Platelet GP IIb/IIIa Inhibition

Release of Soluble CD40L From Platelets Is Regulated by Glycoprotein IIb/IIIa and Actin Polymerization

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OBJECTIVES

The purpose of this study was to examine the effects of glycoprotein (GP) IIb/IIIa antagonists (abciximab, eptifibatide, and tirofiban) and other inhibitors on translocation of CD40L from intraplatelet stores to the platelet surface and on the release of soluble CD40L (sCD40L) from platelets.

BACKGROUND

CD40L is a proinflammatory and prothrombotic ligand in the tumor necrosis factor family.

METHODS

Platelet surface CD40L was measured by flow cytometry, and sCD40L was measured by enzyme-linked immunosorbent assay.

RESULTS

Translocation of CD40L from intraplatelet stores to the platelet surface was not inhibited by GP IIb/IIIa antagonists. However, release of sCD40L from the surface of activated platelets was inhibited by GP IIb/IIIa antagonists in a dose-dependent manner, in concert with inhibition of PAC1 binding to platelets (a surrogate marker for fibrinogen binding). Release of sCD40L from activated platelets was also markedly reduced in Glanzmann platelets (deficient in GP IIb/IIIa). Ethylenediaminetetraacetic acid was an effective inhibitor of sCD40L release, but only when added before platelet activation. Both cytochalasin D (an inhibitor of actin polymerization) and GM6001 (an inhibitor of matrix metalloproteinases [MMPs]) inhibited the release of sCD40L from platelets when added before, as well as 3 min after, platelet activation. However, neither cytochalasin D nor GM6001 affected translocation of CD40L to the platelet surface.

CONCLUSIONS

The GP IIb/IIIa antagonists inhibit release of sCD40L from activated platelets. Release of sCD40L from platelets is regulated, at least in part, by GP IIb/IIIa, actin polymerization, and an MMP inhibitor-sensitive pathway. In addition to their well-characterized inhibition of platelet aggregation, GP IIb/IIIa antagonists may obviate the proinflammatory and prothrombotic effects of sCD40L.

In addition to their well-known function in hemostasis and thrombosis, platelets play an important role in inflammation (1). Platelets contain a variety of inflammatory modulators—including CD40 ligand (CD40L, CD154), platelet-derived growth factor, platelet factor 4, RANTES (regulated upon activation, normal T-cell expressed and secreted), thrombospondin, and transforming growth factor beta—that are released upon platelet activation. Atherosclerosis is a chronic inflammatory disease, and the CD40/CD40L interaction has a central role in the pathogenesis of atherosclerosis (2).

CD40L, a member of the tumor necrosis factor family, is not expressed on the resting platelet surface. However, Henn et al. (3) demonstrated that platelets translocate preformed intraplatelet stores of CD40L to the platelet surface within seconds of activation in vitro and in the presence of thrombus formation in vivo. Furthermore, platelet surface CD40L interacts with endothelial cell CD40 to induce a thromboinflammatory reaction of endothelial cells (3). Activated platelets also release large amounts of soluble CD40L (sCD40L) over a period of minutes to hours (4–6). Indeed, platelets appear to be the predominant source of circulating sCD40L (5). The sCD40L released from platelets during thrombosis has three reported functions (7): 1) induction of the production and release of proinflammatory cytokines from vascular cells and of matrix metalloproteinases (MMPs) from resident cells in the atheroma (although not all studies agree on this point [5]); 2) stabilization of arterial thrombi (8); and 3) inhibition of re-endothelialization of injured blood vessels (9).

In the present study, we examined the effects of GP IIb/IIIa antagonists (abciximab, eptifibatide, and tirofiban) and other inhibitors on the translocation of CD40L from intraplatelet stores to the platelet surface and on the release of sCD40L from platelets.
**Abbreviations and Acronyms**

EDTA = ethylenediaminetetraacetic acid  
ELISA = enzyme-linked immunosorbent assay  
FITC = fluorescein isothiocyanate  
GP = glycoprotein  
HT = HEPES Tyrode’s buffer  
MMPs = matrix metalloproteinases  
FCI = percutaneous coronary intervention  
PE = phycoerythrin  
PerCP = peridinin chlorophyll protein  
PRP = platelet-rich plasma  
sCD40L = soluble CD40 ligand

**METHODS**

**Blood collection.** This study was approved by the institutional review board of the University of Massachusetts Memorial Health Care, and subjects gave written informed consent. Peripheral blood was collected from healthy donors or patients with Glanzmann thrombasthenia ([10](#)) into endotoxin-free 3.2% sodium citrate Vacutainer tubes (Becton Dickinson, San Jose, California), after discarding the first 2 ml of drawn blood. No donor or patient had received any drug known to affect platelet function (including aspirin, cyclooxygenase inhibitors, statins) within the previous 10 days. Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 150 g for 12 min at 22°C. The PRP was then diluted 1:5 (to prevent Fc-mediated effects) incubated at 22°C for 10 min with the FcγRIIa (CD32) blocking antibody IV.3 (Medarex, Princeton, New Jersey) 2.5 µg/ml. The PRP was then incubated at 22°C for 20 min with either HT buffer alone, abciximab 6.4 µg/ml, eptifibatide 1.0 µg/ml, tirofiban 50 ng/ml, ethylenediaminetetraacetic acid (EDTA) 5 mM, or GM6001 (also known as Galardin, an inhibitor of MMPs [11](#)) (Calbiochem, San Diego, California) 30 µM, or cytochalasin D (an inhibitor of actin polymerization [12](#)) (Sigma, St. Louis, Missouri) 60 µM. (Assays with GM6001 and cytochalasin D included 0.3% dimethyl sulfoxide.) Samples were then incubated with or without 50 µM iso(S)FLLRN (iso-thrombin receptor activating peptide) (Multiple Peptide Systems, San Diego, California) and with a saturating concentration of either the CD40L-specific phycoerythrin (PE)-conjugated monoclonal antibody 24-31 (Ancell, Bayport, Minnesota) (or the same concentration of PE-conjugated mouse isotype control IgG; [Pharmingen, San Diego, California]) for 1 h at 37°C or a cocktail of the activated GP IIb/IIIa-specific fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody PAC1 (Becton Dickinson) and the GP IIa-specific peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibody RUU-7F12 (Becton Dickinson) for 3 min at 22°C. Samples were then fixed with 1% formalin (Polysciences, Eppelheim, Germany) for 10 min at 22°C. Samples labeled with 24-31 were then washed by centrifugation at 1,200 g for 7 min, and the formalin supernatant was aspirated. The platelet pellet was then resuspended in HT buffer containing the GP IIIa-specific FITC-conjugated monoclonal antibody Y2/51 (DAKO, Carpinteria, California) 0.5 µg/ml (or, for Glanzmann samples, the GPIX-specific PerCP-conjugated monoclonal antibody Beb1 [Becton Dickinson] 1.25 µg/ml) and incubated at 22°C for 10 min. Samples were then diluted in 1% formalin until analysis by flow cytometry. Flow cytometry was performed in a FACS Calibur (Becton Dickinson) with standard filter configuration and Cell Quest software. Platelets were identified by their characteristic forward and orthogonal light scatter properties and by labeling with a platelet-specific monoclonal antibody (FITC–Y2/51, PerCP–RUU-7F12, or PerCP–Beb1). Platelet surface CD40L and activated GP IIb/IIIa was measured by mean fluorescence intensity (PE and FITC, respectively). Neither Y2/51 nor RUU-7F12 block the binding of abciximab, eptifibatide, tirofiban, or PAC1.

**Enzyme-linked immunosorbent assay (ELISA).** Platelet counts in PRP were normalized to 300,000 platelets per µl. The PRP was then incubated (22°C, 20 min) with various concentrations of a GP IIb/IIIa antagonist, EDTA 5 mM, GM6001 30 µM, cytochalasin D 60 µM, or HT buffer alone. Samples were incubated (37°C, 1 h) with or without 50 µM isoTRAP. In some experiments, EDTA 5 mM, GM6001 30 µM, or cytochalasin D 60 µM was added 3 min after the addition of the 50 µM isoTRAP. After activation, the samples were centrifuged (8,000 g, 3 min, 22°C), the supernatants were transferred to a clean tube and centrifuged again (8,000 g, 3 min, 22°C), and the subsequent supernatants were transferred to a clean tube. These supernatants were then immediately tested for sCD40L by ELISA (Bender MedSystems, San Bruno, California). The ELISA was set up according to the package insert except that plasma samples were diluted 1:1 (50 µl/50 µl) with assay buffer. The average of duplicate samples was recorded. To determine the amount of released sCD40L, the sCD40L concentration of samples without added isoTRAP was subtracted from the sCD40L concentration of samples with added isoTRAP. The range of 0.33 to 4.84 ng/ml in the background sCD40L of our normal donors and Glanzmann thrombasthenia donors is consistent with the sCD40L range of 0.03 to 4.0 ng/ml obtained from a panel of 40 healthy donors (Bender MedSystems, product insert). This normal variability in the background sCD40L of both the normal donors and the Glanzmann thrombasthenia donors does not detract from our conclusions, because each donor and patient acted as his or her own control. The PRP incubated for 40 min in the absence of an agonist resulted in no increase in sCD40L over the background sCD40L in the same donor’s fresh platelet–poor plasma.

**Statistical analysis.** Significance (p < 0.05) was determined by two-tailed t test.
RESULTS

Activation of platelets with 50 μM isoTRAP resulted in a marked increase in the platelet surface expression of CD40L (Fig. 1). This activation-dependent translocation of CD40L from intraplatelet stores to the platelet surface was not inhibited by pharmacologically relevant concentrations of the GP IIb/IIIa antagonists abciximab, eptifibatide, or tirofiban (Fig. 1). However, the release of sCD40L from activated platelets was inhibited in a dose-dependent manner by abciximab, eptifibatide, and tirofiban (Fig. 1), in concert with inhibition of PAC1 binding to platelets (a surrogate marker for fibrinogen binding [13]) (Fig. 2).

To further assess the role of GP IIb/IIIa in the redistribution of CD40L molecules, we next examined the platelets of two patients with Glanzmann thrombasthenia (an inherited deficiency of GP IIb/IIIa [10]). Consistent with our results with GP IIb/IIIa antagonists, the activation-dependent translocation of CD40L from intraplatelet stores to the platelet surface was not reduced in Glanzmann platelets (Fig. 3A), but the release of sCD40L from activated platelets was markedly reduced in Glanzmann platelets (Fig. 3B).

Because sCD40L is released from washed platelets (4,5), no plasma component is required for its cleavage. Members of the tumor necrosis factor family of molecules have been reported to be cleaved by calcium-dependent MMPs and by ADAMs, both of which are inhibited by hydroxamate inhibitors of MMPs (11,14). Therefore, to further examine the mechanism of the redistribution of platelet CD40L and sCD40L release, we assessed the effects of EDTA (a strong calcium chelator) and GM6001 (a hydroxamate inhibitor of...
Platelet rich plasma was measured as in Figure 2. Data are mean activation with 50 μM isoTRAP, was measured as in Figure 1. (B) Released sCD40L in platelet rich plasma was measured as in Figure 2. Data are mean ± SEM, n = 3 for normal control samples and mean of n = 2 for Glanzmann thrombasthenia. Asterisks indicate significance as compared with normal control samples.

DISCUSSION

Our results demonstrated the following: 1) GP IIb/IIIa antagonists (abciximab, eptifibatide, and tirofiban) inhibit the release of sCD40L from activated platelets and therefore, in addition to their well characterized inhibition of platelet aggregation, they may obviate the proinflammatory (15–18) and prothrombotic effects (8) of sCD40L. 2) Release of sCD40L from platelets is regulated, at least in part, by GP IIb/IIIa, actin polymerization, and an MMP inhibitor-sensitive pathway. 3) Translocation of CD40L from intraplatelet stores to the platelet surface is not inhibited by GP IIb/IIIa antagonists and is not regulated by GP IIb/IIIa or actin polymerization.

Soluble CD40L ligand is prothrombotic via stabilization of arterial thrombi by a β3-integrin (GP IIIa)-dependent mechanism (8). The sCD40L has been reported to be proinflammatory via induction of leukocyte chemokine production (15) and endothelial cell adhesive proteins (16). Although the proinflammatory effects of recombinant sCD40L in vitro (15–17) and in vivo (18) are well described, Henn et al. (5) were unable to document such effects with natural sCD40L in vitro. Elevated plasma levels of sCD40L have been demonstrated in thrombotic and/or inflammatory states: unstable angina (4), percutaneous coronary intervention (PCI) (4), cardiopulmonary bypass (19), peripheral arterial occlusive disease (20), essential thrombocythemia (21), reactive thrombocytosis (21), and systemic lupus erythematosus (22). High plasma concentrations of sCD40L may be associated with increased cardiovascular risk in apparently healthy women (23). Platelets appear to be the predominant source of circulating sCD40L (5). The elevated plasma levels of sCD40L in unstable angina and during PCI in the absence of GP IIb/IIIa antagonist therapy (4), therefore, are of particular relevance to the presently described inhibition of sCD40L release from platelets by GP IIb/IIIa antagonists (abciximab, eptifibatide, tirofiban), because these agents are now standard therapy for unstable angina (24) and during PCI (25). The sCD40L is only released when platelets are activated (4–6), that is, at sites of local thrombosis. The presently described inhibitory effects of GP IIb/IIIa antagonists on sCD40L release from activated platelets would therefore occur most prominently in vivo at the site or sites of greatest potential benefit. Thus, the present findings suggest that GP IIb/IIIa antagonist therapy, in addition to its well-known inhibition of platelet aggregation, may be of benefit in patients with unstable angina and during PCI via an additional mechanism of action: inhibition of the prothrombotic (8) and proinflammatory (15–18) effects of sCD40L. Because all three Food and Drug Administration-approved GP IIb/IIIa antagonists (abciximab, eptifibatide, and tirofiban) are able to inhibit sCD40L release, our results do not differentiate between these agents with respect to their ability to produce clinically relevant inhibition of inflammation. Heeschen et al. (26) recently demonstrated that measurement of sCD40L in the first 12 h after the onset of ischemic symptoms in patients with unstable angina identifies a subgroup of patients that has a much greater clinical benefit from abciximab treatment.

Consistent with the present study, Nannizzi-Alaimo et al. (27), in a study published after the completion of the present work, reported that the three Food and Drug Administration-approved GP IIb/IIIa antagonists inhibit
the release of sCD40L from platelets in vitro. These investigators (27) also reported that suboptimal doses of these three antagonists (that resulted in between 0% and 50% platelet aggregation) potentiated the release of sCD40L from platelets in vitro, raising the possibility that the proinflammatory effects of sCD40L could explain the negative results of the Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Arteries (GUSTO IV) trial of abciximab (28). However, our experiments used doses of GP IIb/IIIa antagonist that under our experimental conditions resulted in a similar degree of inhibition of platelet aggregation yet did not potentiate sCD40L release (Fig. 2). This apparent discrepancy between our results and those of Nannizzi-Alaimo et al. (27) may result from technical differences, including anticoagulants (citrate vs. D-phenylalanyl-prolyl-arginine), agonists (50 μM isoTRAP vs. 5 μM thrombin receptor activating peptide), and unstirred versus stirred conditions. The enhanced inhibition of sCD40L release by epftibatide and tirofiban compared with abciximab observed in the study by Nannizzi-Alaimo et al. (27) was likely due to the fact that these investigators initiated platelet aggregation and GP IIb/IIIa inhibition simultaneously by adding PRP to a mixture of platelet agonist and GP IIb/IIIa antagonist. Because abciximab has a slower on rate than the small molecule inhibitors epftibatide and tirofiban (29), processes mediating the release of sCD40L may already be initiated before complete receptor blockade by abciximab. In our experiments, GP IIb/IIIa antagonists were preincubated with platelets for 20 min at room temperature before platelet activation, thereby allowing adequate time for all GP IIb/IIIa antagonists to reach equilibrium. This latter approach may be more relevant to the clinical situation in which GP IIb/IIIa antagonists are dosed at high levels before procedures that activate platelets. Thus, plasma sCD40L in patients after in vivo treatment with abciximab or epftibatide, measured when receptor occupancy was low, was not elevated compared with pretreatment levels (30).

In contrast to both our findings and those of Nannizzi-Alaimo et al. (27) with intravenous GP IIb/IIIa antagonists, Zondlo et al. (31) reported that oral GP IIb/IIIa antagonists (roxiiban and orbofiban) in pharmacologically relevant doses do not affect the release of sCD40L from platelets in vitro. The resultant lack of inhibition of the proinflammatory (15–18) and prothrombotic effects (8) of sCD40L may contribute to the reported lack of clinical effectiveness of oral GP IIb/IIIa antagonists (32). Clopidogrel, a thienopyridine, completely abolishes adenosine diphosphate-induced expression of platelet surface CD40L (33), but the mechanism is via blockade of the P2Y12 adenosine diphosphate receptor, not via a direct effect of clopidogrel on CD40L.

Our results also document a heretofore unrecognized defect in patients with Glanzmann thrombasthenia: a lack of activation-dependent release of sCD40L from platelets (Fig. 3B). The biologic and/or clinical significance of this finding remains to be determined. Patients with Glanzmann thrombasthenia...
thrombasthenia appear to have higher platelet surface expression of CD40L (Fig. 3A). A trivial explanation for this would be that the platelet surface CD40L is more accessible to the detecting antibody owing to the lack of platelet surface GP IIb/IIIa in Glanzmann thrombasthenia. An alternate explanation is that, because translocation of CD40L from intraplatelet stores to the platelet surface and proteolysis of platelet surface CD40L is probably occurring concurrently, the higher platelet surface expression of CD40L in patients with Glanzmann thrombasthenia reflects decreased proteolysis. This latter explanation may also account for the modest increase in the platelet surface expression of CD40L in experiments with EDTA (Fig. 4) and the statistically insignificant increases in the platelet surface expression of CD40L in experiments with abciximab and eptifibatide (Fig. 1).

In human T cells, CD40L is processed into its soluble form in intracellular microsomes (34). However, as independently demonstrated by three groups of investigators (5,27,35), platelet lysates, unlike T-cell lysates, demonstrate full length but not cleaved forms of CD40L. Thus, the intracellular pool of CD40L contains no sCD40L. Although the release of sCD40L from platelets is activation-dependent, sCD40L release is not the direct result of a granule secretion because the time frames are different. The release of CD40L is much slower (maximal at approximately 45 min [5,6,27]) than alpha granule release (maximal at approximately 1 min) (36). Henn et al. (5) reported that CD40 is constitutively expressed on platelets, and that the interaction of CD40 with CD40L on the surface of activated platelets is required for proteolysis of sCD40L. The present study suggests that the GP IIb/IIIa complex integrin αIIbβ3 augments the release of sCD40L. Thus, Glanzmann thrombasthenia platelets and normal platelets treated with fibrinogen-blocking GP IIb/IIIa antagonists have reduced activation-dependent sCD40L release (Figs. 2 and 3B). These findings may reflect the lack of the normal “outside-in” signaling initiated by fibrinogen binding to activated GP IIb/IIIa (37). Alternately, maximal release of sCD40L may require cell–cell contact, and this is disrupted in Glanzmann thrombasthenia and by GP IIb/IIIa antagonist therapy. Although sCD40L has been shown to bind to the activated GP IIb/IIIa complex via the lysine-arginine-glutamic acid (KGD) peptide sequence of CD40L (8), uncleaved platelet membrane CD40L is unlikely to bind to GP IIb/IIIa by this mechanism because the KGD sequence of CD40L is located immediately adjacent to the transmembrane domain (34), whereas the RGD (and KGD) binding site appears to be located in the head region of GP IIb/IIIa (38). However, recent studies by Takagi et al. (39) suggest that the molecule may assume variably bent conformations. Because the orientation of the transmembrane domain and extracellular “legs” are uncertain, it is impossible to assign a height to the ligand binding region. In any event, this three-dimensional arrangement would not preclude CD40 ligand from one platelet from binding to the KGD sequence in uncleaved CD40L on another platelet.

Platelet activation results in fibrinogen binding to the GP IIb/IIIa complex (37), which results in activation of the cytoskeleton via actin polymerization (37) and, we hypothesize based on our results with cytochalasin D (Fig. 5), trafficking of the putative CD40L-cleaving protease to the platelet surface. Consistent with this hypothesis, EDTA, when added before the platelet agonist, inhibits the initial binding of fibrinogen, the subsequent signaling to the cytoskeleton, movement of the protease to the platelet surface, and therefore release of sCD40L (Fig. 5A). When EDTA was added after fibrinogen binding (i.e., 3 min after agonist addition), EDTA was ineffective in inhibiting the release of sCD40L (Fig. 5B), suggesting that: 1) signals initiating trafficking of the protease had already occurred and/or 2) the putative protease is not divalent cation-dependent. Because most metalloproteinases are divalent cation-dependent, an alternate possibility is that, although EDTA inhibits the activation of the putative CD40L-cleaving metalloproteinase, once the enzyme has been activated and engages CD40L at the cleavage site, removal of divalent cations cannot prevent proteolysis. As predicted by our hypothesis, actin polymerization is still required post-activation because addition of cytochalasin D 3 min after the agonist (like addition of cytochalasin D before the agonist) still effectively blocked sCD40L release (Fig. 5). Irrespective of whether it was added before or after the agonist, GM6001 inhibited the release of sCD40L (Fig. 5) because GM6001 is a direct inhibitor of the metalloproteinase that putatively cleaves CD40L. Consistent with these findings, Jin et al. (6) reported that the simultaneous addition of thrombin and KB8301, another metalloproteinase inhibitor, inhibited the thrombin-induced release of sCD40L from platelets.

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