Inhibitory Effects of Glycoprotein IIb/IIIa Antagonists and Aspirin on the Release of Soluble CD40 Ligand During Platelet Stimulation

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Background—Glycoprotein (GP) IIb/IIIa antagonists inhibit platelet aggregation, an activity attributed to the clinical benefits of these drugs in settings that involve acute coronary thrombosis. However, platelet activation and subsequent aggregation are now known to cause the release of a soluble form of CD40 ligand (sCD40L), a prothrombotic and proinflammatory protein with GP IIb/IIIa binding activity and an established role in atherosclerotic lesion progression. The present study was designed to determine what effect GP IIb/IIIa antagonists have on the release of sCD40L.

Methods and Results—Doses of eptifibatide, abciximab, and tirofiban that inhibited platelet aggregation by at least 80% also inhibited sCD40L release in vitro (by 85%, 57%, and 80%, respectively). When platelets were stimulated with a thrombin receptor agonist, inhibition by GP IIb/IIIa antagonists occurred without affecting the release of βTG, an α-granule protein. Unexpectedly, concentrations of the 3 antagonists that blocked aggregation by only 20% to 50% potentiated the release of sCD40L (by 19% to 26%). Platelets from aspirin-treated individuals were partially protected from sCD40L release, but only when the agonist was collagen, an affect augmented by the addition of GP IIb/IIIa antagonists.

Conclusions—These studies suggest that the mechanisms responsible for the clinical benefits of GP IIb/IIIa antagonists (at doses that optimally inhibit aggregation) and of aspirin may not be limited to the inhibition of thrombosis through their blockade of platelet aggregation but may also involve the inhibition of inflammation and thrombosis through their blockade of sCD40L release. These studies also provide a mechanism by which suboptimal doses of GP IIb/IIIa antagonists may be proinflammatory. (Circulation. 2003;107:1123-1128.)

Key Words: platelets ■ thrombosis ■ inflammation ■ drugs

Large clinical trials have established that glycoprotein (GP) IIb/IIIa antagonists are effective for the prevention of acute thrombotic events associated with percutaneous interventions and acute coronary syndromes (reviewed in Lincoff et al1). Arterial thrombosis in these settings is dependent on platelet aggregation, a process mediated by the binding of soluble fibrinogen to GP IIb/IIIa on the surface of stimulated platelets. GP IIb/IIIa antagonists are effective in these indications because they block the binding of soluble fibrinogen to GP IIb/IIIa and therefore inhibit platelet aggregation. Interestingly, some trials suggest less accrual of events such as myocardial infarction and death in the treatment arm than are observed in the placebo arm during the year after an event. Thus, the clinical benefit extends well past the time of acute administration of GP IIb/IIIa antagonists, which is often less than 20 hours.2,3 Although it has been considered that thrombosis-mediated ischemia is a culprit for long-term negative consequences, including atherosclerotic lesion progression after acute arterial coronary thrombosis, it is also possible that thrombosis itself could generate such factors, which could be blocked by GP IIb/IIIa antagonists.

Platelet stimulation and subsequent aggregation are known to cause the expression or release of several factors that could affect vascular pathology. These include TXA2, a costimulator of platelets that has vasoconstrictive activity; P-selectin, an α-granule protein that mediates platelet rolling, leukocyte adhesion, and coagulation; ADP and serotonin, which amplify platelet aggregation; platelet-derived growth factor, a growth factor for vascular cells; and CD40L, a member of the tumor necrosis factor-α family of proteins (reviewed in Gresele et al4). Although any of these factors could contribute to long-term vascular pathologies, CD40L appears to be particularly relevant because this protein is now known to be prothrombotic5 and proinflammatory, to have a proven role in atherosclerotic lesion progression,6 and to be a risk factor for cardiovascular events.7

CD40 ligand (CD40L, CD154, gp39) was originally identified in T lymphocytes, where it has a role in the immune

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response by binding to its receptor on B cells, CD40 (reviewed in Schonbeck and Libby). Both CD40L and CD40 have also been identified on other cells within the vasculature, including endothelial cells, smooth muscle cells, monocytes, and macrophages, where they have been implicated as mediators of inflammation. The pioneering work of Henn and coworkers established that CD40L and CD40 also exist in platelets. They showed that CD40L is cryptic in unstimulated platelets but rapidly becomes exposed on the platelet surface after platelets are activated. They showed further that surface-expressed CD40L is proinflammatory and capable of inducing the expression of chemokines (e.g., interleukin-8 and monocyte chemotactic protein-1), adhesion molecules (intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin), and tissue factor by ligating CD40 on endothelial cells and monocytes. Platelet CD40L has also been shown to inhibit the migration of endothelial cells, which implicates CD40L in restenosis. CD40L expressed on stimulated platelets is subsequently cleaved, generating a soluble hydrolytic fragment termed sCD40L. Elevated levels of sCD40L have been shown in acute coronary syndromes, percutaneous coronary interventions, and cardiopulmonary bypass. Furthermore, elevated levels of sCD40L are associated with risk of future cardiovascular events in otherwise healthy women. Platelets appear to be an important reservoir of this protein in circulation, because platelet count is correlated with levels of plasma sCD40L; essentially all of the sCD40L generated during the clotting of whole blood is derived from platelets. sCD40L is a bifunctional protein, being involved in both thrombosis and inflammation. The thrombotic activity is due to a KGD sequence located near the amino terminus that allows for the protein to bind to GP IIb/IIIa to promote stable platelet aggregation. The inflammatory activity is most likely located in the tumor necrosis factor homology domain, which induces chemokine production in peripheral blood mononuclear cells and adhesive protein expression in endothelial cells. Therefore, sCD40L has the potential of mediating several actions within the vascular space.

Because sCD40L expression correlates with platelet aggregation and because sCD40L is a GP IIb/IIIa binding protein, we asked whether GP IIb/IIIa antagonists affect the release of this prothrombotic and proinflammatory protein from platelets.

**Methods**

**Reagents**

The GP IIb/IIIa inhibitors were eptifibatide (Integrilin Injection, Millennium Pharmaceuticals Inc), abciximab (ReoPro, Eli Lilly & Co.), and tirofiban (Aggrastat, Merck & Co.). PPACK anticoagulant (Phe-Pro-Arg chloromethyl ketone) was purchased from Calbiochem. Platelet agonists were TRAP (TRAP-6: SFFLRN-Gly-Gly-Phe-Pro-Arg chloromethyl ketone) was purchased from Chrono-Log (Corporation), and tirofiban (Aggrastat, Merck & Co.). PPACK anticoagulant was used to inhibit factor Xa activity and to prevent aggregation. Aliquots of PRP were added to tubes with or without 2.5 μmol/L eptifibatide, and 20 μmol/L ADP, 5 μmol/L TRAP-6, or 4 μg/mL collagen was added to initiate aggregation. The samples were rocked or incubated under static conditions ("unstirred"), which did not yield platelet aggregates, and at various times the samples were put on ice and immediately centrifuged at 15 000g for 10 minutes at 4°C. The platelet-poor plasma (PPP) supernatant was removed, and levels of sCD40L were quantified by CD40L ELISA in duplicate. In other experiments, various concentrations of eptifibatide, abciximab, or tirofiban were added to PRP before the addition of TRAP. In eptifibatide experiments, the release of βTG from platelet α-granules was also determined from PPP samples by ELISA. Inhibition of aggregation by GP IIb/IIIa antagonists was determined in PPACK-anticoagulated PRP by light-transmission aggregometry (Chrono-Log) as described previously. To assess the effects of aspirin use on the release of sCD40L from platelets, PRP was obtained from 7 donors, incubated with a single concentration of GP IIb/IIIa inhibitor, and stimulated with 20 μmol/L ADP, 5 μmol/L TRAP, or 4 μg/mL collagen for 30 minutes while rocking at 37°C. Resultant PPP was measured for sCD40L. The same donors then took 325 mg of aspirin for 7 days. PRP was again collected and subjected to the same conditions, and the release of sCD40L was determined. A control reading (no addition of inhibitor) was taken with the agonist 0.5 mmol/L arachidonic acid in citrated PRP to ensure that aspirin had been taken (data not shown).

**Results**

Eptifibatide, a GP IIb/IIIa antagonist, inhibited the hydrolysis of CD40L and release of the soluble form of this protein (sCD40L) from stimulated platelets (Figure 1). Western blot analysis of CD40L immunoprecipitates showed that platelets contained the 33-31 kDa doublet form of CD40L as described previously and that this amount was reduced on TRAP-induced platelet aggregation with the appearance of an 18-kDa CD40L band. The 18-kDa band was sCD40L, the soluble form of CD40L that is released from the aggregated platelets and seen in the platelet supernatant fractions but not in the supernatant from unstimulated platelets.

Multiple platelet agonists, including collagen (Figure 2C), TRAP (Figure 2B and as previously demonstrated), and, to a lesser extent, ADP (Figure 2A), induced the appearance of...
sCD40L protein from aggregated platelets. Maximal CD40L release occurred at 30 minutes for TRAP and ADP (average 2.5 and 1.0 ng/10^8 platelets, respectively) and 45 minutes for collagen (average 4 ng/10^8 platelets). The release of sCD40L from platelets was somewhat slower than the time required for maximal platelet aggregation, which occurred at ~1 to 2 minutes for ADP and TRAP and ~5 minutes for collagen-induced aggregation (data not shown). The inclusion of clinically relevant doses of eptifibatide (2.5 μmol/L) inhibited the release of sCD40L from aggregated platelets by >80% for ADP and TRAP (Figure 2A and B) and 60% for collagen-induced aggregation (Figure 2C).

In Figure 2B, TRAP-activated platelets samples were also incubated under conditions that did not allow for aggregation (unstirred). With these conditions, platelet activation by TRAP occurs as a consequence of inside-out signaling through the thrombin receptor, and events such as α-granule release are not typically affected by GP IIb/IIIa inhibition. sCD40L was indeed shed on platelet inside-out signaling; however, it was unexpectedly inhibited by eptifibatide. The data show that platelet aggregation, and platelet stimulation in the absence of aggregation, caused a time-dependent release of CD40L from platelets, and both events were inhibited by eptifibatide.

It has been shown that sCD40L directly binds to GP IIb/IIIa. Because of this, we compared the ability of GP IIb/IIIa antagonists to inhibit aggregation versus the shedding of sCD40L. Figure 3 illustrates that although the inhibition of platelet aggregation was linear with increasing concentrations of GP IIb/IIIa antagonists, the inhibition of sCD40L did not follow the same pattern. Levels of GP IIb/IIIa antagonist that afford suboptimal platelet aggregation inhibition (eg, <50%) unexpectedly potentiated the release of sCD40L from TRAP-activated platelets. GP IIb/IIIa antagonists caused a 19% to 26% maximal potentiation of sCD40L release from TRAP-stimulated platelets (Table). The release of βTG was secreted from stimulated platelets without the requirement for platelet aggregation; however, this secretion event was not inhibited or potentiated by GP IIb/IIIa inhibition (Figure 3A, inset). All concentrations of GP IIb/IIIa antagonists added to unstimulated platelets had no effect on the release of sCD40L (data not shown).

Aspirin is routinely used in conjunction with GP IIb/IIIa inhibitor therapy and has been shown to modulate the collagen-induced aggregation response by inhibiting the production of thromboxane (TXA2), a costimulator of platelets. Therefore, we examined the effect of aspirin on the release of sCD40L. Platelets were obtained from volunteer donors before and after 7 days of aspirin. Aspirin had no effect on platelet aggregation in response to optimal doses of platelet agonists (data not shown), and Figure 4 shows that the release of sCD40L from platelets stimulated with ADP or TRAP was unaffected by aspirin. In contrast, aspirin use resulted in a 50% decrease in the release of sCD40L from collagen-induced platelet aggregates (P=0.001), which indicates that in this case, TXA2 is a necessary costimulator of platelets. Although all doses of GP IIb/IIIa antagonist partially inhibited the release of sCD40L from ADP- and...
TRAP-stimulated platelets, aspirin addition had no additive effect. In contrast, Figure 4 illustrates that simultaneous GP IIb/IIIa inhibition augmented the inhibitory effects of aspirin by further inhibiting the release of sCD40L from collagen-aggregated platelets.

Discussion

The present study demonstrates that the currently available GP IIb/IIIa antagonists inhibit the cleavage and release of CD40L when used at concentrations that block platelet aggregation by >80%. This inhibition appears to be as robust in reactions that involve platelet aggregation as in those that involve only platelet stimulation in the absence of aggregation. Thus, GP IIb/IIIa antagonists are effective in blocking sCD40L release from stimulated platelets in suspension, a novel activity for GP IIb/IIIa antagonists. Aspirin was also found to inhibit sCD40L release, but only in response to collagen, a platelet agonist dependent on TXA2 production. GP IIb/IIIa antagonists enhanced the inhibitory activity of aspirin. These studies suggest that the clinical benefits of GP IIb/IIIa antagonists, and to a lesser extent aspirin, may be derived not only from their direct inhibition of platelet aggregation but also from their inhibition of inflammation and thrombosis through their blockade of sCD40L release.

The linkage of CD40L to GP IIb/IIIa has been surprising. An initial study showed that sCD40L binds directly to GP IIb/IIIa and is responsible for the formation of stable arterial thrombosis. Aspiration of CD40L knockout mice were found

Figure 2. Kinetics of sCD40L release from platelets stimulated by multiple agonists and inhibition by eptifibatide. PRP stimulated with 20 μmol/L ADP (A), 5 μmol/L TRAP (B), or 4 μg/mL collagen (C) was incubated at 37°C while being stirred on rocking device (solid lines) or under static conditions (B, unstirred, dashed lines) with or without 2.5 μmol/L eptifibatide. At various times, platelets were removed by centrifugation and released sCD40L was measured. Data shown are 1 of 3 experiments, each done in duplicate (±SD).

Figure 3. GP IIb/IIIa antagonists inhibit and potentiate release of sCD40L from TRAP-stimulated platelets. PRP was incubated with various concentrations of eptifibatide (A), abciximab (B), or tirofiban (C) and stimulated with 5 μmol/L TRAP while being stirred for 30 minutes. Platelets were removed by centrifugation and released sCD40L was measured (solid lines). Inhibition of TRAP-induced platelet aggregation was also determined from the same donor (dotted lines). Data are expressed as percent of sCD40L released in absence of inhibitor. βTG release (A, inset) from platelets was also measured.
to be defective in generating stable arterial thrombi, and infusion of sCD40L corrected this deficiency. This activity of sCD40L was mediated by the direct binding of the KGD domain of sCD40L to GP IIb/IIIa. We now report that GP IIb/IIIa antagonists are capable of both inhibiting and potentiating the release of sCD40L from stimulated platelets, effects that are entirely dependent on dose. In considering how GP IIb/IIIa antagonists and aspirin have these effects, one must include observations showing that platelet stimulation is required for the release of sCD40L and that the release is slow, requiring 30 to 60 minutes for completion, lagging considerably behind other stimulatory and aggregation reactions of platelets, which usually are complete within a few minutes. This requirement for platelet stimulation appears to be involved in the mechanism for aspirin inhibition of collagen-induced sCD40L release, because TXA2 production is required for full collagen-induced platelet stimulation.23 GP IIb/IIIa antagonists, however, do not inhibit platelet stimulation induced by platelet agonists.22 The cleavage and release of sCD40L may involve an intermediate step, such as activation of the CD40L protease or activation of the substrate, CD40L. Precedent exists for the interaction of β3-integrins with metalloproteinases,24 the class of proteinases implicated in the shedding of ligands in the tumor necrosis factor family.25 Precedent also exists for the interaction of the KGD motif of CD40L with GP IIb/IIIa.26 It remains for future studies to determine whether either of these interactions is involved in the release of sCD40L from activated platelets and the mechanism by which GP IIb/IIIa antagonists are involved in this process. To the best of our knowledge, this is the first platelet activation signaling event to be identified that is inhibited by a GP IIb/IIIa antagonist.

Inflammation is now known to initiate and/or mediate the progression of atherosclerotic disease, and CD40L is increasingly recognized in this process. This was initially established in mouse models engineered for accelerated atherosclerosis, where disruption of CD40L function by administration of a blocking CD40L antibody27 or targeting of the CD40L gene greatly inhibited lesion progression.28 CD40-CD40L interaction is also involved in plaque stability, most likely because of the CD40L-induced release of matrix metalloproteinases.29,30 Elevated levels of sCD40L are found in patients experiencing vascular inflammation (for example, in acute coronary syndromes,14,15 peripheral arterial occlusive disease,31 and cardiopulmonary and bypass surgery16). Population studies have established that elevated plasma levels of sCD40L are a risk factor for future cardiovascular events.3 This background, it is intriguing that antplatelet agents inhibit the release of sCD40L. In addition to the effects of GP IIb/IIIa antagonists and aspirin described herein, clopidogrel, another commonly used antplatelet drug that inhibits the P2Y12 ADP receptor,32 blocks sCD40L release in response to ADP.33 Future studies are required to determine whether any of the clinical benefits provided by antplatelet agents are mediated by the prevention of sCD40L release or whether such benefits are provided solely by inhibition of platelet aggregation and thrombosis. Recent data showed that abciximab blocks the increased production of CRP that occurs during percutaneous interventions.34 Although this activity was attributed to the cross-reactivity of abciximab with αMβ2, a leukocyte integrin, the current data support an alternative explanation, ie, the inhibition of sCD40L release by a GP IIb/IIIa mechanism.

The data provided herein show that concentrations of GP IIb/IIIa antagonists that give less than robust platelet aggregation inhibition actually potentiate the release of sCD40L. This suggests that a thrombotic event occurring during the use of suboptimal levels of a GP IIb/IIIa antagonist may actually enhance the levels of sCD40L. Orally available GP IIb/IIIa antagonists, because of their pharmacokinetic properties, are typically administered at suboptimal doses to prevent nuisance bleeding. It remains to be determined whether these suboptimal doses caused increased generation of sCD40L in trials of these drugs and contributed toward the observed trend of a negative benefit.35

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