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MINIMAL REGULATION OF PLATELET ACTIVITY BY PECAM-1

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Abstract

OBJECTIVES—PECAM-1 is a member of the superfamily of immunoglobulins (Ig) and is expressed on platelets at moderate level. PECAM-1 has been reported to have contrasting effects on platelet activation by the collagen receptor GPVI and the integrin, $\alpha_{IIb}\beta_3$, even though both receptors signal through Src-kinase regulation of PLC γ 2. The present study compares the role of PECAM-1 on platelet activation by these two receptors and by the lectin receptor, CLEC-2, which also signals via PLC γ 2.

METHODS & RESULTS—Studies using PECAM-1 knockout-mice and cross-linking of PECAM-1 using specific antibodies demonstrated a minor inhibitory role on platelet responses to the above three receptors and also under some conditions to the G-protein agonist thrombin. The degree of inhibition was considerably less than that produced by PGI₂, which elevates cAMP. There was no significant difference in thrombus formation on collagen in PECAM-1^{-/-} platelets relative to litter-matched controls.

CONCLUSIONS—The very weak inhibitory effect of PECAM-1 on platelet activation relative to that of PGI₂ indicate that the Ig-receptor is not a major regulator of platelet activation.

Keywords

PECAM-1; platelet activation; GPVI; integrin $\alpha_{IIb}\beta_3$; CLEC-2

INTRODUCTION

Inhibition of platelet activation is an essential component of physiological blood flow, preventing formation of thrombus in healthy vessels and limiting thrombus growth during haemostasis. The two major, direct inhibitors of platelet function are nitric oxide (NO) and prostacyclin (PGI₂) [1]. NO is constitutively released from endothelial cells and has a short half-life in the vasculature of 3-5 seconds which means that the highest concentration of this gaseous transmitter is found in the vicinity of the endothelial cell, a key site of inhibition of platelet function. Its effects are mediated through activation of guanylyl cyclase [2]. This leads to generation of cGMP and activation of protein kinase G (PKG) which in turn inhibits type-III phosphodiesterase resulting in an elevation of cAMP. PGI₂ is synthesized by endothelial cells from arachidonic acid and activates the Gs-coupled seven transmembrane IP-receptor, which regulates adenylyl cyclase resulting in cAMP formation [3]. Activation

of protein kinase A (PKA) by cAMP and PKG by cGMP inhibit platelet activation through several mechanisms including promoting calcium extrusion and inhibition of phospholipase C (PLC) [4-6].

Recent studies have suggested that Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1 or CD31) is also an important regulator of platelet inhibition [7-10]. PECAM-1 is a 130kDa member of the immunoglobulin superfamily that is expressed on the surface of circulating platelets at a moderate level (~10,000 copies/cell) [11-13]. In addition, PECAM-1 is expressed on endothelial cells, monocytes, neutrophils and in humans, naïve subsets of T-cells and B-cells [14, 15]. PECAM-1 has 6 extracellular Ig-domains, a short transmembrane region and a 118 amino acid cytoplasmic tail that contains 2 immunoreceptor tyrosine-based inhibition motifs (ITIMs) at positions 663 and 686 [16]. The amino terminal Ig-domain mediates homophilic binding between PECAM-1 molecules on adjacent cells. PECAM-1 plays a critical role in many processes within the vasculature including transendothelial migration, apoptosis, angiogenesis, anaphylaxis and wound-healing [14, 15, 17-23].

Many of the actions of PECAM-1 are mediated by upregulation of adhesion through $\beta 1$, $\beta 2$ and $\beta 3$ integrins [17, 24-27]. For example, T-cells treated with bivalent PECAM-1 monoclonal antibodies (mAbs) exhibit increased adherence to plastic wells coated with fibronectin or VCAM-1 [17]. Similarly, ligation of PECAM-1 leads to upregulation of $\beta 2$ integrin function in lymphokine-activated killer cells, monocytes, neutrophils and natural killer cells [18, 24, 25]. Indeed, in initial studies in platelets, a mAb to the 6th Ig-domain of PECAM-1 was observed to augment platelet deposition onto extracellular matrix and to enhance aggregation to ADP and PAF [28].

However more recent studies based on PECAM-1-deficient mice, and with other PECAM-1 antibodies and recombinant PECAM-1 encompassing the six Ig-domains, have provided evidence that the major role of PECAM-1 in platelets is inhibitory [8, 9]. Two studies on PECAM-1 knockout-mice have provided evidence for an inhibitory role for PECAM-1 in aggregation to low but not high concentrations of collagen and the GPVI-specific agonist, collagen-related peptide (CRP) [8, 9]. In addition, a further study on PECAM-1 knockout-mice has provided evidence for a similar inhibitory role of PECAM-1 against platelet spreading and aggregation induced by von Willebrand factor (VWF)-ristocetin, which also signals through the FcR γ -chain [10]. Importantly, thrombus formation on collagen at high shear (1800s^{-1}), but not on VWF at a similar rate of shear (1500s^{-1}), is potentiated in PECAM-1^{-/-} mice. Cross-linking of PECAM-1 using specific antibodies or recombinant PECAM-1 also leads to inhibition of platelet activation by collagen [29]. Interestingly, crosslinking studies using PECAM-1 antibodies have also revealed a weaker inhibitory effect against platelet activation by the G protein-coupled receptor ligands, thrombin and U46619 [29].

Despite the increasing recognition of the inhibitory role of PECAM-1 in platelets, a number of recent observations indicate a more complex picture and have also begun to cast doubt on the molecular basis of the inhibitory action. For example, PECAM-1 has recently been shown to be essential for spreading and to have a major stimulatory role in regulating clot-retraction induced by the major platelet integrin, $\alpha_{IIb}\beta_3$, thereby demonstrating that it also contributes to platelet activation [30]. Furthermore, Wee and Jackson have shown that potentiation of aggregation to CRP in PECAM-1^{-/-} mice is not mediated by an increase in intracellular Ca^{2+} , thereby demonstrating its inhibitory role is not due to inhibition of ITAM regulation of PLC γ [30].

The role of PECAM-1 in the regulation of platelet function in vivo is also less clear in view of a number of contrasting observations and presence of PECAM-1 on endothelial cells. An initial, intravital-microscopy study used a helium-neon laser in the presence of intravascular Evans blue to injure the endothelium of arterioles on the surface of the mouse brain [31]. This study led to the conclusion that exposed PECAM-1 on endothelium promotes platelet adhesion and aggregation. A subsequent intravital study analysing photochemically-induced thrombus in microvessels of the cremaster muscle concluded that PECAM-1 was not critically involved in thrombus formation upon endothelial cell injury [32]. However, more recently it has been reported that thrombi formed in PECAM-1-deficient mice are slightly larger, form more rapidly and are more stable than those in control mice following a heat-induced endothelial injury model which does not lead to collagen exposure [7]. Furthermore, this study used radiation-chimeric mice to demonstrate that this effect was not due to the presence of PECAM-1 on endothelial cells. On the other hand, in the same study, this group demonstrated that PECAM-1 plays a negligible role in a more severe model of thrombus, namely ferric chloride injury of the carotid arteries. Thus, these data point to a growing recognition that the role of PECAM-1 in vivo may vary between vascular beds and with the nature of the injury.

In view of the confusion surrounding the action of PECAM-1 on platelets, the present study was undertaken to compare the role of PECAM-1 in platelet activation by GPVI, integrin $\alpha_{IIb}\beta_3$ and the C-type lectin receptor, CLEC2, which also signals through PLC γ 2 [33]. Importantly studies were performed using both PECAM-1 knockout-mice and crosslinking of PECAM-1 using specific antibodies so as to enable a full comparison to previous studies. The results demonstrate that PECAM-1 has a minor inhibitory effect on platelet activation by tyrosine kinase-linked receptors, which is considerably smaller than of PGI $_2$. Furthermore, at a physiological rate of flow, thrombus formation on collagen is not altered in the absence of PECAM-1, thus questioning its physiological significance.

MATERIALS AND METHODS

Materials and antibodies

The synthetic CRP peptide GCP*(GPP*) $_{10}$ GCP*G (where P* denotes a hydroxyproline amino acid residue) was cross-linked through N- and C-terminal cysteine residues as previously described [34, 35]. Fibrillar-type I Horm collagen from equine tendon was from Nycomed (Axis-Shield, UK). Human fibrinogen, depleted of plasminogen, VWF and fibronectin was from Enzyme Research (Swansea, UK). Rhodocytin was kindly provided by Dr Johannes Eble (Muenster, Germany). Fc γ RIIA specific monoclonal antibody (IV.3) was from Madarex Inc (Annandale, NJ) and F(ab') $_2$ fragments were generated using immobilized pepsin according to manufacturer's instructions. The functional integrity of IV.3 F(ab') $_2$ fragments was confirmed by their inability to induce Fc γ RIIA-receptor-mediated platelet activation after crosslinking using a secondary antibody. PECAM-1 knockout-mice were a gift from Dr Tak Mak (Amgen Institute, Toronto, Ontario, Canada). PECAM-1 $^{-/-}$ mice were housed under pathogen-free conditions at the University of Birmingham Animal SPF facility under National Health and Medical Research Council guidelines and approved animal protocols. PECAM-1 $^{-/-}$ mice were bred from heterozygotes on a C57Bl6 background. The phenotype of the PECAM-1 $^{-/-}$ was confirmed by flow cytometric analysis of leukocytes [21]. Anti-human PECAM1.3 IgG $_1$ mAb was a gift from Prof. Peter Newman (Blood Research Institute, Milwaukee, WI, USA). PECAM-1 mAb AB468 was from Autogen-Bioclear (Wiltshire, UK). Sheep anti-mouse IgG F(ab') $_2$ fragments, a mouse IgG $_1$ isotype control and bovine plasma thrombin were purchased from Sigma (Poole, UK). mAb AB468 and control IgG $_1$ were dialysed prior to experimentation to remove azide. PD0173952 was a gift from Pfizer Global Research & Development (Ann Arbor, MI, USA and Sandwich, UK).

Platelet aggregation studies

Blood was obtained from consenting donors who had not taken any anti-platelet medication within the preceding 2 weeks. To prepare washed platelets, whole blood was anticoagulated with 10% acid-citrate-dextrose (ACD; 80mM citric acid, 120mM sodium citrate, 110mM glucose). The blood was centrifuged at 200 *g* for 20min and the platelet-rich-plasma (PRP) removed. PGI₂ (800nM) was added to the PRP which was then centrifuged at 1000 *g* for 10min to pellet the platelets. The platelet-poor-plasma was decanted and the pellet resuspended in 10ml of the following Ca²⁺-free modified Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 12mM NaHCO₃, 0.34mM Na₂HPO₄, 20mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 5mM glucose, and 1mM MgCl₂, pH 7.3) in the presence of prostacyclin (1μM). The resuspended platelets were centrifuged at 1000 *g*, the supernatant discarded, and the platelets resuspended with the appropriate volume of modified Tyrodes-HEPES buffer to 2×10⁸ platelets/ml. Aggregation studies were performed in siliconized glass cuvettes at 37°C with constant stirring at 1000 rpm in a Born aggregometer. Platelet aggregation was initiated by addition of CRP, collagen, thrombin or rhodocytin and was detected by changes in light transmission. Wild-type C57/BL6 and PECAM-1^{-/-} C57/BL6 litter-matched mice were halothaneasphyxiated and bled by cardiac puncture into 0.1% ACD. Anticoagulated whole blood was then centrifuged at 200 *g* for 5min at room temperature and PRP isolated. Platelets were washed twice using a modified Tyrode-HEPES buffer and resuspended at 2 × 10⁸ /ml.

Platelet Spreading Assay

Washed murine platelets (6 × 10⁷ /ml) were incubated on CRP- or fibrinogen-coated coverslips at 37°C for 45min in a humid environment. Platelet spreading was imaged in real-time using Kohler illuminated Nomarski differential interference contrast optics with a Zeiss 63X oil immersion 1.40 NA axioplan lens on a Zeiss Axiovert 100 Microscope. Time-lapse events were captured by QICAM Mono 10-bit camera (QImaging, Burnaby, BC, Canada) using Openlab software for Macintosh. To compute the surface area of spreading platelets, time-lapse images were manually outlined and quantitated by determining the number of pixels within each outline using a Java plugin for the Image-J software package. Imaging a graticule under the same conditions allowed the conversion of pixels size to microns.

Clot Retraction Assay

Whole murine blood was anti-coagulated with sodium citrate and PRP prepared as above. The platelet count was adjusted to 3 × 10⁸ /ml with Tyrodes-HEPES containing CaCl₂ (2mM) and fibrinogen (2mg/ml). 400μl of the adjusted PRP/Tyrodes-HEPES/Fib/CaCl₂ mix was placed in an aggregometer tube and incubated at 37°C for 5min. 2μl of mouse erythrocytes were added for colour contrast. Thrombin (10U/ml) was added and mixed with a paper-clip and clot retraction was allowed to proceed at 37°C for 2 hours with the paper-clip present. At appropriate time points photographic images of retracting clots were recorded. The clot was pulled out with the paper-clip and the remaining serum volume measured. These experiments were performed blind.

Flow adhesion assay

Mouse blood was taken into 40μM P-PACK with sodium heparin (5U/mL) by cardiac puncture. The blood was perfused through a glass microslide, 1 × 0.1mm inner diameter (Camlab, Cambridge, UK), the luminal surface of which had been coated with 100μg/ml type-I collagen from equine tendon (Horm; Nycomed, Munich, Germany) before blocking with 2% BSA in phosphate-buffered saline. Shear rate of 1800s⁻¹ with perfusion for 3min was generated by a syringe pump (Harvard Apparatus, Southnatick, MA). After perfusion with whole blood, modified Tyrode-HEPES buffer was perfused for 8min at the same shear

rate as the blood. Platelet thrombi that had formed on the surface of the collagen were visualized with an inverted stage videomicroscope system (DM IRB; Leica, Milton Keynes, UK) and surface coverage was analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Subsequently adherent platelets were lysed in ice-cold nonidet P-40 (NP-40) lysis buffer (20 mM Tris [tris(hydroxymethyl)aminomethane], 300mM NaCl, 2mM EGTA, 2mM EDTA, 2% [vol/vol] NP-40, 1mM phenylmethylsulphonyl fluoride, 2mM Na₃VO₄, 10μg/ml leupeptin, 10μg/ml aprotinin, 1μg/ml pepstatin A, pH 7.3) for protein estimation using RC-DC protein assay (Bio-Rad, Hemel Hempsted, UK). These experiments were performed blind.

Cross-Linking Experiments

Washed human platelets (400μl at 2×10^8 /ml) were stimulation with CRP, collagen and thrombin and platelet aggregation was measured with a Born optical platelet aggregometer with continuous stirring (1000 rpm). PECAM-1 was stimulated by incubation with anti-PECAM-1 antibodies, PECAM1.3 or AB468 (1-5μg/ml) for 2min, followed by incubation for 2min with F(ab')₂ fragments of sheep anti-mouse anti-IgG secondary antibodies (30μg/ml) to cross-link. The binding epitope of PECAM1.3 has been mapped to immunoglobulin domain 1 [36]. Control experiments were performed using an equal concentration of mouse IgG₁ isotype-matched control antibody in place of PECAM1.3 or AB468. In all experiments, the low-affinity FcγRIIA receptor for IgG was blocked by incubation with a saturating concentration (1μg/ml) of F(ab')₂ fragments of mAb IV.3 for 2min. Saturating concentrations of mAb IV.3 antibody were established by determining the concentration of F(ab')₂ fragment that completely inhibited subsequent IgG-mediated FcγRIIA crosslinking and platelet activation. Platelet aggregation was determined by optical aggregometry.

Immunoprecipitations and immunoblotting

Platelet stimulation was terminated by the addition of an equal volume of ice-cold lysis buffer. Cell lysates were precleared with protein-A sepharose overnight at 4°C prior to immunoprecipitations. Cell debris was removed by centrifugation for 5min at 13000 *g* at 4°C. Supernatants were collected and PECAM-1 was immunoprecipitated with PECAM1.3 antibody. Control samples were immunoprecipitated with an anti-IgG isotype antibody. Soluble cell lysates and immunoprecipitates were resolved on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer system (Trans-blot SD. BioRad Laboratories, Hertfordshire, UK) and immunoblotted. Proteins were detected by incubating membranes with enhanced chemiluminescence reagents (Amersham Biosciences, Bucks, UK) followed by exposure to hyperfilm MP (Amersham Biosciences, Bucks, UK).

Analysis of Data

All experiments were performed a minimum of 3 times and images shown are representative data from one experiment with data shown as means±SEM. Statistical analysis was conducted using 2-tailed Student unpaired *t* test and statistical significance of differences between means was determined by one-way ANOVA. If means were shown to be significantly different, multiple comparisons by pairs were performed by the *Tukey* test. A chi-squared test was used to determine significance of Mendelian ratios. Probability values of $p < 0.05$ were selected to be statistically significant.

RESULTS

PECAM-1^{-/-} mice birth rate is less than that expected by Mendelian inheritance

We generated PECAM-1^{-/-} mice and litter-matched controls by breeding PECAM-1 heterozygotes on C57B16 mice background. All murine platelet experiments were performed

with littermate-controls generated from breeding C57Bl/6J with PECAM-1^{-/-} generating PECAM-1^{+/-}. Littermates were genotyped by flow cytometry of leukocytes stained with FITC-conjugated rat anti-mouse monoclonal PECAM-1 (MEC13.3) antibody (Figure1a). The frequency of PECAM-1^{-/-} mice was 13 (14%) from 110 births, which was significantly less ($p < 0.01$) than that expected with standard Mendelian inheritance rates (Figure1b). We are not aware of another study which has reported this observation, which may be explained by the critical role of PECAM-1 in capacitation of human spermatozoa prior to fertilization [37].

PECAM-1^{-/-} platelets exhibit a small increase in aggregation to threshold concentrations of GPVI agonists and rhodocytin

To confirm a role of PECAM-1 in platelet function, we examined platelet aggregation in litter-matched wild-type (wt) and PECAM-1^{-/-} platelets to the GPVI-selective agonist, CRP, and to collagen. There was a small potentiation in the magnitude of response to threshold and submaximal concentrations of CRP which began after a delay of 30 sec or more (Figure2a). There was no potentiation in the magnitude or rate of response to higher concentrations of CRP (Figure2a). Furthermore, responses to threshold and intermediate concentrations of collagen (1-3 μ g/ml) were essentially unchanged for the initial 60 sec, although these were then followed by a very minor increase in the extent of aggregation in the absence of PECAM-1 (Figure2b). There was no difference between wt and PECAM-1^{-/-} platelets at a higher concentration of collagen (Figure2b). Mice heterozygous for PECAM-1 expression exhibited near-normal responses to low-dose collagen and CRP (not shown) demonstrating that 50% receptor expression is sufficient to maintain normal function.

We extended our investigation on the role of PECAM-1 in regulating platelet aggregation to agonists which signal through other receptors. The protease thrombin stimulates activation of mouse platelets through the PAR₄ G protein-coupled receptor. Significantly, aggregation to threshold and intermediate concentrations of thrombin was not altered in the absence of PECAM-1 (Figure2c). The snake venom toxin rhodocytin has recently been shown to activate platelets via the lectin receptor, CLEC-2. CLEC-2 signals via Src and Syk family tyrosine-kinases leading to activation of PLC γ 2, but in contrast to GPVI, uses a single YXXL rather than an ITAM sequence to activate Syk [33]. Aggregation of platelets to threshold concentrations of rhodocytin is characterised by a delay of up to several minutes as illustrated in Figure2d. In PECAM-1^{-/-} platelets, the time course of activation by a threshold concentration of rhodocytin (10nM) is significantly reduced and accompanied by a marked potentiation in magnitude of response. Similarly, the delay in activation by a ten-fold higher concentration of rhodocytin (100nM) was also reduced, although there was no increase in magnitude of response. The response to a higher concentration of rhodocytin (1,000nM) was identical in control and PECAM-1^{-/-} platelets.

The present results are in line with those of Patil et al. who reported potentiation of aggregation in response to low concentrations of collagen and CRP but not to thrombin in PECAM-1^{-/-} platelets. However, we have observed a much smaller degree of potentiation, most notably for collagen. In contrast, Jones et al. observed a delay in potentiation of aggregation to a high concentration of collagen (30 μ g/ml) in the absence of PECAM-1, which led to an increase in the overall maximal level of aggregation. In all cases, potentiation was delayed consistent with a feedback action. The present study has also demonstrated potentiation of aggregation to threshold and intermediate concentrations of rhodocytin, which signals through the lectin receptor, CLEC-2. The degree of potentiation was more marked than that observed against CRP for a threshold concentration of rhodocytin, although this may reflect the unusually long delay in response to the snake toxin.

Thus, the present results support a very minor role for PECAM-1 in mediating inhibition after commencement of aggregation induced by agonists which signal through GPVI and lectin receptors.

PECAM-1^{-/-} platelets demonstrate enhanced spreading on CRP and fibrinogen

The major platelet integrin, $\alpha_{IIb}\beta_3$, also regulates PLC γ 2 downstream of Src and Syk tyrosine-kinase, albeit through a pathway that is distinct from those used by GPVI and CLEC-2. Recently, PECAM-1 has been shown to be essential for spreading and to contribute to clot retraction induced by outside-in signalling through $\alpha_{IIb}\beta_3$ [30]. Interestingly, the former result contrasts with the potentiation of spreading observed on botracetin-VWF, which also signals through Src and Syk tyrosine-kinases, leading to activation of PLC γ 2 [10].

To investigate the role of PECAM-1 in 'outside-in' signalling by integrin $\alpha_{IIb}\beta_3$, we investigated spreading of PECAM-1^{-/-} platelets in real-time on fibrinogen, alongside similar studies on CRP, and clot retraction in PRP. Mouse platelets undergo a characteristic series of changes on an immobilised surface of CRP leading to formation of filopodia and lamellipodia. This is exemplified in Figure3a where the majority of platelets have undergone complete spreading by 45min. In contrast, on fibrinogen, the majority of mouse platelets generate filopodia but fail to form distinct lamellipodia. The degree of platelet spreading is potentiated on both surfaces in PECAM-1^{-/-} platelets, albeit to a limited extent and after a delay. This effect is particularly mild on fibrinogen, with a significant difference only observed after 45min (Figure3bi-ii). Interestingly, there was no difference in spreading between control and PECAM-1^{-/-} platelets on fibrinogen in the presence of G protein-coupled receptor agonist, thrombin (Figure3biii). These results therefore demonstrate that PECAM-1 negatively regulates spreading on fibrinogen, although this effect is masked when platelets are stimulated by a powerful G-protein receptor agonist. Further the magnitude of potentiation is relatively minor. These results are in contrast to a recent study which has reported that PECAM-1 is essential for platelet spreading on fibrinogen [30].

The role of PECAM-1 in clot retraction was investigated in PRP stimulated by thrombin (10U/ml) over a course of two hours as illustrated in Figure3ci. Quantitation of the remaining serum volume after this period 2 hours revealed no significant difference relative to litter-matched controls (Figure3ci). In contrast, a Src family kinase inhibitor, PD0173952, that is active in plasma, caused a small, but significant degree of inhibition at 30, 60, 90 and 120 min (Figure3cii and data not shown).

Cross-linking PECAM-1 marginally inhibits platelet aggregation

The above results provide evidence for a very minor feedback role of PECAM-1 in regulating responses to GPVI, integrin $\alpha_{IIb}\beta_3$ and CLEC-2 in platelets. Physiologically, however, the major role of PECAM-1 may not be to mediate feedback activation of platelets, but to prevent activation at appropriate sites in the vasculature. For example, platelet PECAM-1 could undergo a homophilic interaction with endothelial-PECAM-1, where it is expressed at 1 million copies per endothelial cell, leading to inhibition of platelet function [38, 39].

The effect of crosslinking of PECAM-1 on platelet activation was investigated using two anti-PECAM-1 mAbs, PECAM-1.3 and AB468, in combination with a secondary antibody, F(ab')₂ fragment. mAb AB468 was dialysed prior to experimentation to remove azide, which was found to inhibit platelet activation. Experiments were performed in the presence of a F(ab')₂ fragment of mAb IV.3, which blocks the low-affinity immune receptor, Fc γ RIIA, on the platelet surface. Both antibodies induced a marked increase in tyrosine phosphorylation

of PECAM-1, which was similar or slightly less than that induced by thrombin or CRP (Figure4a). On their own, neither had an effect on platelet aggregation.

Crosslinking of PECAM-1 for varying times prior to agonist addition inhibited platelet aggregation induced by relatively low concentrations of CRP (0.03 μ g/ml), thrombin (0.01U/ml) and rhodocytin (10nM) that were just sufficient to induce near maximal platelet aggregation. This is illustrated in Figure4bi for mAb AB468, with a similar set of data obtained with mAb PECAM-1.3 (not shown). Cross-linking of PECAM-1 had little or no inhibitory effect on aggregation to higher concentrations of CRP, thrombin, and rhodocytin (Figure4bii). This is particularly striking for rhodocytin, where the concentration was only increased by 3-fold.

The relatively weak nature of inhibition of platelet aggregation to all three agonists induced by crosslinking of PECAM-1 prompted us to investigate the magnitude of this effect relative to PGI₂, which mediates platelet inhibition via elevation of cAMP. For these experiments, platelets were pre-incubated with varying concentrations of PGI₂ prior to stimulation with CRP and thrombin. As illustrated in Figure5, PGI₂ completely inhibited aggregation to both agonists, with an approximately ten-fold increase in concentration being required to fully block aggregation to the higher concentration of the two ligands. These results therefore demonstrate that PECAM-1 mediates platelet inhibition only to very low concentrations of CRP and thrombin, whereas PGI₂ causes a more profound level of inhibition to higher concentrations of the two stimuli. Importantly, PGI₂ also markedly inhibits aggregation to rhodocytin and spreading on fibrinogen (not shown) thereby demonstrating that it inhibits platelet activation by the major classes of tyrosine kinase-linked receptors.

Thrombus formation is maintained in PECAM-1^{-/-} platelets

The role of PECAM-1 in regulating platelet activation under shear conditions was investigated using collagen-coated microcapillary tubes in a capillary-tube flow chamber at a shear rate of 1800s⁻¹. Blood was perfused for 3min and platelet-rich-aggregates/thrombi imaged after perfusion by DIC microscopy before extraction for protein assay. These conditions were chosen because they are similar to those used by Jones et al. in which a small, potentiatory role of PECAM-1 was recorded [8]. However, in contrast, to this study, we observed a reduction in thrombus formation in the absence of PECAM-1^{-/-} (Figure6a), although this did not reach statistical significance (Figure6b). Similar results were obtained at a lower shear rate of 1000s⁻¹ (data not shown). The lack of effect on thrombus formation, however, is in line with a similar result for thrombus formation on VWF in the presence of botrocetin at a similar rate of shear, 1500s⁻¹. Furthermore, thrombus formation under shear was reduced by approximately 90% in the presence of PGI₂ (Auger & Watson unpublished data).

DISCUSSION

The aim of the present study was to further explore the role of PECAM-1 in platelets using both PECAM-1^{-/-} mice and receptor crosslinking studies, and to extend this work to the lectin-like receptor, CLEC-2, which also mediates platelet activation through PLC γ 2. Studies using PECAM-1^{-/-} mice demonstrate that the Ig-receptor has a minor inhibitory effect against platelet activation induced by receptors that signal through Src and Syk family tyrosine-kinases, namely GPVI, integrin $\alpha_{IIb}\beta_3$ and CLEC-2, but that it has no effect on responses to the G protein-coupled receptor agonist, thrombin. A previous study has also shown that PECAM-1 weakly inhibits platelet activation by GPIb, which also signals through Src and Syk family tyrosine-kinases. The inhibitory effect of PECAM-1 against all of these receptors occurs after an initial delay consistent with a feedback mechanism. On the other hand, crosslinking of PECAM-1 mediates inhibition to low concentrations of both

tyrosine kinase-linked and G protein-coupled receptors, with the inhibitory effect being overcome by higher concentrations of agonists. The relatively weak nature of these inhibitory effects is put into perspective by studies with PGI₂, which activates the G protein-coupled IP-receptor leading to elevation of cAMP. PGI₂ completely inhibited activation by concentrations of CRP and thrombin, which were not altered in the absence of PECAM-1 or following crosslinking of the Ig-receptor. Together, therefore, the present results emphasise that the inhibitory role of PECAM-1 in platelets is extremely mild, thereby questioning its likely physiological relevance. This is consistent with the observation that thrombus formation on collagen at a high rate of shear was not altered in the absence of PECAM-1.

In qualitative terms, many of the present observations (with the exception of the critical role of PECAM-1 in outside-in signalling by integrin $\alpha_{IIb}\beta_3$) are in general agreement with previous reports, but in contrast, the overall conclusion from this study is that PECAM-1 is unlikely to play a major role in regulating platelet activity. This conclusion is based on the very weak nature of the potentiation that has been observed in light of the more powerful inhibition of activation by PGI₂, which elevates cAMP. Furthermore, there is also the possibility that the weak inhibitory effect observed upon crosslinking of PECAM-1 is due to a steric effect in view of the similar inhibition in response to thrombin and the fact that the Ig-receptor is expressed at a very high level on the platelet surface.

To minimise differences within experimental design relative to other published studies, the PECAM-1 knockout mice were bred on the same C57Bl/6 background as used in these studies [8, 9]. Information on the age and sex of the mice used in other studies was not provided [7-9]. We have performed our study using litter-matched mice of equal gender with ages ranging from 8 to 10 weeks. Importantly, however, pilot studies demonstrated no differences between platelet aggregation and spreading between older and younger mice.

The present study extended investigation of the role of PECAM-1 in platelet activation to rhodocytin, which activates the lectin receptor CLEC-2. This receptor has recently been shown to mediate platelet activation through a similar pathway to that used by GPVI, although it requires only a single YXXL motif. Overall, the profile of inhibition of responses to rhodocytin is similar to that of the GPVI-selective agonist, CRP, although the effect is more dramatic, most likely because of the unusually long lag time required for platelet activation by low concentrations of the snake venom toxin. Significantly, however, the response to higher concentrations of rhodocytin are also not altered in the absence of PECAM-1 or upon crosslinking of the receptor. This study has also investigated the effect of PECAM-1 on spreading on fibrinogen and clot retraction, in view of the recent observation that PECAM-1 is essential for filopodia formation downstream of integrin $\alpha_{IIb}\beta_3$ and also plays a major role in mediating clot retraction [30]. We observed, however, weak potentiation in the absence of PECAM-1 of spreading on fibrinogen, which was masked in the presence of thrombin. Furthermore, we observed a slight potentiation rather than inhibition of clot retraction in the absence of PECAM-1. The explanation for these contrasting observations is not known, although it is noteworthy that potentiation of spreading of PECAM-1^{-/-} platelets was also observed in the present study against CRP and previously against VWF-batractin [10]. Thus, overall PECAM-1 weakly inhibits responses to four distinct classes of receptors that signal through Src and Syk family tyrosine-kinases, namely GPVI, GPIb, CLEC-2 and integrin $\alpha_{IIb}\beta_3$. The underlying basis of this inhibition, however, is unclear in view of the recent observation that PECAM-1 does not inhibit Ca²⁺ elevation by the collagen receptor GPVI, thereby demonstrating that it does not interfere with PLC γ activation [30].

In conclusion, the present study has added to the growing recognition that PECAM-1 has a very weak feedback effect against responses to tyrosine kinase-linked receptors, but has

brought into the question the physiological significance of this effect in view of the lack of effect on thrombus formation, especially in light of the powerful inhibitory effect of PGI₂. The functional significance of PECAM-1 in platelets therefore remains unclear.

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Figure 1a above

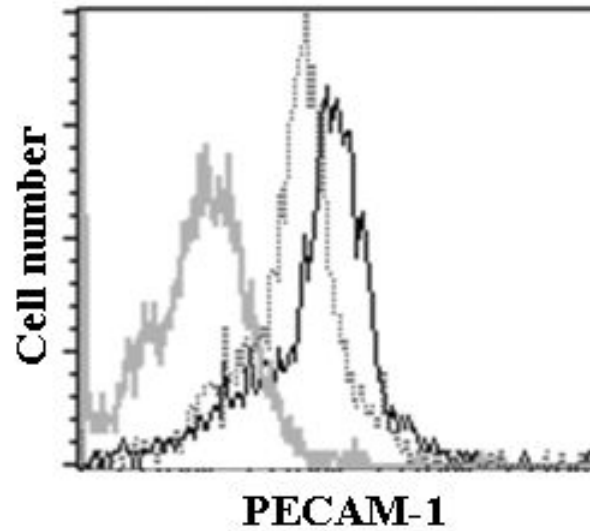
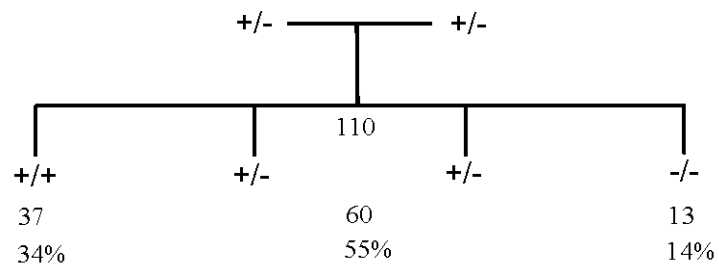


Figure 1b above

**Figure 1.**

Genotyping of PECAM-1^{-/-} mice.

(a) PECAM-1 expression on leukocytes stained with FITC-conjugated rat anti-mouse monoclonal PECAM-1 antibody (MEC13.3) assessed by flow cytometry. PECAM-1^{+/+} (bold line), PECAM-1^{+/-} (dashed line) and PECAM-1^{-/-} (grey line). 10000 viable lymphocytes were gated in each case. (b) Interbreeding heterozygote mice generated knock-out mice with a significant reduction in the expected Mendelian frequency of PECAM-1^{-/-} mice ($p < 0.01$).

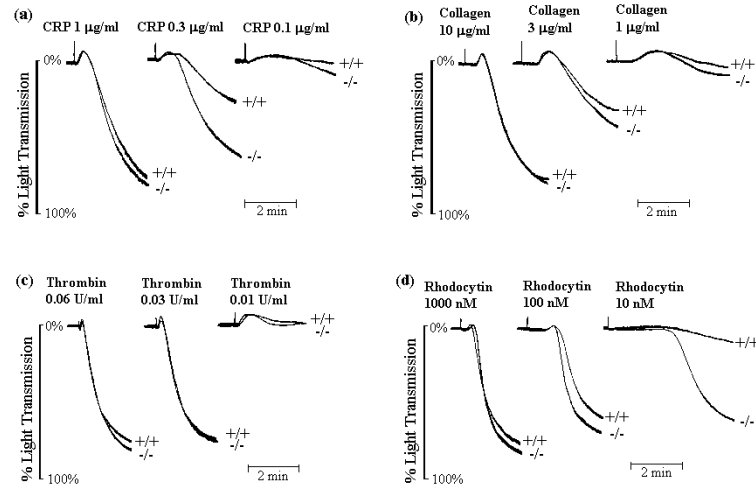


Figure 2. PECAM-1^{-/-} platelets show hyper-aggregation in response to stimulation with low concentrations of CRP and rhodocytin. Platelets from PECAM-1^{-/-} mice and litter-matched controls were exposed to CRP, collagen, thrombin and rhodocytin. Results are representative of 3 separate experiments.

Figure 3a above

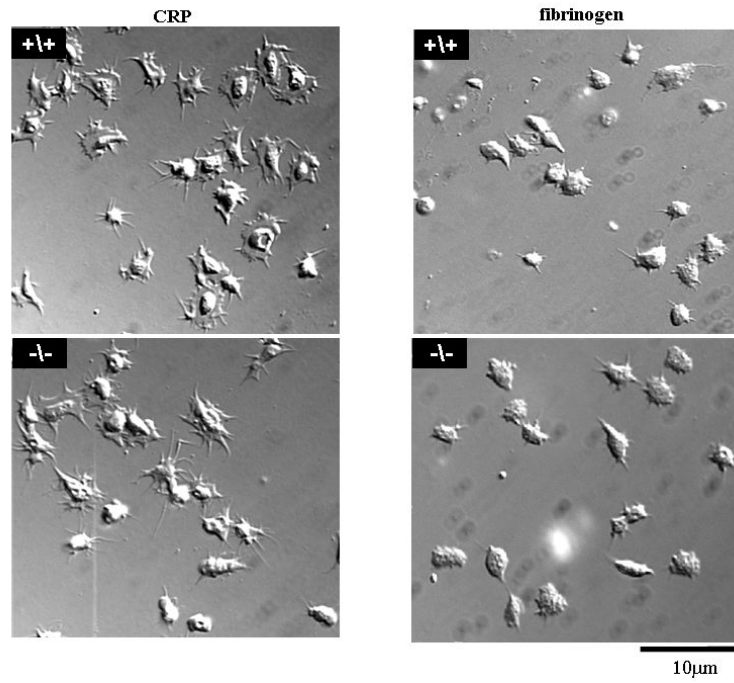


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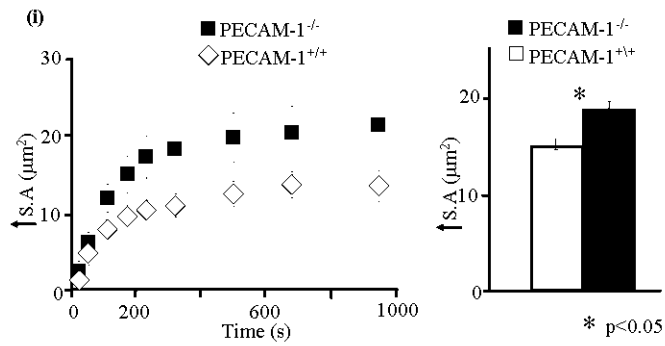


Figure 3bii above

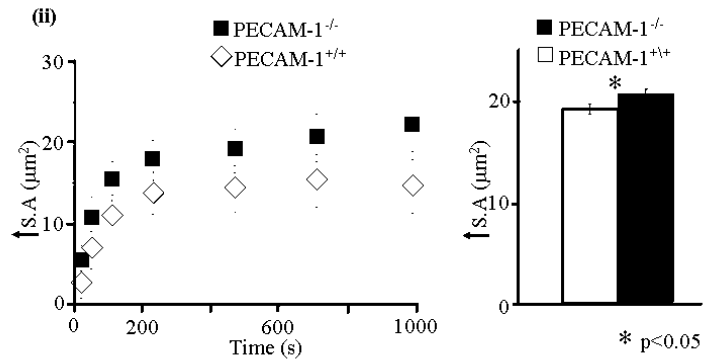


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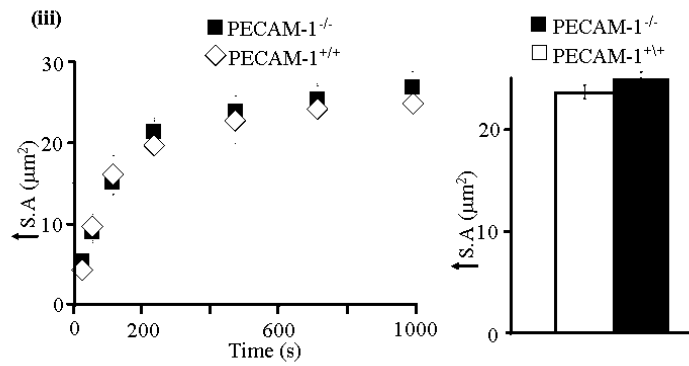


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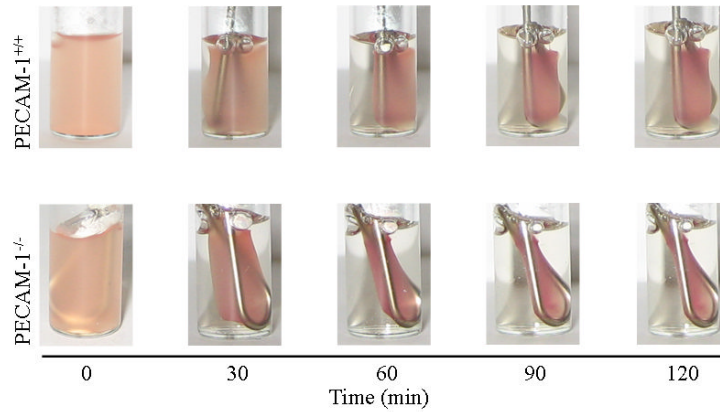
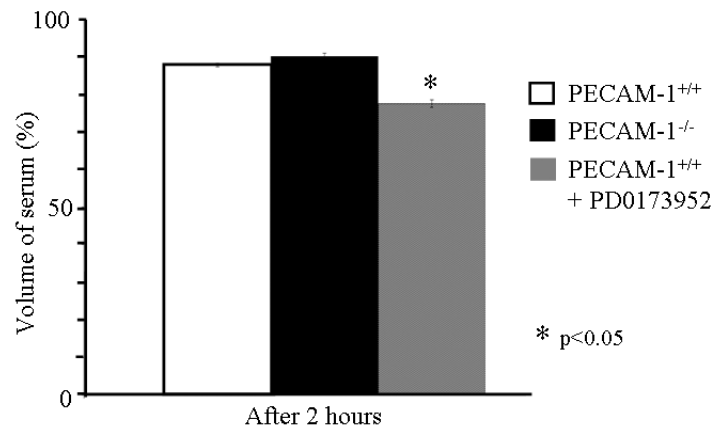


Figure 3cii above**Figure 3.**

Spreading and clot retraction in PECAM-1^{-/-} platelets.

(a) Spreading of PECAM-1^{-/-} and litter-matched control platelets were analysed using DIC microscopy after being allowed to spread on fibrinogen and CRP for 45min. (b) Time course of spreading of PECAM-1^{-/-} and litter-matched control platelets on (i) CRP and (ii) fibrinogen, in the absence and presence of thrombin (iii). Increase in mean surface area (SA) of platelets after 45min; values are shown as mean±SEM of between 115–209 platelets from one experiment that is representative of three; * P < 0.05 relative to the corresponding control. (c) Clot retraction in PECAM-1^{-/-} and litter-matched control platelets: (i) time course of contraction; and (ii) volume of extracted serum after 2 hours (n = 4).

Figure 4a above

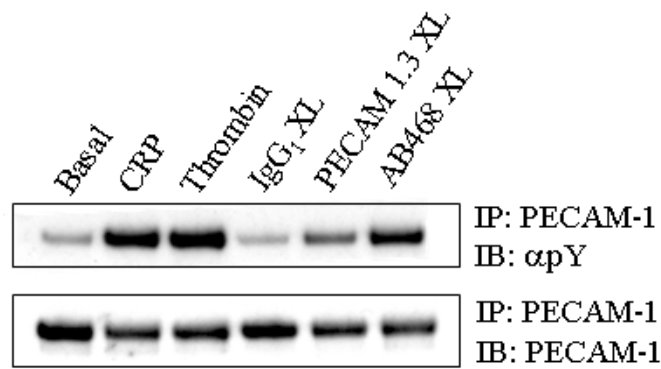


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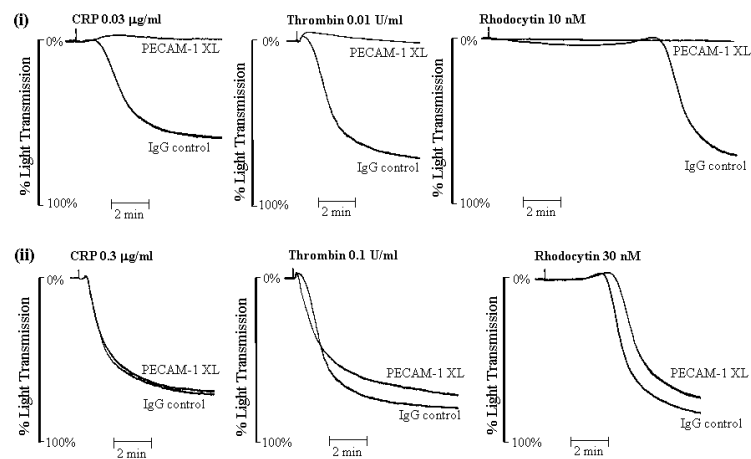


Figure 4.

Cross-linking of PECAM-1 inhibits platelet aggregation to low but not high concentrations of platelet agonists.

(a) Tyrosine phosphorylation of PECAM-1 induced by CRP, thrombin and crosslinking (XL) using two different anti-PECAM-1 antibodies, namely PECAM-1.3 or AB468.

PECAM-1 was immunoprecipitated and western-blotted for tyrosine phosphorylation as described in the methods.

(b) Crosslinking of PECAM-1 inhibits platelet aggregation induced by low but not intermediate concentrations of CRP, thrombin and rhodocytin. Data are representative of 3 separate experiments.

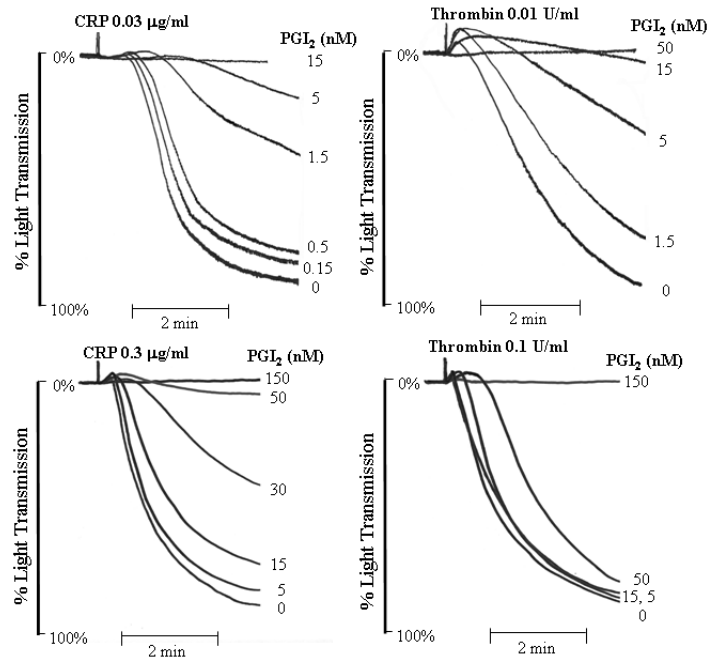


Figure 5. Prostacyclin (PGI₂) mediates powerful inhibition of platelet aggregation. PGI₂ inhibits platelet aggregation induced by low and intermediate concentrations of CRP and thrombin. Results are representative of three experiments.

Figure 6a above

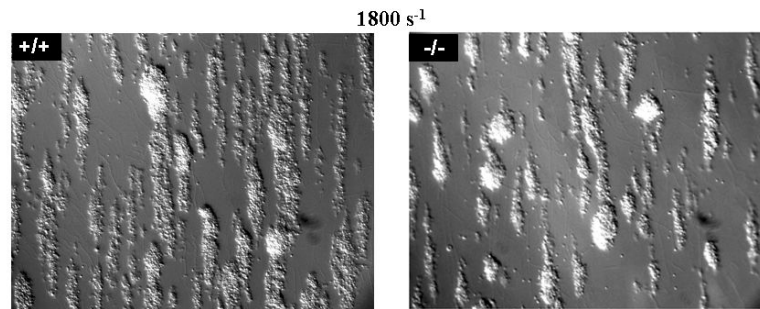
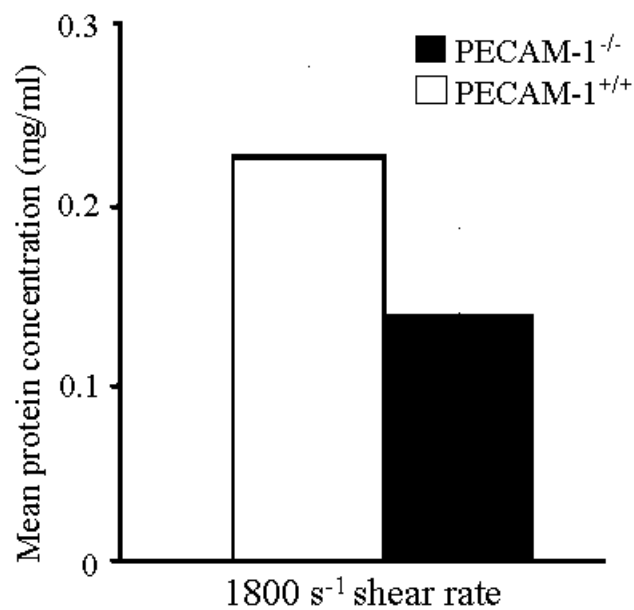


Figure 6b above

**Figure 6.**

Thrombus formation in PECAM-1^{-/-} platelets.

PECAM-1^{-/-} and litter-matched control platelets were flowed over collagen at a shear rate of 1800s⁻¹ for 3min and thrombi visualized with an inverted-stage videomicroscope. (a) Representative images showing thrombus formation. (b) Thrombus formation was measured using RC-DC protein assay as described in the methods. Results are shown as mean±SEM of four experiments.