelet activation-dependent complications of CPB, thus providing the framework for future studies on the mechanisms responsible for the systemic thrombotic and inflammatory responses induced by this procedure.

The present study of sCD40L was facilitated by a procedure to optimize detection of this protein in plasma with standard ELISA protocols. CD40L is trimeric, consisting of subunits of Mr=33 and 31 kDa, and is rapidly expressed on the platelet surface after platelet stimulation by agonists such as thrombin. Although there is not an internal pool of sCD40L in platelets, CD40L is cleaved to a soluble 18-kDa form (sCD40L) and subsequently released from stimulated platelets in a time-dependent manner. Clinical studies have used both plasma and serum samples, but we have now found that there was a time-dependent release of sCD40L during the preparation of serum (Figure 1A), which indicates that potential artifacts are seen with this procedure. This is most likely caused by the thrombin generated in serum preparation, which can induce platelet stimulation. We also found that EDTA prevented platelet activation-induced hydrolysis of CD40L and release of sCD40L (Figure 1B). Thus, to minimize postvenipuncture CD40L hydrolysis, EDTA anticoagulation was used, including a rapid preparation of samples on ice, conditions that minimized further platelet stimulation.

Although CD40L is widely distributed and has been detected both in hematopoietic and vascular cells, the data indicate that platelets can be implicated as a major source of the sCD40L generated during CPB. First, the amount of CD40L contained within platelets is sufficient to account for the increase in plasma sCD40L generated during CPB. Platelets contain ~2.5 ng of CD40L per 10^9 platelets. Data presented here show that stimulation of platelets in plasma with TRAP causes the release of most of this protein (Figure 4).
1B). Given that platelet counts average $2.5 \times 10^9$ platelets/mL, sufficient platelet-associated CD40L is available to account for the $\approx 1$ ng/mL sCD40L observed in plasma after CPB. Second, the stimulation of platelets that occurred during CPB, typically monitored either by quantification of $\alpha$-granule proteins in plasma (eg, PF4 and $\beta$-TG)\(^{24}\) or activation markers on circulating platelets (eg, P-selectin, CD63, or activated GP IIb/IIIa),\(^{26-27}\) provides a mechanism for the release of sCD40L. The patient cohort examined herein also showed procedurally induced platelet stimulation, as detected by a rise in plasma PF4 and $\beta$-TG. We additionally showed that the content of these 2 proteins in circulating platelets was also reduced, which suggests that the released $\alpha$-granule proteins were derived not only from the platelets cleared by the extracorporeal circuit but also from the stimulated platelets that remained in circulation. We thus conclude that the primary source of the sCD40L found in plasma of patients undergoing CPB is platelets. This conclusion, however, does not rule out endothelial cells and other vascular cells as additional sources of circulating sCD40L. Other clinical conditions associated with thrombosis and inflammation have also been shown to involve elevated levels of sCD40L, eg, in acute coronary syndromes\(^{20}\) and angioplasty,\(^{19}\) peripheral arterial occlusive disease,\(^{21}\) and systemic lupus erythematosus.\(^{22}\) Additionally, the increase in plasma sCD40L correlates with increased cardiovascular risk in women.\(^{23}\) It remains to be determined whether platelets are also the primary source of the sCD40L observed in these conditions.

Untoward complications of CPB include events that are derivative of thrombosis and inflammation. Recent data have demonstrated that sCD40L is involved in both of these processes. The role in thrombosis was established in CD40L\(^{-/-}\) mice, which have defective thrombosis that can be corrected by infusions of recombinant sCD40L.\(^{17}\) In addition, sCD40L has been shown to promote the aggregation of human or mouse platelets under conditions of high shear, an activity that results from sCD40L binding to GP IIb/IIIa.\(^{17}\) Thus, sCD40L appears to be a platelet agonist and necessary for thrombi stability under conditions of arterial shear. The sCD40L generated during CPB would therefore be expected to participate in the aggregates formed in the extracorporeal circuit during this procedure that result in platelet clearance and thrombocytopenia. The systemic thrombotic complications after CPB (eg, stroke and acute myocardial infarction) may also be augmented by the increased plasma sCD40L in circulation, which peaks at 2 hours after this procedure, in addition to the presence of activated platelets.

The function of sCD40L in inflammation is controversial\(^{16,19}\); however, it has been shown that sCD40L is capable of inducing chemokine production in peripheral blood mononuclear cells,\(^{18,19}\) PGE2 production in lung fibroblasts,\(^{28}\) and adhesive protein expression in endothelial cells.\(^{11}\) In addition, CD40L expressed on the surface of activated platelets potently stimulates inflammatory responses from endothelial cells and monocytes and could provide one mechanism.\(^{16}\) Because surface-expressed CD40L is transient on platelets,\(^{15}\) whereas sCD40L accumulates in plasma until the time of its clearance, and because the appearance of IL-6 correlates with the rise in sCD40L, it would appear that the sCD40L generated during CPB is an important contributor to the inflammatory response that occurs during this procedure.

GP IIb/IIIa antagonists\(^{29}\) have been shown to prevent the release of sCD40L from platelets in response to several agonists, whereas clopidogrel, a P2Y12 inhibitor,\(^{30}\) has been shown to block release in response to ADP.\(^{31}\) The precise roles of platelet-derived sCD40L in mediating the thrombotic and inflammatory complications of CPB await future studies examining the abilities of such antiplatelet agents to block the release of sCD40L and the generation of thrombotic and inflammatory responses during this procedure.

Conclusions

CPB is both proinflammatory and prothrombotic.\(^{32}\) The mechanisms responsible for these 2 complex, potentially interdependent responses remain poorly understood. CD40L has a well-established role in the immune response but also has recently emerged not only as an important mediator of inflammation but as being essential for the formation of stable thrombi. The present data suggest that CPB induces the release of sCD40L, primarily from platelets. Future studies are required to determine the role that activation of the platelet CD40L system during CPB has in the thrombotic and inflammatory events that occur in this procedure.

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References


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