CD40 Is Constitutively Expressed on Platelets and Provides a Novel Mechanism for Platelet Activation

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Abstract—CD40 is a 48-kDa phosphorylated transmembrane glycoprotein belonging to the TNF receptor superfamily. CD40 has been demonstrated on a range of cell types, and it has an important role in adaptive immunity and inflammation. CD40 has recently been described on platelets but platelet activation by CD40 has not been described. In the present study, we use flow cytometry and immunoblotting to confirm that platelets constitutively express surface CD40. CD40 mRNA was undetectable, suggesting that the protein is synthesized early in platelet differentiation by megakaryocytes. Ligation of platelet CD40 with recombinant soluble CD40L trimer (sCD40LT) caused increased platelet CD62P expression, α-granule and dense granule release, and the classical morphological changes associated with platelet activation. CD40 ligation also caused β3 integrin activation, although this was not accompanied by platelet aggregation. These actions were abrogated by the CD40L blocking antibody TRAP-1 and the CD40 blocking antibodies M2 and M3, showing that activation was mediated by CD40L binding to platelet CD40. β3 integrin blockade with eptifibatide had no effect, indicating that outside-in signaling via αIIbβ3 was not contributing to these CD40-mediated effects. CD40 ligation led to enhanced platelet-leukocyte adhesion, which is important in the recruitment of leukocytes to sites of thrombosis or inflammation. Our results support a role for CD40-mediated platelet activation in thrombosis, inflammation, and atherosclerosis. (Circ Res. 2003;92:1041-1048.)

Key Words: CD40/CD40L  ■  platelets  ■  atherosclerosis  ■  inflammation  ■  thrombosis

CD40 is a 48-kDa phosphorylated membrane glycoprotein belonging to the tumor necrosis factor (TNF) receptor superfamily. It was first described on human bladder carcinoma cells1 but is also expressed on B lymphocytes, monocytes, dendritic cells, and endothelial cells. The ligand for CD40, CD40L or CD154, is a 39-kDa glycoprotein belonging to the TNF family. The importance of CD40L-CD40 interactions in the adaptive immune response was highlighted by the discovery that mutations in the CD40L gene are responsible for X-linked hyper IgM syndrome, a rare immunodeficiency disease characterized by the absence of both class-switched B cells and high-affinity antibody production.2

Although the CD40L/CD40 interaction has been recognized to be of critical importance in the adaptive immune response for some time, it has now also been demonstrated to have an important role in inflammation. Ligation of CD40 on endothelial cells,3–5 monocytes,6–8 and dendritic cells9 results in activation with adhesion molecule and tissue factor expression and production of proinflammatory cytokines and chemokines. Recent work has demonstrated that CD40L-CD40 signaling is critical in the pathogenesis of atherosclerosis.10 In addition to its role in inflammation and atherosclerosis, CD40L is involved in thrombosis: at high shear stress, CD40L binds directly to platelet αIIbβ3 via the integrin binding sequence KGD, enhancing thrombus formation and inducing platelet spreading via outside-in integrin signaling.11

Platelets express membrane-bound CD40L on activation, which induces proinflammatory changes in endothelial cells via endothelial CD40.12,13 Platelets are the major source of soluble CD40L in the circulation, and the protean effects of CD40L have led some to suggest that platelet CD40L may be a pivotal link between the processes of thrombosis, inflammation, and atherosclerosis.14 Clinical research has demonstrated that platelet CD40L expression is enhanced in acute coronary syndromes15,16 and that soluble CD40L is released after cardiopulmonary bypass.17

Soluble CD40L is released from platelets following activation by thrombin, ADP, or collagen.15 Henn et al18 have demonstrated that this binds to CD40, also expressed on platelets, leading to further cleavage of membrane-bound CD40L and release of soluble CD40L. However, platelet activation by CD40L was not observed in this study. In the present study, we confirm that platelets constitutively express CD40 and demonstrate that platelet CD40 also provides a mechanism for platelet activation.
Materials and Methods

Patients and Subjects
The hospital research ethics committee approved the study protocol and informed consent was taken. Patients were 3 children with X-linked hyper IgM syndrome confirmed by mutation analysis and by absence of CD40L expression on activated T cells and platelets. One patient with Glanzmann’s thrombasthemia with complete absence of αIbβ3 expression was also investigated. All 4 patients were seen at Great Ormond Street Hospital for Children, United Kingdom. Controls were young adults working in the laboratory. No subject was taking any drug affecting platelet function at the time of the study.

Antibodies and Reagents
Recombinant hirudin was from Aventis. Adenosine diphosphate (ADP), arachidonic acid, aspirin, EGTA, probenecid, and chlorimipranine hydrochloride were from Sigma, UK. Collagen was from Organon Teknika and thrombin receptor agonist peptide 1 to 6 (SFLLRN) from BACHEM. Recombinant trimeric human CD40L (sCD40LT) was from Dr Richard Armitage (Amgen, Seattle, Wash). [3H]-5-HT (1.85 MBq/mL) were from Amersham International, Amersham, UK. Scintillation fluid was from BDH Laboratories Supplies. Fluo-3-AM from Molecular Probes was prepared as a 500 μmol/L solution in anhydrous dimethylsulphoxide (DMSO). HEPES/Tyrode’s (HT) buffer was 129 mmol/L NaCl, 8.9 mmol/L NaHCO3, 2.8 mmol/L KCl, 0.8 mmol/L KH2PO4, 5.6 mmol/L dextrose, and 10 mmol/L HEPES, pH 7.4.

The following directly conjugated monoclonal mouse anti-human antibodies were obtained from Becton Dickinson: CD342 FITC phycoerythrin (PE), CD40L PE (TRAP-1 clone), IgG1 PE isotype-matched control, Pac-1 FITC, CD42a PerCP, and an isotype-matched IgG1 PerCP control. CD40 FITC (clone 14G7) and an isotype-matched control antibody were obtained from Caltag. Unconjugated CD40 monoclonal antibody clones G28.5 (Professor Ed Clark, Department of Microbiology, University of Washington, Seattle, Wash), Mab89 (Dr Francine Briere, Schering Plough, Dardilly, France), and M2 and M3 (Dr Richard Armitage, Amgen, Seattle, Wash) were also used. Unconjugated isotype-matched control antibodies (Serotec and Becton Dickinson) were used in all experiments. FITC conjugated goat anti-mouse Ig (Dako) was used as the secondary antibody. Antibodies used in the blocking experiments were TRAP-1 (Becton Dickinson), which binds to the extracellular domain of CD40L and blocks its binding, and son, which can be found in the online data supplement available at http://www.circresaha.org.)

Platelet-Dense Granule and α-Granule Release
Dense granule release was assessed by determining platelet [3H]-5-HT release as previously described (see online data supplement).24 α-Granule release was assessed by determining platelet β-thromboglobulin release in the supernatant plasma from these experiments using a commercially available ELISA according to the manufacturer’s instructions (Roche Diagnostics).

Assessment of Platelet-Neutrophil Complexes
Platelet-neutrophil complexes were assessed as previously described23 (see online data supplement).

Electron Microscopy
PRP was fixed in 3% glutaraldehyde in sodium cacodylate buffer (0.1 mol/L sodium cacodylate, 2 mmol/L calcium chloride, pH 7.4) for 30 minutes and spun at 800g for 5 minutes to obtain a platelet pellet. The pellets were postfixed in osmium tetroxide, dehydrated in ethanol, and embedded in araldite resin before sectioning. Sections (70 nm) were cut, mounted on copper grids, and stained with uranyl acetate and lead citrate. Transmission electron microscopy was performed on a JEOL 1200EX electron microscope (JEOL).

Ca2+ Flux Measurements
Calcium flux was assessed as previously described24 (see online data supplement).

Platelet Aggregometry
The residual blood after PRP collection was centrifuged at 1200g for 15 minutes at room temperature to obtain platelet-poor plasma.
platelets compared with $7.5\pm0.4\%$ in unstimulated controls ($P=0.002$, $n=5$).

Despite the presence of CD40 on the platelet surface, CD40 mRNA was not detected in platelets by reverse transcription PCR (Figure 2), suggesting that CD40 protein is preformed by megakaryocytes during platelet production.

### CD62P Expression

Platelet activation with the agonists ADP, SFLLRN, or collagen results in expression of $\alpha$-granule proteins, including CD62P. sCD40LT was similarly found to induce CD62P expression on platelets. A dose-response curve is shown (Figure 3A). Incubation of platelets with $1\ \mu g/mL$ sCD40LT increased the percentage of CD62P-positive cells from $15.2\pm0.9$ to $46.7\pm2.8$ ($P=0.0002$ and $P=0.0001$, $n=5$). This was completely abrogated by the CD40L-blocking antibody TRAP-1 ($P=0.0002$) and the CD40-blocking antibodies M2 ($P=0.0001$) and M3 ($P=0.0001$). There was no inhibition by isotype-matched control antibody (Figure 3B).

These results are consistent with platelet activation caused by ligation of CD40 by CD40L. However, CD40L has the integrin binding sequence KGD and Andre et al.\textsuperscript{11} have reported that sCD40L binds to platelets through interactions with $\alpha_{IIb}\beta_{3}$. To investigate the possibility that sCD40LT-induced platelet activation is mediated by outside-in signaling through $\alpha_{IIb}\beta_{3}$, sCD40LT-mediated platelet CD62P expression was investigated in the presence of the $\alpha_{IIb}\beta_{3}$-blocking peptide eptifibatide. In these experiments, there was no reduction in the level of CD62P expression induced by sCD40LT (Figure 3B). Additionally, platelets from a patient with Glanzmann’s thrombasthenia with complete absence of $\alpha_{IIb}\beta_{3}$ were normally responsive to sCD40LT (not shown).

### α-Granule and Dense Granule Release

sCD40LT caused both platelet dense granule and $\alpha$-granule release in a CD40-dependent manner. sCD40LT increased $\beta$-thromboglobulin release from platelet $\alpha$-granules from $2258\pm151$ to $3692\pm211$ IU/mL ($P=0.002$, $n=5$) (Figure 4A). This response was inhibited by the CD40-blocking antibodies M2 ($P=0.002$) and M3 ($P=0.01$) and the CD40L
blocking antibody TRAP-1 (P<0.01) but not by isotype-matched control antibody or by eptifibatide. Eptifibatide alone had no effect. sCD40LT increased [14C]-5-HT release from platelet-dense granules from 3.8±0.2% to 8.9±1.1% (P<0.03, n=5) (Figure 4B). This response was inhibited by the CD40L blocking antibody TRAP-1 or CD40L blocking antibodies M2 and M3, but not isotype-matched control antibody. Eptifibatide did not inhibit CD62P expression caused by sCD40LT. Eptifibatide alone had no effect. Effect of stimulation with 100 μmol/L thrombin receptor agonist (SFLLRN) peptide is shown for comparison. Mean±SEM of 5 representative experiments shown.

Platelet-Leukocyte Complexes
In whole blood, CD62P expressed on platelets binds to neutrophils and monocytes, which express the specific counter-receptor, P-selectin glycoprotein ligand 1 (PSGL-1). This adhesive interaction activates leukocytes and allows the formation of platelet-leukocyte complexes, which are important in the recruitment of leukocytes to sites of thrombosis and tissue injury. Because sCD40LT preferentially causes platelet CD62P expression rather than αIIbβ3 activation, we investigated the capacity of sCD40LT to cause the formation of platelet-neutrophil complexes in whole blood. Without platelet activation, 14.1±0.6% of neutrophils formed complexes with adherent platelets. This increased to 31.6±4.3% after stimulation with sCD40LT. Platelet-neutrophil complex formation was inhibited by the CD40L blocking antibody TRAP-1 (P<0.02), the CD40L blocking antibodies M2 (P<0.03) and M3 (P<0.03), and by the CD62P blocking antibody G1 (P=0.01) but not by control antibody, showing that the effect requires a CD40L-CD40 interaction and that it depends on expression of platelet CD62P (Figure 5). Similar results were obtained with platelet-monocyte complexes (not shown).

Platelet Shape Change
Platelet activation by sCD40LT also induced pseudopodia formation and other morphological changes typically associated with activation (Figure 6).

CD40 Ligation Does Not Induce Intracellular Calcium Flux
sCD40LT did not induce intracellular calcium flux, in contrast to SFLLRN, which is known to cause calcium flux via
activation of the G protein–coupled thrombin receptor PAR-1 (Figure 7).

**β3 Integrin Activation**

Platelet activation with the agonists ADP, SFLLRN, or collagen results in a conformational change in αIIbβ3, causing it to expose its high-affinity ligand binding site, which is recognized by the monoclonal antibody Pac-1.29 We found that sCD40LT caused a small but significant increase in Pac-1 antibody binding from an MFI of 19.9 ± 1.8 to 28.1 ± 2.9 \((P = 0.004, n = 5)\). This was inhibited by the CD40L blocking antibody TRAP-1 \((P = 0.03)\) and the CD40 blocking antibodies M2 \((P = 0.04)\) and M3 \((P = 0.06)\). There was no inhibition by isotype-matched control antibody (Figure 8A).

To determine if sCD40LT can induce platelet aggregation, we exposed normal platelets to sCD40LT and measured aggregation in an aggregometer. Despite the modest increase in platelet surface Pac-1 binding indicating αIIbβ3 activation, sCD40LT did not induce aggregation of platelets (Figure 8B). sCD40LT did not alter the capacity of both ADP and arachidonic acid to induce platelet aggregation at a range of concentrations (not shown) and CD40L blocking antibody TRAP-1 had no effect on aggregation of platelets from normal controls (not shown). Moreover, platelets from patients with CD40L deficiency, which we have previously shown do not express CD40L on stimulation,26 aggregated normally (Figure 8). These experiments demonstrate that CD40L is not involved in homotypic platelet adhesion via αIIbβ3 at the low shear rates encountered in an aggregometer.

**Discussion**

Platelets are important mediators of inflammation as well as hemostasis. They possess adhesion molecules, which facilitate interactions with neutrophils, monocytes, and endothelial cells, and can activate these cells through direct contact or through the release of soluble mediators. Platelets express the transmembrane signaling protein CD40L within seconds of activation,12 and after platelet activation with thrombin, ADP, or collagen, a soluble form of CD40L is also released. Henn et al18 have demonstrated that this binds to platelet CD40, leading to further cleavage of membrane-bound CD40L and release of soluble CD40L. However, platelet activation through CD40 has not been reported. In this present study, we show for the first time that platelets can be activated through ligation of constitutively expressed CD40.

Ligation of platelet CD40 by sCD40LT causes platelet CD62P expression, α-granule and dense granule release, morphological changes typically associated with activation, and modest increases in αIIbβ3 activation.

Platelets constitutively express CD40 and on stimulation with SFLLRN, 23.1% are induced to coexpress CD40L. Other cells also have the capacity to coexpress CD40L and CD40, including vascular endothelial cells and macrophages in vitro30,31 and in vivo in the context of atherosclerosis.31 SFLLRN mimics the physiological action of thrombin; therefore, platelet CD40L-CD40 interactions should occur during thrombus formation—an everyday event in healthy individuals. Coexpression of CD40 and CD40L has been demonstrated in human thrombus,18 and platelet activation with
CD40L expression occurs even in the absence of thrombus formation in conditions such as sickle cell disease,
acute coronary syndromes, and hypercholesterolemia. Platelet-derived CD40L may have the capacity to stimulate resting platelets by binding to CD40 during direct cell-cell contact or, as platelets are a major source of soluble CD40L, via release of soluble CD40L. Concentrations of sCD40L of up to 50 ng/mL have been reported in peripheral blood in humans in disease states. Although this is at the lower end of the range required for platelet activation in vitro (we found platelet activation occurred at concentrations of 100 ng/mL), much higher concentrations might be generated at sites of platelet activation and tissue injury.

The biological activity of platelet sCD40L is likely to depend not only on local concentration but also on whether it exists as a trimer or monomer. The soluble CD40L used in this investigation was a trimeric form held together by an isoleucine zipper motif, which is far more active than sCD40L monomer. Whereas Henn et al suggested that 18-kDa platelet sCD40L is monomeric, other workers have found that 18-kDa sCD40L sediments on a sucrose gradient with an apparent molecular mass of 56 kDa, suggesting that it spontaneously forms trimers. Molecular modeling and crystallography suggest that membrane bound CD40L is also trimeric and therefore membrane bound CD40L is likely to have the capacity to cause platelet activation.

Andre et al demonstrated that at high shear (1000/s) and during the formation of arterial thrombus in an animal model, CD40L can bind to the platelet ligand binding site via the KGD integrin binding sequence, and promote platelet spreading and thrombus formation. However, CD40L had no effect on aggregation at low shear. All our aggregometry experiments were also conducted at low shear (100/s), and the results indicate that CD40LT does not modulate platelet aggregation under these conditions. However, we did find that sCD40LT caused a modest increase in platelet activation. The increase in platelet activation caused by sCD40LT was abrogated by both CD40 and CD40L blocking antibodies, indicating that activation was occurring as a result of a CD40L-CD40 interaction. This is an additional mechanism for CD40L-induced platelet activation to that proposed by Andre et al and may also become important at high shear rates.

The principal effects of platelet CD40 ligation were therefore to induce platelet shape change and α-granule release. In contrast, activation was minimal. This pattern of activation is distinct from that seen with other platelet agonists. An explanation for this observation may be that ADP, thrombin, and thromboxane A2 are G protein–coupled receptors, whereas CD40 is not. αIIbβ3 activation requires G protein–mediated inside-out signaling, which is always associated with intracellular calcium flux. CD40 signals through protein kinase cascades, including protein kinase C (PKC).
PKC signaling also occurs in platelets but usually in combination with increases in cytosolic calcium. Interestingly, phorbol esters, which induce PKC signaling alone, have been reported to cause platelet secretion without raising cytosolic calcium levels and take several minutes to induce a weak aggregation response. The pattern of platelet activation caused by CD40, with substantive granule release but minimal platelet aggregation, is consistent with this explanation. Further work is required to clarify the signaling pathways activated by CD40 ligation in the platelet.

The biological importance of platelet CD40-mediated platelet activation is likely to stem from the release of α-granule adhesion molecule CD62P which is also of critical importance in the recruitment of leukocytes to sites of thrombosis and inflammation and in the formation of circulating platelet-leukocyte complexes. These complexes represent a subpopulation of neutrophils and platelets primed for adhesion, phagocytosis, and bacterial killing, and their significance has been demonstrated in a number of clinical conditions including sepsis, multiple organ failure, and SIRS. The enhancement of platelet-leukocyte adhesion caused by ligation of platelet CD40 and the proinflammatory stimulus caused by the release of granule contents may be particularly important where platelets are in close proximity to cells expressing CD40L, such as platelets in thrombi, and T cells, macrophages, and endothelial cells in atherosclerotic plaques. Induction of platelet-leukocyte adhesion through CD40-mediated platelet CD62P expression is likely to provide a mechanism whereby leukocytes can be directed to sites of thrombosis, inflammation, or tissue injury and in the initiation and propagation of atherosclerosis. Our findings suggest that CD40-mediated platelet activation is likely to provide a pivotal link between thrombosis and inflammation.

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