Shear stress augments the enhanced adhesive phenotype of cells expressing the Pro33 isoform of integrin β_3

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Abstract Adhesion of platelets to the exposed extracellular matrix proteins at sites of vascular injury is partly regulated by the local fluid shear stress. Because the Leu33Pro (Pl^A) polymorphism of integrin β_3 confers only a modest increase in adhesion under static conditions, we used CHO and 293 cells expressing the Leu33 or Pro33 isoform of β_3 in flow chamber experiments to test whether shear forces would alter the Pl^A adhesive phenotype. We found that shear force augmented the Pro33-mediated enhanced adhesion to fibrinogen. This Pro33-dependent enhancement was aspirin-sensitive and was also observed on immobilized von Willebrand factor and cryoprecipitate, but not fibronectin. Thus, shear stress enhances the adhesive phenotype of the Pro33 cells to multiple physiologic substrates.

Key words: β_3 integrin; Pl^A polymorphism; Shear stress

1. Introduction

Ischemic myocardial and cerebral injury occurs when a platelet thrombus occludes a vessel at the site of a ruptured or eroded atherosclerotic plaque [1]. Platelets initiate hemostasis by adhering to extracellular matrix (ECM) proteins including von Willebrand factor (vWF), collagen, laminin and fibronectin in the subendothelium [2]. Firm platelet adhesion to the subendothelium first requires a pronounced deceleration via the interaction of the platelet glycoprotein (GP) Ib–IX complex with vWF immobilized to ECM. Stable adhesion is mediated through platelet integrins $\alpha_{IIb}\beta_3$ (GPIIb–IIIa) binding to immobilized fibrin/fibrinogen and vWF, $\alpha_2\beta_1$ binding to collagen, and $\alpha_5\beta_1$ binding to fibronectin [3]. Subsequent thrombus formation depends upon platelet aggregation and the crosslinking of platelets via the interaction of $\alpha_{IIb}\beta_3$ with fibrinogen and vWF.

Blood flow through the vasculature imposes mechanical shear stress on the vessel wall. Shear stress regulates vascular endothelial cell cytoskeletal reorganization, activation of intracellular signaling pathways, gene expression and protein synthesis [4,5]. Endothelial integrin receptors act as biosensors for the mechanotransduction of shear stress into biochemical signals [6]. In addition, attachment of circulating platelets (and leukocytes) to the vessel wall depends on the balance between the shearing forces and the adhesive forces generated by the interactions between cell surface receptors and their ligands. As in the case of endothelial cells, blood flow and the associated shear stress have been shown to modulate platelet function and signaling. For example, under high flow conditions such as those encountered in arterioles (wall shear rates $>1000\ s^{-1}$), an interaction between platelet GPIb and immobilized vWF is required for initial tethering, whereas at lower shear rates, stable adhesion proceeds without this requirement [7]. Under pathological high stress conditions, platelets aggregate in the absence of modulators like ristocetin and botrocetin and involves tyrosine phosphorylation of α-actinin [8]. Furthermore, under the effect of high shear stress vWF can substitute for fibrinogen as the $\alpha_{IIb}\beta_3$ ligand during platelet aggregation [9,10].

The β_3 subunit of $\alpha_{IIb}\beta_3$ is polymorphic at residue 33 (Leu33Pro), and the Pro33 variant (often referred to as the Pl^{A2} antigen) has been shown to be a risk factor for myocardial infarction in some, but not all studies (for review see reference [11]). There have been conflicting findings in in vitro studies assessing the functional consequences of the Leu33Pro polymorphism, perhaps due to differences among the assays used, the design of the studies, and donor-to-donor variations in normal platelet function [12]. However, it is not known whether fluid shear stress will modify the impact of the Leu33-Pro polymorphism on cell adhesion to fibrinogen and other $\alpha_{IIb}\beta_3$ ligands. Since adhesive differences in our previous static assays [13] between the Leu33 and Pro33 cells to fibrinogen were modest, we wondered if part of the confusion in genetic epidemiological and in vitro functional studies might be related to Leu33Pro functional differences under shear. We now report that compared to static adhesion assays, fluid shear stress further amplifies the enhanced adhesive phenotype of Pro33 cells in a ligand-dependent fashion. Thus, the thrombotic potential associated with the polymorphism may be underestimated when tested under static conditions.

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Abbreviations: ECM, extracellular matrix; vWF, von Willebrand factor; ASA, acetylsalicylic acid; COX, cyclooxygenase

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2. Methods

2.1. Reagents

Anti-$\alpha_{IIb}\beta_3$ monoclonal antibody (mAb) P2 was from Immunotech (Marseilles, France). Blocking mAb 10E5 against $\alpha_{IIb}\beta_3$ was a gift from Dr. Barry Coller (The Rockefeller University, New York, NY, USA). Human fibronectin, αMEM media, 0.05% trypsin, and G418 (Geneticin) were obtained from Life Technology Inc. (Gaithersburg, MD, USA). Human fibrinogen was from Enzyme Research Laboratories Inc. (South Bend, IN, USA). vWF at 100% of normal plasma concentration purified from normal human cryoprecipitate [14] was a gift from Dr. Joel Moake (Baylor College of Medicine, Houston, TX, USA). Normal human cryoprecipitate was from the Blood Bank at Johns Hopkins Hospital. Biochemical analysis of the cryoprecipitate was determined by using STA analyzer kit (Diagnostica Stago, France), and showed 0.60 mg/ml fibrinogen and vWF at 44% normal plasma concentration.

2.2. Cell lines and flow cytometry

Stable Chinese hamster ovary (CHO) cell lines overexpressing the Leu33 and Pro33 isoforms of $\alpha_{IIb}\beta_3$ (designated Leu33 CHO and Pro33 CHO) and the 'vector only' control CHO cells (designated LK) were generated by flow cytometric sorting using mAbs specific for $\alpha_{IIb}\beta_3$ as previously described. A second set of cell lines was also generated in the 293 human embryonal kidney cell line and described previously [13]. Cell surface expression of $\alpha_{IIb}\beta_3$ was analyzed by flow cytometry using P2 mAb followed by an anti-mouse FITC-labeled antibody using a Beckman Coulter flow cytometer, within 24 h of each experiment to assure equivalent expression between the Leu33 and Pro33 cell lines. Cells were not used if there was a greater than 10% difference in the expression of Leu33 and Pro33 isoforms.

2.3. Cell adhesion under flow conditions

Cells were grown to 70–80% confluence and detached using 0.05% trypsin. After neutralization, cells were suspended in Tyrode's or Hanks' balanced salt solution for adhesion studies to fibrinogen or fibronectin respectively, as described previously [13]. For adhesion studies under shear, glass coverslips (No. 1, 24×50 mm; Dow Corning, Corning, NY, USA) were coated with 12.5 μg/ml fibrinogen, fibronectin, cryoprecipitate or 100% of normal vWF plasma concentration and incubated for 3 h in a humidified chamber. The parallel-plate flow chamber was assembled with the cover slip and the adhesion assays under flow performed as we have previously described [15]. Briefly, the flow chamber was mounted on an inverted-stage microscope (Nikon eclipse TE300, Nikon, Melville, NY, USA) equipped with a 20× phase objective and a high-speed digital camera (Quantix Photometric, Photometric Ltd, Tuscon, AZ, USA) connected to a computer. In most experiments, 5×10^5 cells were perfused through the chamber for 5 min at a constant flow rate that corresponds to the wall shear rates of 25, 50, 100 or 125 s^{-1}. In some experiments, cells were pretreated for 30 min with 10 μg/ml of 10E5 mAb or 300 μg/ml of aspirin (Sigma, St. Louis, MO, USA) prior to perfusion. Four fixed fields of observation were identified and the number of cells adhering onto the substrate in each region was counted using the Metamorph imaging software (Universal Imaging Corp., Downingtown, PA, USA).

In some experiments cells were fluorescently labeled with BCECF dye (Molecular probes, Oregon) and perfused over fibrinogen (100 μg/ml) in the parallel-plate perfusion chamber at wall shear rates of 25, 50 or 100 s^{-1} for 5 min, as described [16]. Epifluorescence was used to directly visualize cell adhesion throughout the perfusion period and experiments were recorded in real time with an sVHS video cassette recorder. At the end of the perfusion, videotape images were digitized and quantitative information on cell adhesion was obtained by digital image processing, as previously described [16].

2.4. Immunofluorescence

Integrin $\alpha_{IIb}\beta_3$ distribution on CHO cells was examined in the presence and absence of fibrinogen binding. Adherent cells on coverslips were fixed with 3.5% paraformaldehyde for 8 min and blocked with PBS containing 10% FCS for 20 min at 22°C. Cells were immunostained for $\alpha_{IIb}\beta_3$ using integrin complex specific P2 (20 μg/ml) antibody for 1 h at 22°C followed by FITC-labeled anti-mouse IgG (1:50) for 1 h at 22°C. The coverslips were washed twice and mounted in permafluor (Lipshaw immunon, Pittsburgh, PA, USA). Fluorescent images were obtained and analyzed using Metamorph imaging system.

2.5. Statistics

Results were expressed as mean ± S.E.M. of 8–24 observations from different experiments, and then analyzed by Student's '*t*' test.

3. Results

3.1. Shear amplifies the enhanced adhesion of Pro33 cells to fibrinogen

To determine whether fluid shear stress would modify the adhesive phenotype of cells based on the Pl^A genotype, we examined the adhesion of Leu33 and Pro33 cells to fibrinogen in the presence of shear. Significantly more Pro33 CHO cells adhered on 100 μg/ml fibrinogen than did the Leu33 cells over a range of wall shear rates ($P \leq 0.05$; Fig. 1A). The number of CHO cells adhering to fibrinogen was inversely proportional to the wall shear rate with negligible adhesion occurring at >250 s^{-1} (not shown). Adhesion was inhibited by the 10E5 mAb, indicating $\alpha_{IIb}\beta_3$ dependence (Fig. 1B). Surface expression of $\alpha_{IIb}\beta_3$ was not detectably different between cell lines and could not account for the observed differences in cell adhesion (Fig. 1C).

Since plasma fibrinogen levels have been shown to modify the enhanced Pro33-dependent platelet aggregation [17], we tested if a lower concentration of immobilized fibrinogen would modify the adhesion of Leu33 or Pro33 CHO cells. Fig. 2A shows that the Pro33 cells adhered to a significantly greater extent ($P < 0.001$) on 12.5 μg/ml fibrinogen compared to the Leu33 cells at all shear rates tested. Surface expression of $\alpha_{IIb}\beta_3$ between cell lines was not detectably different (Fig. 2B), and this adhesion was $\alpha_{IIb}\beta_3$ mediated (not shown). The

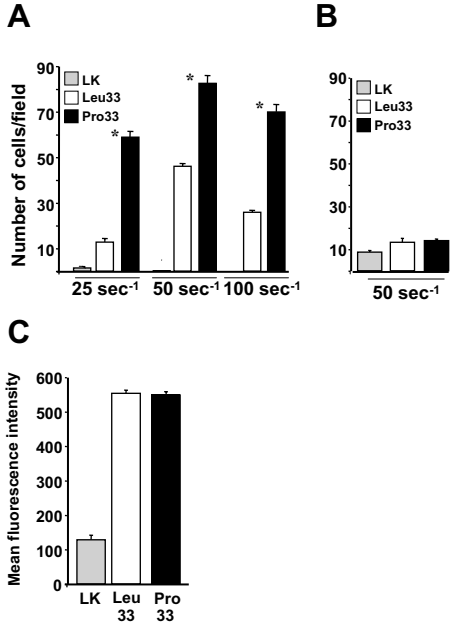

Fig. 1. Adhesion of Leu33 and Pro33 CHO cells on 100 μg/ml fibrinogen. A: Cells were perfused over 100 μg/ml fibrinogen in a parallel-plate flow chamber and the number of adherent cells quantified from three independent experiments. Compared to Leu33 cells, Pro33 cells show 4.5-fold increased adhesion at 25 s^{-1}, 1.7-fold increased adhesion at 50 s^{-1}, and 2.6-fold increased adhesion at 100 s^{-1}. *$P < 0.001$ for Leu33 vs. Pro33. B: Cells were pretreated with 10 μg/ml 10E5 and perfused as in panel A at a wall shear rate of 50 s^{-1}. C: Mean fluorescence intensity of P2 binding to the three cell lines.

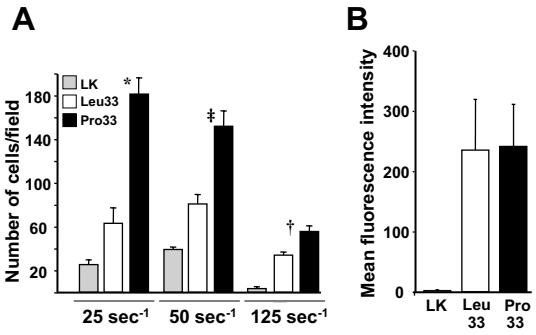

Fig. 2. Adhesion of Leu33 and Pro33 CHO cells on 12.5 μg/ml fibrinogen. A: Cells were perfused over 12.5 μg/ml fibrinogen and the number of adherent cells quantified from two to three independent experiments. Compared to Leu33 cells, Pro33 cells show 2.8-fold increased adhesion at 25 s^{-1}, 1.8-fold increased adhesion at 50 s^{-1}, and 1.5-fold increased adhesion at 125 s^{-1}. $^{*}P<0.001$, $^{‡}P=0.05$; $^{†}P=0.007$ for Leu33 vs. Pro33. B: Mean fluorescence intensity of P2 binding to the three cell lines.

distribution patterns of integrin $\alpha_{IIb}\beta_3$ were similar in Leu33 and Pro33 cells regardless of whether they were adhered to fibrinogen (Fig. 3). This suggests that the enhanced adhesion of Pro33 cells under flow was most likely not caused by increased integrin clustering. The observation that more cells adhered at 12.5 μg/ml compared to 100 μg/ml fibrinogen is consistent with a previous study [18] in which the binding of an anti-fibrinogen antibody linearly increased up to about 10 μg/ml fibrinogen and then declined at higher fibrinogen concentrations, and also with our previous static adhesion study [13].

Table 1
Adhesion (in fold units) of Pro33 cells relative to Leu33 cells[a]

Shear rate	CHO cells	293 cells
0 (static)	1.2 ($P<0.001$)	1.2 ($P=0.006$)
25 s^{-1}	2.8 ($P<0.001$)	1.4 ($P=0.005$)
50 s^{-1}	1.8 ($P=0.05$)	2.2 ($P=0.008$)
125 s^{-1}	1.5 ($P=0.007$)	1.5 ($P=0.07$)

[a]Adhesion to 12.5 μg/ml fibrinogen.

Although the CHO cells were generated by cell sorting and should not exhibit clonal variations, we examined the influence of shear on adhesion to fibrinogen in a second set of cell lines generated in 293 cells. Table 1 summarizes the effect of shear on adhesion to fibrinogen mediated by Leu33 or Pro33 $\alpha_{IIb}\beta_3$ expressing CHO and 293 cells. Similar results were obtained with the 293 cells except that the Pro33-dependent adhesive differences were greatest at 50 s^{-1} for the 293 cells, and at 25 s^{-1} for the CHO cells, perhaps reflecting cell type specificities. However, both cell types showed an enhanced Pro33 adhesion under shear compared to static conditions.

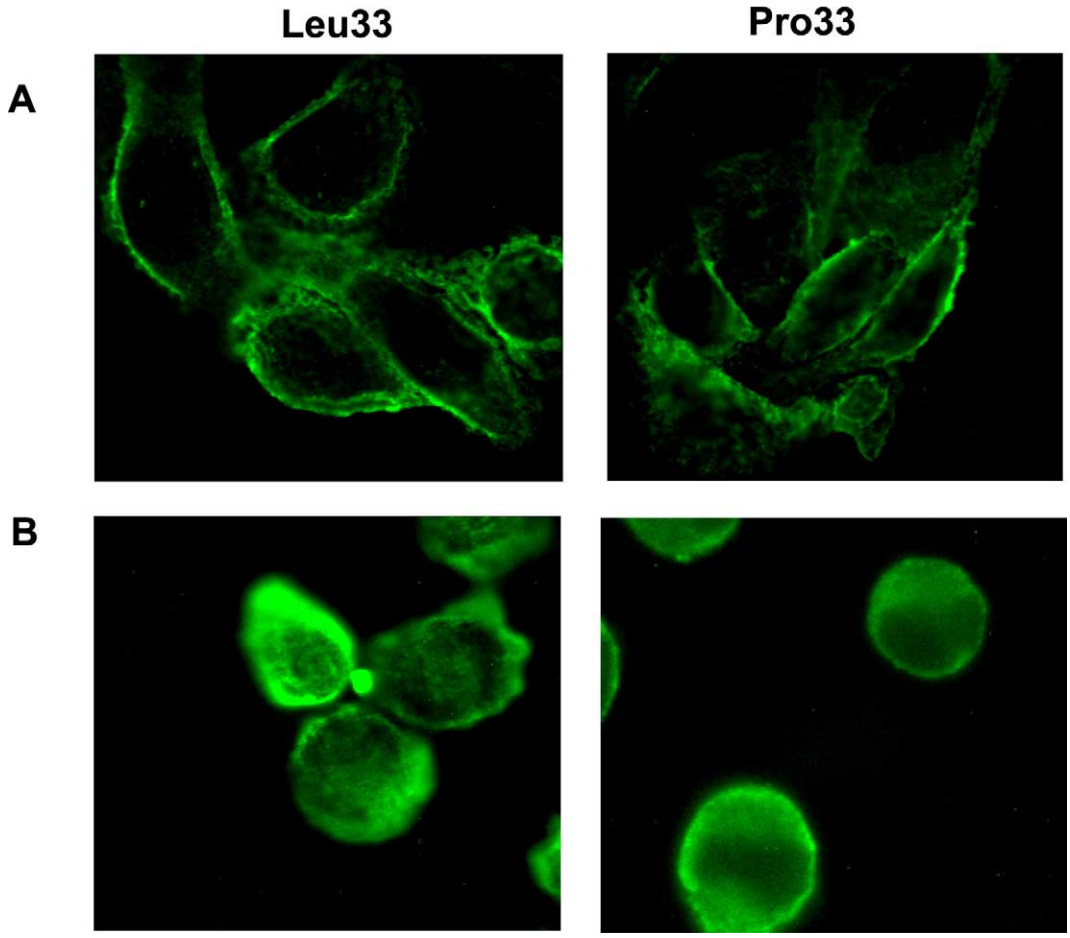

Fig. 3. Fluorescence microscopy of integrin $\alpha_{IIb}\beta_3$ distribution in CHO cells. A: Cells grown on coverslips were stained with P2 antibody followed by FITC-labeled anti-mouse IgG. B: Cells that adhered to 12.5 μg/ml fibrinogen under shear (25 $s^{-1)}$ were stained for integrin $\alpha_{IIb}\beta_3$. LK (vector cells) did not adhere to fibrinogen and are not shown.

Table 2a
Relationship of fibrinogen (FGN) concentration and aspirin on CHO cell adhesion

FGN	Ethanol			ASA (300 μg/ml)		
	LK	Leu33	Pro33	LK	Leu33	Pro33
12.5 μg/ml	18.33 ± 5.16	42 ± 5.91	89 ± 5.91, $P < 0.001$*	15.33 ± 4.27	44.22 ± 5.91, $P = 0.784^{\dagger}$	56.11 ± 6.74, $P = 0.06$*
50 μg/ml	0.06 ± .06	18.18 ± 1.64	41.68 ± 2.85, $P < 0.001$*	0.375 ± .25	24.25 ± .972, $P = 0.005^{\dagger}$	25.43 ± 1.75, $P = 0.437$*
100 μg/ml	0.33 ± .18	19.53 ± 2.10	46.93 ± 2.27, $P < 0.001$*	0.6 ± .23	28.13 ± 1.54, $P = 0.0009^{\dagger}$	31.13 ± 2.04, $P = 0.078$*

Numbers in table are number of cells per microscopic field at a shear rate of 25 s^{-1}. *P values for Leu33 vs. Pro33. $^{\dagger}P$ values for Leu33 ethanol vs. Leu33 aspirin.

3.2. Enhanced adhesion of Pro33 CHO cells to fibrinogen depends on the cyclooxygenase (COX) pathway

Previous studies have demonstrated that aspirin (acetylsalicylic acid, ASA) inhibition of platelet function varies with Leu33Pro genotype, with Pro33-positive platelets showing greater sensitivity to inhibition by ASA [19–23]. These studies were all conducted under static conditions, so we studied the effect of aspirin on the enhanced adhesion of Pro33 CHO cells to fibrinogen. Aspirin selectively inhibited the adhesion of Pro33 cells and not Leu33 cells at all concentrations of fibrinogen (Table 2A) and all shear stresses (Table 2B). Interestingly, at high fibrinogen concentration and low shear stress, ASA (300 μg/ml) consistently enhanced the adhesion of Leu33 cells. However, the adhesion of Leu33 cells remained unaffected at a low dose ASA (100 μg/ml) while the enhanced adhesion of Pro33 cells was selectively blocked (not shown). Thus, the outcome of aspirin on the effect of the Leu33Pro polymorphism on cell adhesion is dependent on the substrate density and the shear force.

3.3. Pro33 CHO cells demonstrate greater adhesion to vWF and cryoprecipitate, but not to fibronectin

Platelet $\alpha_{IIb}\beta_3$ interacts with multiple physiologic substrates in vivo. Compared to the Leu33 cells, we also found that the Pro33 variant exhibited significantly more adhesion to vWF (1.5-fold) (Fig. 4A), but there was no significant difference in adhesion on fibronectin (Fig. 4B). This data indicates that the increased adhesion of the Pro33 variant of β_3 is substrate-specific. Cryoprecipitate is composed of vWF and fibrinogen, and is used clinically in the management of bleeding in certain deficiency states. We observed significantly greater adhesion for Pro33 cells compared to Leu33 cells on normal human cryoprecipitate (Fig. 5A). This adhesion was $\alpha_{IIb}\beta_3$ dependent since it was blocked by 10E5 antibody (Fig. 5B).

4. Discussion

Fluid shear force is an important determinant of vascular

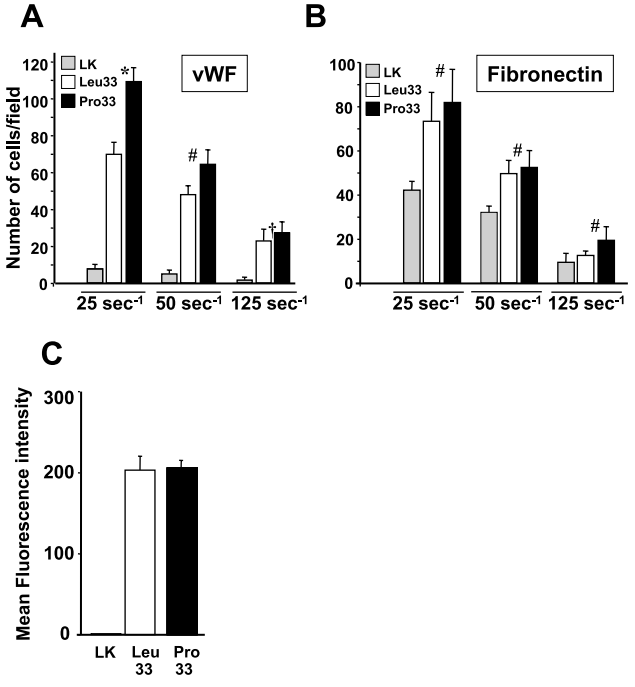

Fig. 4. Adhesion of Leu33 and Pro33 CHO cells on vWF and fibronectin. Cells were perfused over vWF or fibronectin in a parallel-plate flow chamber and the number of adherent cells quantified from two to three experiments. A: On a vWF substrate, the increased adhesion of Pro33 cells is most prominent at 25 s^{-1}. *$P = 0.007$, $^{\#}P = 0.105$; $^{\dagger}P = 0.59$ for Leu33 vs. Pro33. B, Adhesion of Leu33 and Pro33 cells to 12.5 μg/ml fibronectin from three experiments. $^{\#}P > 0.28$. C: Mean fluorescence intensity of P2 binding to the three CHO cell lines determined within 24 h of the adhesion assay. Surface expression of $\alpha_{IIb}\beta_3$ was not detectably different between the Leu33 and Pro33 cells.

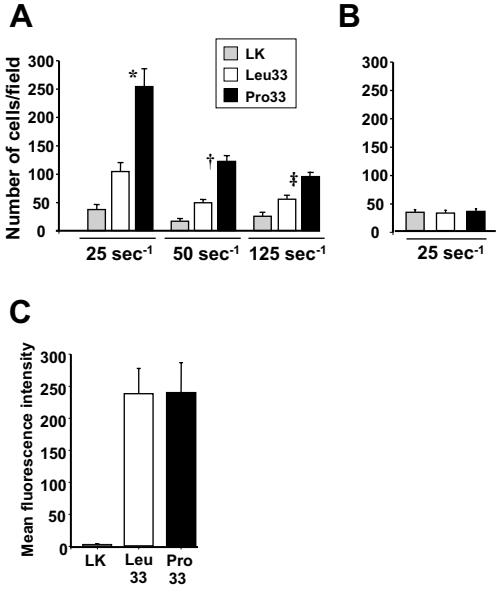

Fig. 5. Adhesion of Leu33 and Pro33 CHO cells to normal human cryoprecipitate. A: Cells were perfused over 12.5 μg/ml cryoprecipitate in a parallel-plate flow chamber and the number of adherent cells quantified from two to three experiments. Compared to Leu33 cells, Pro33 cells show 2.5-fold increased adhesion at 25 s^{-1}, 2.4-fold increased adhesion at 50 s^{-1}, and 1.7-fold increased adhesion at 125 s^{-1}. *$P = 0.003$, $^{\dagger}P = 0.002$; $^{\ddagger}P < 0.001$ for Leu33 vs. Pro33. B, The 10E5 mAb (10 μg/ml) blocked adhesion of both cell lines at a wall shear rate of 25 s^{-1}. C: Mean fluorescence intensity of P2 binding to the three CHO cell lines.

Table 2b
Relationship of shear rate and aspirin on CHO cell adhesion

Shear	Ethanol			ASA (300 μg/ml)		
	LK	Leu33	Pro33	LK	Leu33	Pro33
25 s^{-1}	0.43 ± .18	20.56 ± 1.76	53.81 ± 3.39, $P < 0.001$*	0.5 ± .18	30.31 ± 1.18, $P < 0.001^{\dagger}$	33.4 ± 1.81, $P = 0.068$*
50 s^{-1}	0.31 ± .17	21.06 ± 1.30	35.18 ± 1.40, $P < 0.001$*	0.25 ± .11	23 ± .98, $P = 0.29^{\dagger}$	16.8 ± 1.46, $P = 0.046$*
125 s^{-1}	0	8.63 ± .79	13.6 ± 1.01, $P = 0.006$*	0.1 ± .09	8.7 ± 1.07, $P > 0.05^{\dagger}$	10.09 ± .62, $P = 0.175$*

Numbers in table are number of cells per microscopic field adhering to 100 μg/ml fibrinogen. *P values for Leu33 vs. Pro33. $^{\dagger}P$ values for Leu33 ethanol vs. Leu33 aspirin.

and blood cell function and plays an essential role in arterial thrombosis. In this study, we have evaluated the impact of shear stress on the adhesion to ECM proteins of CHO and 293 cells expressing the Leu33 or Pro33 isoforms of integrin $\alpha_{IIb}\beta_3$. Using a parallel-plate perfusion chamber, we showed that compared to the Leu33 cells, Pro33 cells exhibited enhanced adhesion to fibrinogen and vWF. This enhanced Pro33 adhesion was mediated through integrin $\alpha_{IIb}\beta_3$, was substrate specific, and required signaling through the COX pathway. In addition, our findings suggest that aspirin may enhance adhesion in Pro33-negative cells under low shear/high fibrinogen conditions. The enhanced adhesive phenotype of Pro33 cells under shear may have particular relevance in arterial thrombosis.

In previous studies we have shown that CHO and 293 cells expressing the Pro33 variant of $\alpha_{IIb}\beta_3$ demonstrated a small (~20% greater) but very reproducible increased adhesion to immobilized fibrinogen under static conditions [13]. Considering this modest effect under static conditions, we wondered whether shear might abolish the Pro33-mediated enhanced adhesion to immobilized fibrinogen. However, our studies showed a clear enhancement of Pro33-mediated cell adhesion to immobilized fibrinogen under shear conditions compared to static conditions. The shear effect on Pro33-mediated increased adhesion was observed in two independent sets of cell lines (Table 1), demonstrating that clonal variation cannot account for the different phenotype observed. Cadroy et al. [24] assessed the relationship of shear and Pro33 for platelet adhesion on tissue factor and collagen, and found that compared to the Pro33-positive platelets, more Pro33-negative platelet deposited on tissue factor, but not collagen at 650 s^{-1}. The apparent differences between our studies may be due to the fact that neither of these substrates are ligands for $\alpha_{IIb}\beta_3$ and/or the different shear forces used. The Pro33-dependent enhanced adhesion in our study was observed with the other major $\alpha_{IIb}\beta_3$ substrate, vWF, both in its purified form and in cryoprecipitate. Comparing the relative effects across the various substrates suggests that the Pro33 effect under shear is greatest on fibrinogen and that cryoprecipitate favors more cell adhesion, perhaps due to cooperativity between the multiple $\alpha_{IIb}\beta_3$ ligands.

In this study we used a range of wall shear rates typically chosen to study adhesive interactions between a suspension of flowing leukocytes (cells of size similar to CHO) and cultured endothelial cells [25]. We found that rather low shear rates were required to observe the Pro33-dependent effect on adhesion, since little or no cell adhesion was observed at flow rates above 125 s^{-1}. It is difficult to directly correlate shear forces in the parallel-plate flow chamber with arterial or venous shear because the heterologous cells used in this study express only integrin $\alpha_{IIb}\beta_3$ and lack the GPIb–IX complex that slows platelets in vivo. This is analogous to the situation in which human platelets lacking GPIb–IX adhere to collagen substrates at venous but not arterial shear forces [26]. Furthermore, the large size of the CHO cells compared to smaller platelets substantially increase the drag force applied to the $\alpha_{IIb}\beta_3$–ligand bond. Despite these inherent limitations in our model system, the key finding from our studies is that shear force augments the Pro33-dependent increase in cell adhesion.

It is not clear how shear would selectively confer enhanced adhesiveness to Pro33 cells. Immunofluorescence studies in Fig. 3 indicate that a difference in integrin $\alpha_{IIb}\beta_3$ clustering does not explain the enhanced Pro33 cell binding to fibrinogen under shear. The increased relative fluid velocities during flow could increase the rate of $\alpha_{IIb}\beta_3$–fibrinogen bond formation (kinetic effect) in Pro33 cells, as demonstrated for L-selectin-mediated rolling [27]. Alternatively, shear forces could cause a high-affinity conformation for the receptor $\alpha_{IIb}\beta_3$ in Pro33 cells as shown for $\alpha_v\beta_3$ [6] and decrease the bond off-rate. Our experiments suggest that the bond off-rate may not be influenced by the Leu33Pro polymorphism, since both the Leu33 and Pro33 cells that adhered on fibrinogen at 25 s^{-1} could not be dislodged even after increasing the shear rate up to 1000 s^{-1} at the end of the perfusion (data not shown). Studies using surface plasmon resonance spectroscopy suggest that $\alpha_{IIb}\beta_3$–fibrinogen binding consists of two consecutive processes: an initial fast reaction, with a reversible low affinity complex and a subsequent slower high affinity complex [28]. It is plausible that the combined presence of shear and the β_3-helix disrupting residue proline could change the kinetic rates (and increase the on-rate) of integrin $\alpha_{IIb}\beta_3$ to fibrinogen at one or both these steps.

Previous studies have demonstrated that integrin-dependent adhesion is regulated by post-receptor signaling through the COX pathway, both in flowing leukocytes [29] and static endothelial cells [30]. Our study indicates that under shear the $\alpha_{IIb}\beta_3$ Pro33-mediated enhanced adhesion also depends on COX (Table 2). We have observed a similar dependence under static studies (data not shown). Our studies led to an interesting and unexpected finding: aspirin enhanced the adhesion of Leu33 cells. This effect was observed primarily under low shear stress (25 s^{-1}) and not higher shear stresses (50 or 100 s^{-1}) and under the higher fibrinogen concentrations (50 μg/ml and 100 μg/ml) and not the lower fibrinogen concentration (12.5). Note that aspirin only inhibited and never enhanced Pro33 cell adhesion under any condition. This effect of aspirin on Leu33 cells was very reproducible. Furthermore, this data is consistent with a study demonstrating an increased platelet aggregation with a higher plasma fibrinogen concentration only in Pro33-negative but not Pro33-positive subjects [17].

What is the functional significance for a shear-enhanced adhesiveness of the Pro33 cells? Pro33 platelets have been reported to be prothrombotic in some but not all clinical

epidemiology studies [11] and in this in vitro study we show shear forces amplify by two- to four-fold the Pro33-mediated enhanced adhesion to fibrinogen. Perhaps some part of the inconsistency in clinical studies stems from variability in arterial shear forces among the heterogeneous vascular lesions among different patients. Our findings that shear force enhances the adhesive phenotype of Pro33 cells are consistent with the fact that many clinical epidemiology studies have found an association between the Pro33 polymorphism and arterial thrombosis, but none have found an association with venous thrombosis [31–34]. According to our presented data the biggest risk of the Pro33 polymorphism would be at moderate shear stress likely to be seen in atherosclerotic intermediate sized vessels. Hence a possible consequence of the Pro33 polymorphism could be to increase the platelet recruitment and the speed of growth of a thrombotic lesion. Aspirin has been reported to be of some benefit in the prevention of venous thromboembolic disease [35]. Since our study shows enhanced adhesion of Leu33 cells under low shear/high fibrinogen conditions, it would be very interesting to assess whether plasma fibrinogen levels and the Leu33Pro polymorphism interact to modulate the effects of aspirin on the prevention or development of venous thrombosis. In conclusion, the Leu33Pro polymorphism is shown to regulate the adhesive behavior of cells to the ECM proteins in a substrate-specific manner, and fluid shear stress can further enhance the adhesive phenotype of Pro33 cells. Future clinical pharmacogenetic studies will be required to consider whether Pro33-positive patients may derive more prothrombotic benefit from cryoprecipitate infusions and more antithrombotic benefit from aspirin compared to Pro33-negative patients.

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