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Platelet endothelial cell adhesion molecule-1 signaling inhibits the activation of human platelets

Milenko Cicmil, Joanne M. Thomas, Mireille Leduc, Cassian Bon and Jonathan M. Gibbins
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Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is a 130-kd transmembrane glycoprotein and a member of the growing family of receptors with immunoreceptor tyrosine-based inhibitory motifs (ITIMs). PECAM-1 is expressed on platelets, certain T cells, monocytes, neutrophils, and vascular endothelial cells and is involved in a range of cellular processes, though the role of PECAM-1 in platelets is unclear. Cross-linking of PECAM-1 results in phosphorylation of the ITIM allowing the recruitment of signaling proteins that bind by way of Src-homology domain 2 interactions. Proteins that have been implicated in the negative regulation of cellular activation by ITIM-bearing receptors include the tyrosine phosphatases SHP-1 and SHP-2. Tyrosine phosphorylation of immunoreceptor tyrosine-based activatory motif (ITAM)–bearing receptors such as the collagen receptor GPVI-Fc receptor γ-chain complex on platelets leads to activation. Increasing evidence suggests that ITIM- and ITAM-containing receptors may act antagonistically when expressed on the same cell. In this study it is demonstrated that cross-linking PECAM-1 inhibits the aggregation and secretion of platelets in response to collagen and the GPVI-selective agonist convulxin. In these experiments thrombin-mediated platelet aggregation and secretion were also reduced, albeit to a lesser degree than for collagen, suggesting that PECAM-1 function may not be restricted to the inhibition of ITAM-containing receptor pathways. PECAM-1 activation also inhibited platelet protein tyrosine phosphorylation stimulated by convulxin and thrombin; this was accompanied by inhibition of the mobilization of calcium from intracellular stores. These data suggest that PECAM-1 may play a role in the regulation of platelet function in vivo.

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Introduction

Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) is a 130-kd membrane-spanning glycoprotein whose expression is restricted to several hematopoietic cell types including platelets, monocytes, neutrophils, certain T lymphocytes, and vascular endothelial cells. The functions of PECAM-1 are diverse and include angiogenesis, vasculogenesis, integrin regulation, trans-endothelial migration of leukocytes, and T- and B-cell antigen receptor function, though the role of this molecule in platelets is unclear. When PECAM-1 was cloned, it was assigned to the family of cell adhesion molecules on the basis of structural similarities. PECAM-1 is involved in adhesion, though much attention has been directed recently to studying its ability to participate in signal transduction. The cytoplasmic tail of PECAM-1 contains a conserved motif called an immunoreceptor tyrosine-based inhibitory motif (ITIM), which underlies its signaling properties and is shared by a growing family of inhibitory receptors. These include immunoglobulin G (IgG) receptor FcγRIIB, killer inhibitory receptors, and signal regulatory proteins, but PECAM-1 is the only ITIM-bearing receptor reported to be expressed in platelets. Therefore, it has been proposed that PECAM-1 be assigned to the immunoglobulin–ITIM family of receptors. The ligand-binding properties of PECAM-1 are complex. It has the capacity for homophilic interactions and heterophilic interactions with a number of molecules that include integrin αβ, and CD38. PECAM-1 becomes phosphorylated on tyrosine residues in response to a variety of stimuli that include PECAM-1 cross-linking, activation of the high-affinity receptor for IgE (FcεRI), shear stress, and oxidative stress. Furthermore, we have recently reported that platelet activation through the collagen receptor glycoprotein VI (GPVI) and through thrombin receptors results in PECAM-1 tyrosine phosphorylation and that this is not dependent on platelet aggregation and secretion, though tyrosine phosphorylation is enhanced by aggregation. The tyrosine residues that become phosphorylated in PECAM-1 have been mapped and fall within the ITIM. Phosphorylated ITIMs recruit signaling molecules, such as the tyrosine phosphatases SHP-1 and SHP-2, which bind to the motif through Src-homology 2 domain interactions. Indeed, both SHP-1 and SHP-2 have been shown to associate with tyrosine-phosphorylated PECAM-1 and phosphotyrosine phosphatases activate these phosphatases in vitro. Generally, these protein tyrosine phosphatases inhibit growth factor receptor signaling. Immune receptor tyrosine-based activatory motif (ITAM)–bearing receptors have a critical role in the regulation of platelet function. Indeed, the collagen receptor GPVI-FcR γ-chain complex signals through an ITAM on the cytoplasmic tail of the FcR γ-chain. Several studies in other cell systems have...
provided evidence of an antagonistic relationship between ITAM- and ITIM-containing receptors when they are expressed on the same cell. An example of this is the receptor for IgG FcγRIIB (ITIM), which negatively regulates cell activation stimulated by FcγRIIA (ITAM).34

In this study we examined the effect of PECAM-1 signaling on the activation of human platelets. We demonstrate that the activation of PECAM-1 signaling by antibody-mediated cross-linking results in the inhibition of collagen-mediated activation; similar results were obtained using a GPVI-selective agonist convulxin (Cvx). Furthermore, we present evidence to indicate that the inhibitory functions of PECAM-1 may not be restricted to the inhibition of ITAM-containing receptor signaling pathways because thrombin-stimulated activation was also inhibited. Inhibition of platelet activation is accompanied by a concomitant inhibition of platelet protein tyrosine phosphorylation and decreased levels of calcium mobilization from intracellular stores. Our data support the notion that PECAM-1 signaling may be involved in the regulation of platelet function in vivo.

Materials and methods

Materials

Horn-Chemie collagen (collagen fibers from equine tendons) was purchased from Nycomed (Munich, Germany). Cvx was purified from the venom of the rattlesnake (Crotalus durissus terrificus), as described previously.25 Thrombomxane mimetic U46619 and adenosine diphosphate (ADP) was purchased from Sigma (Poole, United Kingdom); and monoclonal antibody PECAM-1, PECAM-1 (C-20) and monoclonal antibody AB468, from Autogen Bio-Sciences, United Kingdom. Anti-PECAM-1 antibodies were obtained as follows: monoclonal antibody HC1/6 (mAb (4G10) was obtained from Upstate Biotechnology (TCS Biologicals, Buckinghamshire, United Kingdom). Anti-PECAM-1 antibodies were obtained as follows: monoclonal antibody HC1/6, from Serotec (Oxford, United Kingdom); polyclonal anti-PECAM-1 (C-20) and monoclonal antibody AB468, from Autogen Bio-Sciences, Wiltshire, United Kingdom; and monoclonal antibody PECAM-1.3, kindly provided by Professor Peter Newman (The Blood Center of Southeastern Wisconsin, Milwaukee). Control mouse IgG1 was purchased from Sigma (Poole, United Kingdom). Monoclonal antibody IV.3 was generated by pepsin digestion using reagents purchased from Pierce (Perbio Science, Chester, United Kingdom). Horseradish peroxidase-conjugated secondary antibodies were diluted at 1:10 000.

Preparation and stimulation of platelets

Human platelets from drug-free donors were prepared on the day of the experiment by differential centrifugation, as described previously, and suspended in modified Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 20 mM HEPES, 5 mM glucose, 1 mM MgCl2, pH 7.3) to a density of 2 × 10^10 cells/mL. For protein precipitation experiments, platelets were resuspended at 8 × 10^9 cells/mL in buffer containing 1 mM EGTA to prevent aggregation. Stimulation of platelets (450 μL) with collagen, Cvx, and thrombin (delivered in 50 μL) was performed at 37°C in an optical platelet aggregometer (Chrono-log, Havertown, PA) with continuous stirring (1200 rpm). PECAM-1 activation was stimulated by incubation with anti-PECAM-1 antibodies (AB468, 1 μg/mL or PECAM 1.3, 10 μg/mL (μg/mL)) for 5 minutes, followed by incubation for 90 seconds with F(ab')2 fragments of anti-IgG secondary antibodies (30 μg/mL) to cross-link. Monoclonal antibody AB468 was generated against full-length PECAM-1 ectodomain, and the precise binding epitope has not been mapped. The binding epitope of PECAM 1.3 has been mapped to immunoglobulin domain 1.12 Control experiments were performed using an irrelevant isotype-matched antibody in place of AB468 or PECAM 1.3 and were used at the same concentration. In some experiments, the low-affinity receptor for IgG was blocked by incubation with a saturating concentration (1 μg/mL) of F(ab')2 fragments of mAb IV.3 for 5 minutes. Saturating concentrations of mAb IV.3 antibody were established by determining the concentration of F(ab')2 fragment that completely inhibited subsequent whole IgG-mediated FcγRIIA cross-linking and platelet activation (as described previously). Platelet aggregation was determined by optical aggregometry.

Immunoprecipitation studies

Platelet stimulation was terminated by the addition of an equal volume of ice-cold lysis buffer (2% (vol/vol) Nonidet P40, 20 mM Tris, 300 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 10 μg/mL leupeptin, 10 μg/mL aprozin, 1 μg/mL pepstatin A, pH 7.5). Detergent-insoluble debris was removed, and the lysates were preclared by mixing with protein A-Sepharose for 1 hour at 4°C (20 μL of a 50% (wt/vol) suspension of protein A–Sepharose in Tris-buffered saline–Tween (TBS-T; 20 mM Tris, 137 mM NaCl, 0.1% (vol/vol) Tween 20, pH 7.6)). Protein A–Sepharose was removed from the lysates before the addition of anti–PECAM-1 antibody (HC1/6, 1 μg). After rotation at 4°C for 1 hour, 0.5 μL secondary antisera was added (rabbit anti–mouse IgG) and mixed for a further 30 minutes. Protein A–Sepharose suspension (25 μL) was added to each sample, and mixing continued for 1 hour before the Sepharose pellet was washed in lysis buffer; this was followed by a wash with TBS-T and by the addition of Laemml soluble treatment buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using 10% gels and were transferred to polyvinylidene difluoride membranes by semidy Western blotting.

Immunoblotting

Membranes were blocked by incubation in 10% (wt/vol) bovine serum albumin dissolved in TBS-T. Primary and secondary antibodies were diluted in TBS-T containing 2% (wt/vol) bovine serum albumin and were incubated with membranes for 1 hour at room temperature. Blots were washed for 2 hours in TBS-T after incubation with antibodies for 1 hour at room temperature and were developed using an enhanced chemiluminescence detection system. Primary antibodies were used at a concentration of 1 μg/mL (anti-phosphotyrosine; 4G10; anti-PECAM-1, C-20), and horseradish peroxidase–conjugated secondary antibodies were diluted at 1:10 000.

5-Hydroxytryptamine secretion assay

Platelets were loaded with [3H]5-hydroxytryptamine (5-HT) by incubation with 0.5 μCi/mL (18.5 kBq) platelet-rich plasma for 1 hour at 37°C. Platelets were prepared from the platelet-rich plasma as described above. Stimulation of platelets was terminated by the addition of an equal volume of 6% glutaraldehyde and microcentrifugation, and the level of [3H]5-HT release into the supernatant was determined by scintillation spectrometry. [3H]5-HT release was expressed as a percentage of the total tissue content after subtraction of release under basal conditions.

Measurement of [Ca2+]i by spectrofluorimetry

Washed human platelets (prepared as above) were incubated at 2 × 10^9 cells/mL in calcium-free Tyrode HEPES buffer with 3 μM Fura-2 am for 45 minutes. Platelets were washed once and resuspended at 2 × 10^9 cells/mL in modified Tyrode HEPES buffer. Stimulation of platelets (450 μL) in the presence of 2 mM EGTA with Cvx and thrombin (delivered in 50 μL) was performed with constant stirring at 37°C in a luminescence spectrophotometer (LS-50B; Perkin-Elmer) with excitation wavelengths of 340 nm and 380 nm. Fluorescence emission was measured at a wavelength of 510 nm. Where required, PECAM-1 was cross-linked before stimulation with agonist as described above. The ratio of emission values (excitation at 340/380 nm) was calculated and converted to calcium concentration.
using FLWinLab software (Perkin-Elmer) using the equation 
\[ [Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times SFB, \]
where \( R \) is emission ratio value (340/380 nm). \( R_{max} \), the maximum 340/380 ratio, was determined by lysing platelets with 25 \( \mu \)M digitonin in the presence of 1 mM CaCl_2. The \( R_{min} \) 340/380 nm ratio was obtained by adding 2 mM EGTA. \( K_d \) was the dissociation constant of the Fura-2/Ca^{2+} complex (224 nM), and SFB was the fluorescence ratio at 340/380 nm \( R_{min} \) and \( R_{max} \).

**Statistical analysis**

Determination of statistical significance was performed using the Student paired \( t \) test. Results are expressed as mean ± SEM.

**Results**

**Cross-linking PECAM-1 inhibits collagen-stimulated platelet aggregation**

An antagonistic relationship has been reported between ITIM- and ITAM-containing receptors when they are expressed in the same cell.\(^{12,13,14}\) Because the platelet collagen receptor GPVI signals through an ITAM on the Fcγ-chain with which it is associated, we investigated the effect of PECAM-1 signaling on platelet activation with collagen. PECAM-1 was activated by incubation with antibodies specific for the ectodomain of PECAM-1 (AB468 (Figure 1) or PECAM1.3, not shown) and was cross-linked with a secondary antibody, (Fab')\(_2\) fragment. Consistent with findings from other reports,\(^{19}\) this resulted in increased tyrosine phosphorylation of the protein and did not result in the stimulation of platelet aggregation (Figure 1A). Tyrosine phosphorylation of PECAM-1 was maintained on cross-linking in the presence of EGTA (1 mM), RGDS peptide (0.5 mM), and \( \gamma \)-chain peptide of fibrinogen (100 \( \mu \)M), added separately or together (not shown). This, together with fact that these experiments were performed on washed platelets, indicates that the tyrosine phosphorylation of PECAM-1 on cross-linking is not dependent on integrin \( \alpha_{IIb}\beta_3 \) engagement. The effect of PECAM-1 cross-linking for 90 seconds before stimulation with collagen was found to have a marked inhibitory effect on collagen-stimulated platelet aggregation. At lower concentrations of collagen (eg, 10 \( \mu \)g/mL), cross-linking of PECAM-1 before agonist addition completely abolished aggregation (data not shown). Figure 1Bii shows the marked inhibitory effect of PECAM-1 cross-linking on a high concentration of collagen (100 \( \mu \)g/mL). The use of an isotype-matched IgG control and cross-linker F(ab')\(_2\) had no effect on PECAM-1 tyrosine phosphorylation (not shown) and collagen-stimulated platelet aggregation (Figure 1Bi). Results are representative of 3 separate experiments. Similar results were obtained using the alternative anti-PECAM-1 antibody, PECAM 1.3. In some experiments, the low-affinity receptor for IgG FcγRIIA was blocked before PECAM-1 cross-linking and agonist stimulation using a saturating concentration of F(ab')\(_2\); fragments of mAb IV.3. The inhibitory effect of PECAM-1 cross-linking was unaltered under these conditions, indicating that the inhibitory effect of PECAM-1 using antibodies was not caused by the activation of FcγRIIA. This result was not surprising given that the activation of FcγRIIA by cross-linking with antibodies results in platelet activation.\(^{15}\)

**PECAM-1 cross-linking inhibits GPVI- and thrombin receptor-mediated platelet aggregation**

Given the marked effect of PECAM-1 signaling on collagen-mediated platelet aggregation, we investigated whether this was restricted to GPVI-mediated signaling. GPVI was stimulated using the selective agonist Cvx, a protein purified from the venom of the rattlesnake, *C. durissus terrificus*. Aggregation stimulated with 15 ng/mL Cvx was completely inhibited by prior activation of PECAM-1 (Figure 2Ai), and a partial inhibitory effect was observed at higher concentrations of Cvx (31.25 and 62.5 ng/mL; not shown and Figure 2Aii, respectively). Similar results were observed on the stimulation of platelets with the G protein–coupled receptor agonist thrombin. Complete inhibition of aggregation at 90-second stimulation was observed at a thrombin concentration of 0.05 U/mL (Figure 2Bi) and a partial effect at 0.1 U/mL (Figure 2Bii). The use of an isotype-matched IgG control and cross-linker F(ab')\(_2\) had no effect on Cvx- or thrombin-stimulated platelet
aggregation (Figure 2Ai-Bi). No inhibitory effect of PECAM-1 activation was observed at higher concentrations of thrombin (e.g., 0.5 and 1 U/mL; data not shown). Results are representative of 5 separate experiments. Similar results were obtained using the alternative anti-PECAM-1 antibody PECAM 1.3 and when FcRIIA was blocked before PECAM-1 cross-linking.

Platelet secretion is inhibited by PECAM-1 signaling

Platelet activation is accompanied by secretion from dense granules. Dense granule secretion was assessed by measuring the release of \(^{3}\)H]-5-HT from preloaded washed platelets. Figure 3 shows the results of experiments to determine the effect of PECAM-1 cross-linking on \(^{3}\)H]-5-HT secretion. A significant reduction in secretion was observed in platelets in which PECAM-1 was activated before stimulation with Cvx (81.9% ± 2.9% to 39.8% ± 4.1%; \(P = 0.02; n = 3\)) or thrombin (70.9% ± 4.6% to 37.0% ± 8.0%; \(P = 0.01; n = 3\)). The use of an isotype-matched IgG control and cross-linker F(ab\(^{2}\)) had no effect on Cvx- or thrombin-stimulated dense granule secretion (not shown). Experiments performed in the presence of mAb IV.3 to block the Fc receptor FcRIIA produced similar results.

PECAM-1 inhibits platelet protein tyrosine phosphorylation

The effect of PECAM-1 cross-linking on GPVI- and thrombin receptor–stimulated signaling was investigated. Platelets were stimulated with Cvx (15 ng/mL) or thrombin (0.05 U/mL) with or without prior cross-linking of PECAM-1 for 90 seconds. Whole-cell protein tyrosine phosphorylation levels were determined by immunoblotting. Cross-linking PECAM-1 alone had no effect on basal platelet tyrosine phosphorylation levels (Figure 4). Stimulation with Cvx (15 ng/mL) or thrombin (0.05 U/mL) caused an increase in the level of tyrosine phosphorylation of a broad range of proteins, consistent with previous reports.\(^{32,37}\) In samples in which PECAM-1 signaling was stimulated by cross-linking before incubation with Cvx or thrombin, total tyrosine phosphorylation was reduced (Figure 4). The use of an isotype-matched IgG control and cross-linker F(ab\(^{2}\)) had no detectable effect on Cvx- or thrombin-stimulated total tyrosine phosphorylation levels.

PECAM-1 inhibits the mobilization of calcium from intracellular stores

Stimulation of the collagen receptor GPVI and thrombin receptors leads to rapid intracellular mobilization of calcium, an effect that is essential for secretion and aggregation.\(^{38}\) Intracellular calcium
Discussion

Rapid and complete activation of platelets at sites of tissue damage is ensured through numerous positive feedback pathways, mainly through the actions of mediators such as thromboxane A2 and ADP that are released from activated platelets. The existence of such a rapid and reactive system emphasizes the need for effective regulation of platelet function to prevent disorders such as thrombosis and hemorrhage. Platelet reactivity is a controlled balance between positive and negative regulatory factors and signaling mechanisms. Much attention has recently been focused on the identification of the receptors and signaling pathways that lead to platelet activation, particularly on exposure to collagen, thrombin, and ADP. Negative regulatory mechanisms in platelets are less well understood. The effects of endothelium-derived prostacyclin and nitric oxide are recognized for their roles in inhibiting platelet activation in healthy blood vessels. These molecules inhibit activation through cyclic adenosine monophosphate- and cyclic guanosine monophosphate-dependent signaling mechanisms, respectively. We report here on a third negative regulation system that is mediated through a cell-surface ITIM-bearing adhesion receptor, PECAM-1.

Platelet collagen receptor GPVI signals through an ITAM present on the cytoplasmic tail of the FcRγ-chain with which it is associated. The basis for the hypothesis that PECAM-1 may be a negative regulator of platelet activation was the presence of an ITIM because in some contexts ITAM- and ITIM-containing receptors have been reported to function in an antagonistic fashion. Indeed, the activation of PECAM-1 by cross-linking has been reported to inhibit signaling by T-cell and B-cell antigen receptors, each of which signals through an ITAM. We have reported previously that activation of GPVI or thrombin receptors on platelets leads to tyrosine phosphorylation of PECAM-1 in an integrin-independent manner, and other reports suggest that thrombin receptor activatory peptide-mediated PECAM-1 tyrosine phosphorylation is enhanced by integrin engagement. In addition, platelet activation with Cvx or thrombin results in increased cell surface exposure of PECAM-1. If PECAM-1 signaling is inhibitory in platelets, this may form part of a negative feedback pathway.

PECAM-1 was stimulated through cross-linking using antibodies directed to the extracellular domain of the receptor. This strategy was chosen as the most specific manner through which to activate PECAM-1, and activation was confirmed because cross-linking stimulated its tyrosine phosphorylation. Confusion surrounds the natural endogenous ligand for PECAM-1 on platelets in vivo as described earlier, PECAM-1 has been shown to participate in homophilic and heterophilic ligand binding. One would anticipate that results similar to those described here would be obtained using the recombinant extracellular domain of PECAM-1 as ligand; however, results of such experiments may be complicated by the ability of PECAM-1 to bind to other cell surface proteins. As has been reported previously, PECAM-1 cross-linking stimulates tyrosine phosphorylation (Figure 1) and association of SHP-2 (not shown) but did not in itself cause platelet activation. Furthermore, tyrosine phosphorylation of PECAM-1 on cross-linking appeared not to be dependent on integrin αIIbβ3 engagement, though we cannot rule out the possibility that in vivo it may enhance this effect. Cross-linking PECAM-1 for 90 seconds before stimulation with collagen caused the inhibition of platelet aggregation. At
lower concentrations of collagen, aggregation was inhibited completely, but even at high concentrations of collagen (100 μg/mL) PECAM-1 activation caused a substantial inhibition of aggregation. Because collagen is also able to bind other receptors on the platelet, including the integrin αIIbβ3, we examined the effect of PECAM-1 cross-linking on GPVI-mediated platelet aggregation using the specific agonist Cvx. Similar results were obtained, with complete inhibition of aggregation at lower concentrations of agonist (15 ng/mL) and partial effects at higher concentrations (62.5 ng/mL). This suggests that PECAM-1 is a potent inhibitor of GPVI-mediated (ITAM) platelet activation. This is consistent with the findings of Patil et al., who have recently reported that PECAM-1–deficient mouse platelets are hyperresponsive to collagen and display enhanced collagen adhesion.

Experiments were conducted to determine whether the inhibitory effect of PECAM-1 is restricted to signaling through ITAM-containing receptors. On the contrary, PECAM-1 cross-linking was also found to inhibit thrombin-stimulated platelet aggregation. It is not possible to compare directly the potency of 2 different agonists that mediate their effects through different receptors and signaling pathways. However, no inhibitory effect of PECAM-1 was reported recently that PECAM-1–deficient platelets are hyperresponsive to collagen and collagen-related peptide (CRP), but not to thrombin. In assays of platelet aggregation and secretion, hyperresponsiveness was almost completely lost at high concentrations of collagen and CRP (10 μg/mL and 5 μg/mL, respectively). The concentrations of thrombin used in their experiments were 0.1
PECAM-1 inhibits the activation of human platelets

Antigen receptor

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