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Preferential binding of platelets to monocytes over neutrophils under flow

Kyung C. Ahn [1], Andy J. Jun [1], Parag Pawar [1], Sameer Jadhav, Susan Napier, Owen J.T. McCarty, Konstantinos Konstantopoulos *

Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, Baltimore, MD 21218, USA

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Abstract

This study was undertaken to systematically investigate the binding kinetics of platelet recruitment by monocytes relative to neutrophils in bulk suspensions subjected to shear as well as the molecular requirements of leukocyte–platelet binding. Hydrodynamic shear-induced collisions augment the proportion of monocytes with adherent platelets more drastically than that of neutrophils with bound platelets. These heterotypic interactions are further potentiated by platelet activation with thrombin or to a lesser extent by monocyte stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). Monocyte–platelet heteroaggregation increases with increasing shear rate and shear exposure time. Platelet P-selectin binding to monocyte P-selectin-glycoprotein-ligand-1 is solely responsible for maximal platelet adhesion to unstimulated monocytes in shear flow. However, the enhanced platelet binding to fMLP-treated monocytes involves a sequential two-step process, wherein P-selectin-PSGL-1 interactions are stabilized by CD18-integrin involvement. Blocking platelet $\alpha_{IIb}\beta_3$ or monocyte β_1-integrin function had no effect. This study underscores the preferential recruitment of platelets by monocytes relative to neutrophils in shear flow, and demonstrates that the shear environment of the vasculature coupled to the state of cell activation modulates the dynamics and molecular constituents mediating monocyte–platelet adhesion.

Keywords: Monocyte; Neutrophil; Platelet; Aggregation; Shear; P-selectin; PSGL-1; CD18; fMLP; Thrombin

Accumulating evidence suggests that enhanced leukocyte–platelet adhesion occurs in the circulation of patients with unstable angina [1], acute myocardial infarction (AMI) [2], stroke [3] or after coronary angioplasty [4]. These heterotypic adhesive interactions are thought to promote thrombosis and vascular occlusion, thereby impairing blood flow and exacerbating ischemia. Indeed, disruption of leukocyte–platelet binding has been shown to effectively reduce the incidence of reocclusion in animal models of vascular injury [5,6].

To date, most of the previous work has focused on delineating the binding kinetics and molecular mechanisms by which neutrophils interact with platelets, since neutrophils represent the largest leukocyte subpopulation in blood [7–11]. Consequently, very little is known about the kinetics and molecular constituents mediating attachment of other leukocyte subpopulations to platelets. For instance, monocytes, although they comprise <10% of circulating leukocytes in blood, may have a competitive advantage over other leukocyte subtypes including neutrophils, in binding activated platelets. Indeed, clinical observations suggest that in the setting of percutaneous coronary intervention, the number of circulating monocyte–platelet aggregates is significantly larger than that of neutrophil–platelet complexes [12]. Moreover, the in vivo half-life of circulating monocyte–platelet aggregates in patients with AMI is longer than that of neutrophil–platelet aggregates [12].

* Corresponding author. Fax: +1 410 516 5510.
E-mail address: kkonsta1@jhu.edu (K. Konstantopoulos).
[1] These authors have contributed equally.

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Consequently, it has been suggested that the quantification of circulating monocyte–platelet aggregates may be a robust and sensitive marker of acute coronary syndromes [12,13].

In accord with the neutrophil model, prior work has revealed that the molecular interaction between CD162 (P-selectin glycoprotein ligand-1; PSGL-1) on monocytes and platelet CD62P (P-selectin) was primarily responsible for monocyte tethering, rolling, and adhesion to surface-adherent, activated platelets in shear flow [14]. Moreover, CD18-integrins were requisite for optimal monocyte firm adhesion, as evidenced by increased numbers of rolling monocytes on platelet layers upon CD18-integrin blockade [14]. The aforementioned in vitro model in which monocytes interact with surface-adherent platelets simulates events that take place at sites of vascular injury, as may occur after coronary angioplasty, in which platelet deposition to denuded endothelial cell surfaces had occurred. However, monocytes are more likely to bind to free-flowing platelets throughout the vascular system rather than to limited sites of immobilized platelets. The fundamental physical and molecular requirements of monocyte binding to either resting or activated platelets in free-cell suspensions as opposed to immobilized, activated platelet substrates remain largely obscure. Although evidence suggests that in the absence of flow activated platelets attach to monocytes more extensively than do resting platelets [15], no quantitative comparisons have been reported under well-defined hydrodynamic shear conditions in the absence of homotypic platelet aggregation which could interfere with the heterotypic adhesion process. Moreover, the effects of hydrodynamic shear and cell activation on the comparative binding kinetics of monocytes versus neutrophils to platelets have yet to be resolved. Consequently, this study was undertaken to systematically investigate the dynamics of monocyte–platelet and neutrophil–platelet heteroaggregation in reconstituted free-cell suspensions containing monocytes, neutrophils, and platelets suspended in plasma, subjected to controlled levels of hydrodynamic shear by the use of a cone-and-plate rheometer. Experimental data were analyzed using a mathematical model based on Smoluchowski's two-body collision theory that yields numerical estimates of capture efficiency, an index that reflects the binding affinity of interacting cells [16–18]. Moreover, we wished to investigate the influence of shear rate (0–800 s^{-1}), shear exposure time (15–120 s), and exogenous cell activation on the molecular requirements of monocyte–platelet heteroaggregation.

Materials and methods

Reagents and monoclonal antibodies. The IgG murine monoclonal antibodies (mAbs) 6.7 (blocking anti-CD18), KPL-1 (blocking anti-CD162 (anti-PSGL-1)), AK-4 (blocking anti-CD62P (anti-P-selectin), purified as well as conjugated with fluorescein isothiocyanate (FITC)), Dreg-56 (anti-CD62L (anti-L-selectin) conjugated with FITC), and MOPC-21 (an irrelevant control IgG antibody conjugated with FITC) were from BD-Pharmingen (San Diego, CA). The Fab anti-$\alpha_{IIb}\beta_3$ mAb c7E3 was obtained from Centocor (Malvern, PA). The nonpeptide small-molecule platelet $\alpha_{IIb}\beta_3$ (CD41/CD61) antagonist XV454 [19] and the blocking anti-CD29 (anti-β1-integrin) mAb 13 were kind gifts of Dr. S.A. Mousa (Albany College of Pharmacy, Albany, NY) and Dr. S.K. Akiyama (National Institutes of Health, Research Triangle Park, NC), respectively. The fibrin polymerization inhibitor Gly-Pro-Arg-Pro-amide (GPRP-NH_2), thrombin, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), PKH26 Red Fluorescent Cell Linker Kit for cell membrane labeling (PKH26 Red), Vybrant DiD, and a Fluoro FITC Conjugation Kit, which was used to conjugate c7E3 with FITC, were from Sigma (St. Louis, MO).

Isolation of monocytes and staining. Human venous blood was drawn by venipuncture from healthy volunteers into 10 mM ethylenediaminetetraacetic acid (EDTA) anticoagulant. Ten milliliter aliquots of whole blood specimens were diluted by addition of 25 ml of 0.9% NaCl saline, then layered on 9 ml of 1.079 g/ml Percoll solution, and centrifuged at 500*g* for 20 min at room temperature (RT). Mononuclear cells aspirated from the interface were further diluted with Dulbecco's phosphate-buffered saline (D-PBS) lacking Ca^{2+}/Mg^{2+} containing 2.5 mM EDTA, layered on Accuprep (Axis-Shield PoC, Oslo, Norway), and centrifuged at 160*g* for 20 min at 4 °C. The upper layer containing residual platelets was removed by suction, and mononuclear cells were further centrifuged at 350*g* for 20 min at 4 °C. After centrifugation, lymphocytes and monocytes (i.e., mononuclear cells) harvested from the interface using a Pasteur pipette were stained with PKH26 dye (1×10^{-7} M) according to manufacturer's instructions. Mononuclear cells were washed three times at 400*g* for 5 min at 4 °C with filtered D-PBS without Ca^{2+}/Mg^{2+} containing 0.1% bovine serum albumin (BSA). Monocytes were negatively isolated from a mononuclear cell sample by using the Dynal Monocyte Negative Isolation Kit (Dynal Biotech, Lake Success, NY). Monocytes were centrifuged at 225*g* for 8 min at 4 °C, resuspended in D-PBS containing 0.1% BSA at a concentration of 1×10^7 cells/ml, and stored at 4 °C for no longer than 3 h before use in aggregation assays or flow cytometry. Monocyte purity, determined by flow cytometry, was >90%, and monocyte viability, assessed by trypan blue exclusion, was >97%. Moreover, the percentage of monocytes with adherent platelets was <10% as detected by flow cytometry using a platelet specific anti-$\alpha_{IIb}\beta_3$ mAb, c7E3, conjugated with FITC.

Isolation of neutrophils and staining. Human neutrophils were obtained from citrate phosphate dextrose (Sigma–Aldrich) anticoagulated venous blood of healthy volunteers (1.4 ml citrate phosphate dextrose/10 ml blood) by centrifugation through a neutrophil isolation medium (Robbins Scientific, Sunnyvale, CA). Isolated neutrophils were then mixed with sterile filtered water for 30 s to lyse erythrocytes. The lysis reaction was stopped by adding 10× pipes buffer [20]. Neutrophils were then washed once, resuspended in D-PBS lacking Ca^{2+}/Mg^{2+} at a concentration of 10^7 cells/ml, and then incubated with 2.5 μM Vybrant DiD per 1×10^6 neutrophils/ml for 60 s at RT. Vybrant-DiD-stained neutrophils were washed once, resuspended in Ca^{2+}/Mg^{2+}-free D-PBS/0.1% BSA, and stored at 4 °C for no longer than 3 h before use in aggregation assays or flow cytometry. Neither the expression levels of L-selectin on resting neutrophils nor the extent of homotypic neutrophil aggregation in response to hydrodynamic shear and chemotactic stimulation was affected by DiD (data not shown). It is of note that both monocytes and neutrophils were isolated from the same blood donor in all experiments reported herein.

Platelet preparation. Human blood was drawn by venipuncture from healthy volunteers into sodium citrate (0.38% wt/v) anticoagulant. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described [18]. The final platelet count of the PRP was adjusted to the desired levels with PPP. Specimens were

stored at RT in capped polypropylene tubes and used within 2 h of isolation.

Cone-and-plate rheometry assays. Platelets (5×10^7 cells/ml) and PKH red-stained monocytes (1×10^6 cells/ml) were allowed to equilibrate separately to 37 °C for 1 min, and then placed onto the stationary plate of a cone-and-plate rheometer (RS150; Haake, Paramus, NJ) pretreated with Sigmacote (Sigma) for 1 min to prevent non-specific cell binding. Shear rates varied from 100 to 800 s^{-1} (typical of microcirculation) for prescribed periods of time ranging from 15 to 120 s. Static conditions were achieved by setting the shear rate to 0 s^{-1}. Upon termination of shear or static incubation, samples (50 μl) were obtained and instantly fixed with 1% formaldehyde. Specimens were then allowed to incubate with a FITC-labeled platelet-specific mAb directed against $\alpha_{IIb}\beta_3$ (5 μg/ml, C7E3 FITC) for 30 min in the dark at RT. The labeling reaction was then stopped by further dilution with 1% formaldehyde, and specimens were subsequently analyzed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The 1° cone and plate of the rheometer were maintained at 37 °C during the entire experiment.

In triple-color assays, the aforementioned procedure was performed by using platelets (5×10^7 cells/ml), PKH-stained monocytes (1×10^6 cells/ml), and Vybrant-DiD-stained neutrophils (4×10^6 cells/ml).

Cell treatment with fMLP, thrombin, and mAbs. In selected runs, to potentiate platelet activation, PRP specimens were incubated with thrombin (2 U/ml) in the presence of the fibrin polymerization inhibitor GPRP-NH_2 (2 mM) for 10 min at 37 °C before shear exposure [18]. In other experimental runs, monocytes and neutrophils were stimulated with fMLP (1 μM) 1 s before the application of shear.

For inhibition studies, platelets were pretreated for 10 min with a function-blocking anti-P-selectin mAb (20 μg/ml AK4) or an $\alpha_{IIb}\beta_3$ antagonist (100 nM XV454) at RT prior to being mixed with monocytes in the shear field of a cone-and-plate rheometer. Alternatively, monocytes were preincubated with 20 μg/ml function-blocking mAbs specific for PSGL-1 (KPL-1), CD18-integrins (6.7) or CD29-integrins (mAb 13) for 10 min at 37 °C before the application of shear. In parallel, matched control experiments were performed in which platelets and monocytes were treated exactly as stated above, but in the absence of any receptor antagonist. Monocyte–platelet adhesive interactions in response to hydrodynamic shear were unaltered by the presence of an irrelevant IgG control mAb. For instance, the proportion of fMLP-stimulated monocytes with bound platelets in the absence and presence of an IgG control mAb was 60.5% ± 3.5 and 64.5% ± 6.5, respectively, after 60 s of shear exposure at 400 s^{-1}. Similarly, no significant differences were detected for all other experimental conditions tested.

Quantitation of L-selectin expression. Monocytes collected after Percoll isolation and after the final step of isolation were incubated with a FITC-labeled anti-L-selectin mAb, Dreg-56-FITC, for 45 min at 4 °C. Thereafter, specimens were fixed with 1% formaldehyde and analyzed by flow cytometry. Monocytes treated with chymotrypsin (0.4 U/10^6 cells) for 10 min at RT were used for the determination of background FITC-fluorescence [21]. The background FITC-fluorescence intensity was subtracted from anti-L-selectin-FITC-labeled monocytes. Results were expressed as the percentage of anti-L-selectin-FITC specific fluorescence intensity of monocytes after Percoll isolation. A similar procedure was used to quantify L-selectin expression levels in neutrophils.

Quantitation of aggregation. PKH26 Red-stained monocytes, Vybrant-DiD stained neutrophils, and FITC-labeled platelets were identified on the basis of their characteristic forward-scatter, side-scatter, and fluorescence profiles in a FACSCalibur flow cytometer (Fig. 1). PKH26 Red, DiD, and FITC are excited efficiently at 488 nm by the argon laser of a flow cytometer, and their emission spectra are well separated (567 nm for PKH26 Red, 665 nm for DiD, and 515 nm for FITC), thereby allowing simultaneous three-color immunofluorescence measurements. Acquisition and processing of 5000 PKH26 Red-stained monocytes was then used to determine (1) the population distribution of platelets in heterotypic aggregates (Figs. 1C, D, G, and H), and (2) the percent of monocytes bound to platelets (Figs. 1A and B), the percent of neutrophils bound to platelets (Figs. 1E and F), and the percent of monocytes and neutrophils in three-cell aggregates (containing platelets, monocytes, and neutrophils). The following strategy was used to determine the number of platelets bound to monocytes and neutrophils. The mean and standard deviation of a single platelet FITC-fluorescence (green) intensity was computed. Six times the observed standard deviation provides the range of a single platelet fluorescence event with a 99% confidence [18]. The same methodology was used to calculate the green-fluorescence background range of a single monocyte and that of a single neutrophil (Populations 'M' and 'N' in Figs. 1C and D and G and H, respectively). Superimposition of integral multiples of the fluorescence intensity range for single platelets to the computed threshold value of monocyte or neutrophil green fluorescence gives the fluorescence ranges for heteroaggregates containing progressively higher number of platelets (Fig. 1). For instance, P_1M and P_2M represent a monocyte with one and two adherent platelets, respectively. Using this strategy, heterotypic aggregates containing up to four platelets and one monocyte and/or one neutrophil were detected and enumerated. Heterotypic aggregates containing four or more adherent platelets represented <3% of the total monocyte population and were therefore lumped together. A similar procedure was used to quantify the potential presence of multiple monocytes and neutrophils bound to platelets. For instance, M_2-P corresponds to a monocyte doublet with adherent platelets. However, homotypic leukocyte aggregates were rare events.

Simultaneous three-color immunofluorescence measurements and CellQuest analysis allowed us to detect monocyte–neutrophil–platelet triple aggregates. Three-cell aggregates exhibited all three different fluorescence values of monocytes, neutrophils, and platelets. Hence, it was possible to distinguish this population from the pool of singlets and two-cell aggregates.

Confocal microscopy. PKH-red-labeled monocytes, DiD-labeled neutrophils, and FITC-labeled platelets in the presence and absence of a chemical agonist were subjected to prescribed levels of shear rate and shear exposure time in a cone-and-plate rheometer. Upon termination of shear, samples were immediately fixed and processed as previously described [22]. Images were obtained on a Zeiss Meta 510 scanning confocal microscope (Fig. 2).

Adhesion efficiencies. The probability with which colliding cells adhere and form stable aggregates is termed adhesion efficiency. This index is a function of the intrinsic biological characteristics of the cells that are pertinent to their aggregation behavior such as number and affinity of receptors and their response to applied shear. The two-body collision frequency per unit volume, $f_{[(i,j),(k,l)]}$, is a function of the physical parameters of the experimental system, and can be calculated by the Smoluchowski equation:

$$f_{[(i,j),(k,l)]} = \frac{4}{3}\frac{G[r(i,j)+r(k,l)]^3 C(i,j)C(k,l)}{(1+\delta_{i,k}\delta_{j,l})}, \quad (1)$$

in which $r(i,j)$ and $r(k,l)$ are the radii of the two colliding particles, one composed of i platelet + j monocyte or neutrophil singlets and the other one composed of k platelet + l monocyte or neutrophil singlets, $C(i,j)$ and $C(k,l)$ are their respective concentrations, G is the shear rate, and $\delta_{i,k}$ is the Kronecker delta function. The radii of monocytes and neutrophils were measured by light microscopy and found to be 4.2 and 3.9 μm, respectively, whereas the radius for a platelet singlet was set to 1.34 μm [18].

The rate of stable heterotypic aggregate formation is obtained by fitting the aggregation data over the first 15 s after application of shear with a mathematical model based on Smoluchowski's two-body collision theory. This model (Eq. (2)) describes the temporal change of the concentrations of aggregates $C(k,l)$ composed of k platelet and l monocyte or neutrophil singlets [16–18,23]:

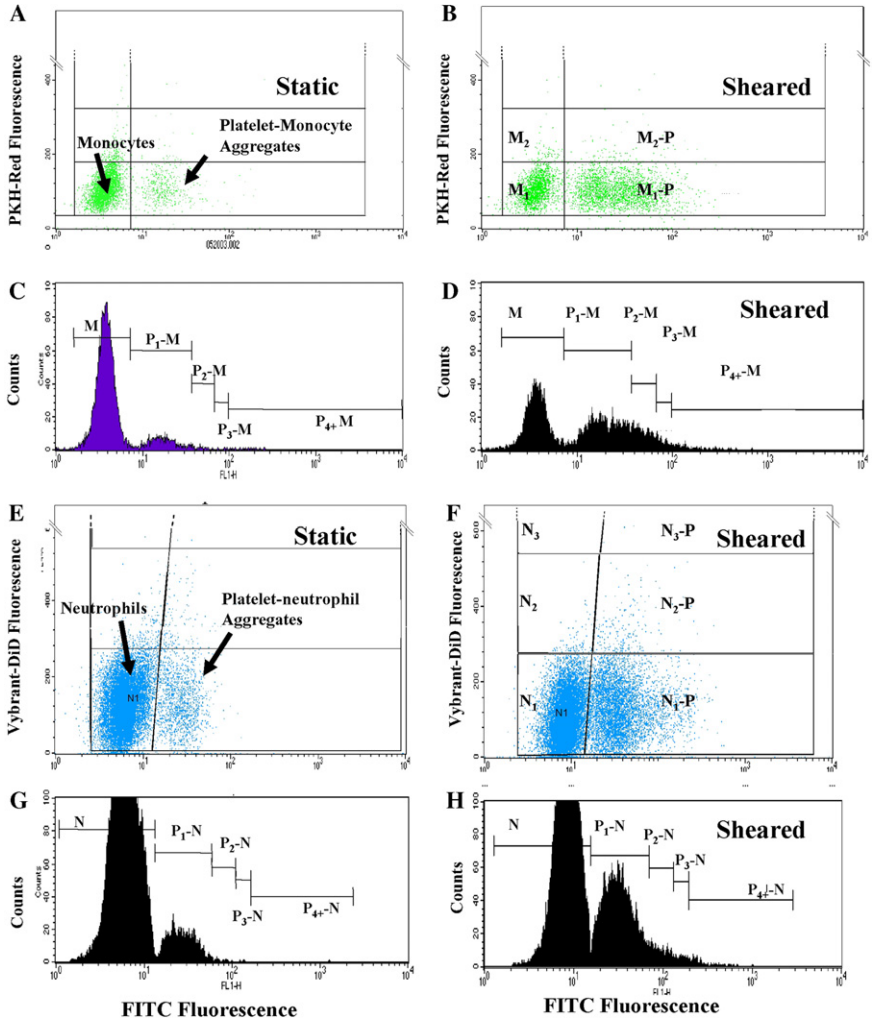

Fig. 1. Detection of monocyte–platelet and neutrophil–platelet heterotypic aggregates by flow cytometry. PKH-Red-stained monocytes (1×10^6 cells/ml), Vybrant DiD-stained neutrophils (4×10^6 cells/ml), and platelets (5×10^7 cells/ml) were subjected to either $0\ s^{-1}$ or $800\ s^{-1}$ for 15 s at 37 °C. Upon termination of shear, aliquots were obtained, immediately fixed with 1% formaldehyde, post-labeled with a FITC-conjugated platelet specific antibody (c7E3-FITC), and subsequently analyzed by flow cytometry. (A) Unsheared monocyte–platelet suspensions after 15 s, (B) monocyte–platelet suspensions subjected to $800\ s^{-1}$ for 15 s, (E) unsheared neutrophil–platelet suspensions after 15 s, (F) neutrophil–platelet suspensions subjected to $800\ s^{-1}$ for 15 s. The vertical lines in (A, B, E, and F) correspond to the FITC-fluorescence threshold that separates nonadherent monocytes (A, B, left) or nonadherent neutrophils (E, F, left) from those bound to platelets (right). (C, D, G, and H) show corresponding mean platelet fluorescence histograms that were used to determine the platelet size distribution in aggregates.

$$\frac{dC(k,l)}{dt} = \sum_{i=0}^{k}\sum_{j=0}^{l}[0.5(1+\delta_{i,k-i}\delta_{j,l-j})K_{[(i,j),(k-i,l-j)]}C(i,j)C(k-i,l-j)] - \sum_{i=k}^{k_{max}}\sum_{j=l}^{l_{max}}[(1+\delta_{k,i-k}\delta_{l,j-l})K_{[(k,l),(i-k,j-l)]}C(k,l) \times C(i-k,j-l)], \quad (2)$$

in which $K_{[(i,j),(k,l)]}$ is the aggregation rate coefficient, k_{max} represents the maximal number of platelets in an aggregate, and l_{max} represents the maximal number of monocytes (or neutrophils) in an aggregate, respectively. It is to be noted that the aggregation of monocytes and neutrophils with no bound platelets was negligible under the conditions of this study. k_{max} was set equal to 4 and l_{max} was set equal to 2 for monocytes and neutrophils. The first term in the right-hand side of Eq. (2) accounts for the formation of the combination from two smaller aggregates, whereas the second term represents the depletion of the combination due to the formation of a higher order aggregate. The set of coupled differential equations represented by Eq. (2) was integrated using the fourth order Runge–Kutta–Gill method, and the adhesion efficiency given by Eq. (3) was estimated by minimizing the difference between calculated and experimental values of singlet and aggregate species concentrations using the Nelder Mead Simplex method [24].

$$E_{[(i,j),(k,l)]} = \frac{K_{[(i,j),(k,l)]}C(i,j)C(k,l)}{f_{[(i,j),(k,l)]}}. \quad (3)$$

At the beginning of each time step, concentrations of singlets and aggregates were set equal to those obtained from the static assay to ensure that all aggregates formed after a shear exposure time of 15 s were shear-induced. The calculations were performed twice—once each for calculating E_{PM} and E_{PN}, where E_{PM} denotes the adhesion efficiency of a platelet binding to a monocyte, and E_{PN} that of a platelet binding to a neutrophil. The model does not take into account the depletion of platelets by binding to neutrophils while calculating E_{PM} (or the depletion of platelets by binding to monocytes while calculating E_{PN}). This is a valid approximation because the percentage of platelets bound to

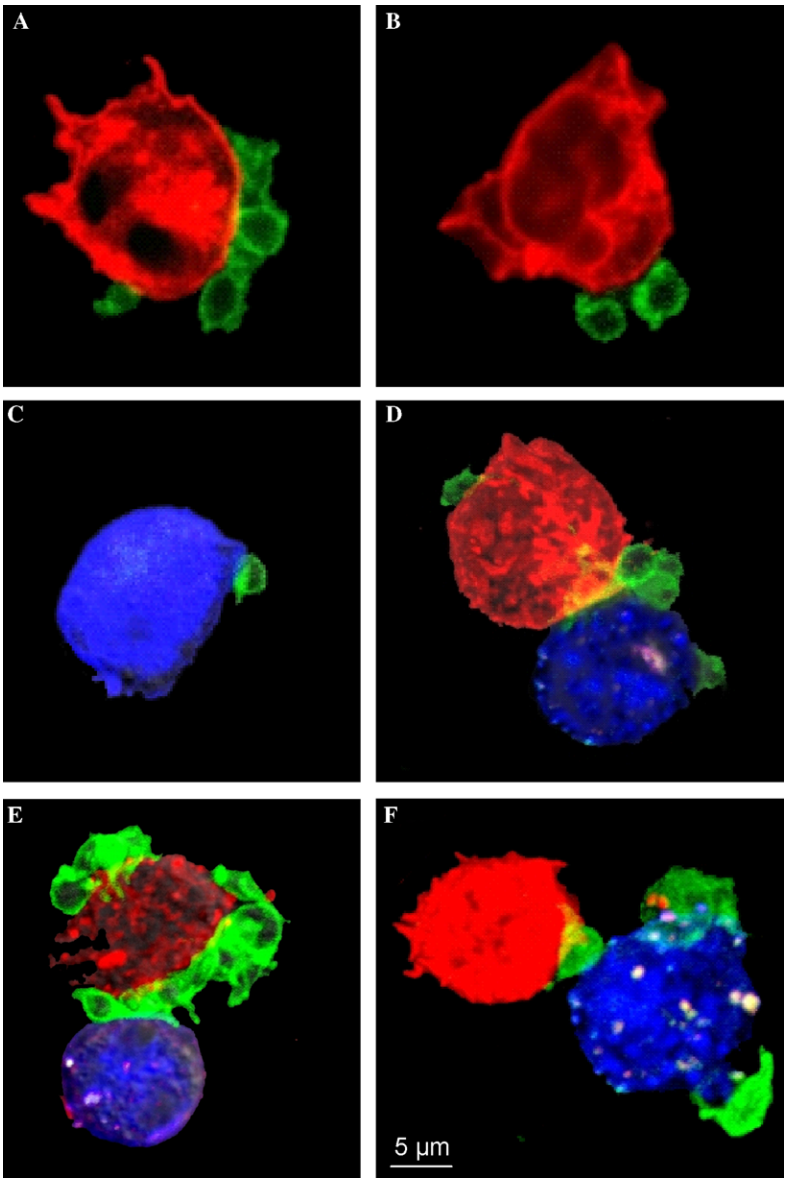

Fig. 2. Visualization of monocyte–platelet, neutrophil–platelet, and monocyte–neutrophil–platelet complexes. PKH-red-stained monocytes (1×10^6 cells/ml), DiD-stained neutrophils (4×10^6 cells/ml), and FITC-labeled thrombin-activated platelets (5×10^7 cells/ml) were subjected to a uniform shear rate of 800 s^{-1} for 60 s in a cone-and-plate rheometer. Confocal microscopy images show (A) a monocyte with two adherent platelets, (B) a monocyte with five adherent platelets, (C) a neutrophil with one adherent platelet, (D–F) a monocyte and neutrophil bridged by multiple platelets. (Scale bar = 5 μm).

either monocytes or neutrophils is negligible compared to the number of platelet singlets (<5% of the total platelet number even under the most extreme conditions analyzed in this study (800 s^{-1}, fMLP-stimulated monocytes and neutrophils)). Thus, the probability of a collision of a platelet in an aggregate with either monocytes or neutrophils is negligible compared to that of a collision of a platelet singlet with either monocytes or neutrophils. As a way of taking into account this temporal depletion of platelets, we considered the extreme case where all platelets bound to monocytes were neglected and the values of E_{PN} were recalculated. This approach yielded values of E_{PN} with less than 5% difference between the original value of E_{PN} and the modified one, even for the most extreme conditions studied (data not shown).

Statistics. Data are expressed as means ± SEM unless otherwise stated. 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 Tukey test.

Results

Monocytes and neutrophils retain L-selectin surface expression levels after isolation

L-selectin expression levels on the monocyte surface were measured by flow cytometry after the first (Percoll) as well as the final isolation step to check for possible cell activation during the separation process (Fig. 3).

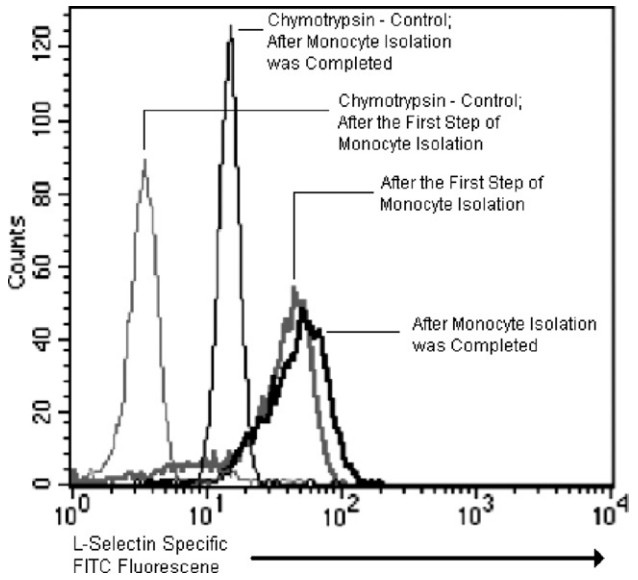

Fig. 3. L-selectin levels of monocytes measured after the first (Percoll) step of monocyte isolation and after monocyte isolation was completed. Surface L-selectin levels of monocytes were measured after the first step of monocyte isolation (gray histogram) and after monocyte-isolation was completed (black histogram). Dreg-56 FITC was used to label L-selectin on monocytes. Monocytes treated with chymotrypsin (0.4 U/10^6 cells) for 10 min at RT were used for background fluorescence.

Results show small differences in the L-selectin levels of finally isolated monocytes (89.3% ± 4.6% ($n = 7$) of the L-selectin expression levels of monocytes after Percoll isolation). Similar L-selectin retention levels (81.0% ± 5.8% SEM ($n = 12$)) were detected after neutrophil isolation.

Hydrodynamic shear-induced collisions preferentially augment monocyte–platelet heteroaggregation

In the present study, we examined two major cell types in the leukocyte family, monocytes and neutrophils, and their adhesive behavior with platelets. To investigate how the hydrodynamic shear environment of the vasculature modulates adhesive interactions among these three blood cell types, isolated monocytes and neutrophils were combined with platelets suspended in plasma in a cone-and-plate rheometer, and subjected to controlled levels of shear for prescribed periods of time. For all shear experiments, cell concentrations of 1×10^6 cells/ml monocytes, 4×10^6 cells/ml neutrophils, and 5×10^7 cells/ml platelets were maintained. The neutrophil and monocyte concentrations were chosen to match those found in vivo. The subphysiological concentration of platelets was selected to prevent homotypic platelet–platelet aggregation which might interfere with leukocyte–platelet heterotypic interactions.

In the absence of shear, baseline levels of platelet–monocyte binding (~12% of the total monocyte population) and platelet–neutrophil binding (~8% of the total neutrophil population) were detected at the 15 s shear exposure time (Fig. 4A). Application of shear in the absence of any exogenously added chemical agonist augmented monocyte–platelet heteroaggregation more drastically than neutrophil–platelet heteroaggregation (Fig. 4A). This preferential recruitment of platelets by monocytes was noted at all shear levels and shear exposure times studied here. The extent of monocyte–platelet heterotypic aggregation increased with increasing shear exposure time over a wide range of shear rates (Fig. 4A). Moreover, platelet binding to monocytes increased with increasing shear rate at early time points while smaller differences were detected at longer shear exposure times.

Effects of exogenous cell activation on leukocyte–platelet aggregate formation

The recruitment of platelets by monocytes (Fig. 4B) and neutrophils (Fig. 4C) was potentiated over a wide range of shear rates at both shear exposure times upon activation of platelets with thrombin in the presence of fibrin polymerization inhibitor GPRP-NH_2. It is noteworthy that upon thrombin/GPRP-NH_2 stimulation, heterotypic monocyte–platelet or neutrophil–platelet aggregates containing multiple (four or more) platelets were consistently observed as opposed to the smaller aggregates observed in the absence of thrombin activation (Figs. 4B and C). Interestingly, thrombin/GPRP-NH_2 stimulation in the presence of hydrodynamic shear resulted in the formation of triple aggregates containing monocytes, neutrophils, and multiple platelets (Fig. 2). These triple aggregates were more evident for the longer shear exposure of 60 s at all shear rates (Fig. 4D).

To investigate how monocyte and neutrophil activation affects the extent of heteroaggregation, cells were stimulated with the chemotactic bacterial peptide fMLP (1 μM) 1 s prior to the application of shear. Formation of monocyte–platelet aggregates was drastically enhanced at all shear rates for 15 s of shear exposure time (Fig. 4B). Smaller differences were noted for the longer shear exposure time of 60 s (Fig. 4B). Interestingly, fMLP-stimulated monocytes were able to recruit more than one platelet more efficiently than untreated monocytes at all shear rates at short shear exposure times. In marked contrast, neutrophils displayed no significant change in their ability to recruit platelet upon stimulation with fMLP (Fig. 4C).

The adhesion efficiency of platelet capture by monocytes and neutrophils provides a measure of the biological properties of the cells that control their aggregation behavior, and was computed by fitting the aggregation data over the first 15 s after application of shear [17,18,23]. Fig. 4E shows the shear rate dependence of adhesion efficiencies in the absence and presence of

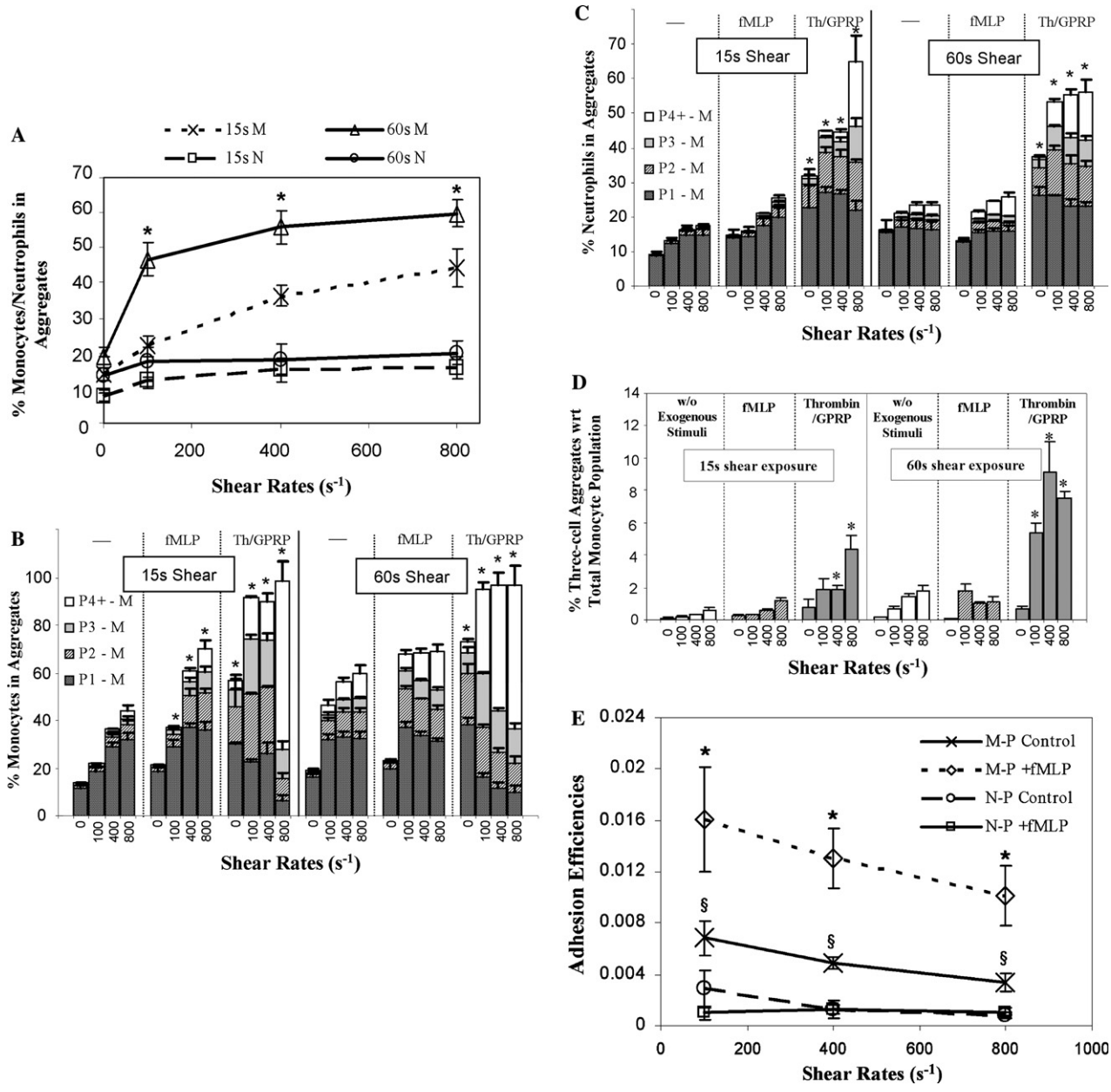

Fig. 4. Kinetics of monocyte–platelet and neutrophil–platelet aggregate formation. (A) PKH-Red-stained monocytes (1×10^6 cells/ml), Vybrant-DiD-stained neutrophils (4×10^6 cells/ml), and platelets (5×10^7 cells/ml) were subjected to well-defined levels of shear. Plot shows the percentage of monocytes or neutrophils with adherent platelets for a shear exposure time of 15 s (monocytes, × and neutrophils, □) or 60 s (monocytes, △ and neutrophils, ○). *$p < 0.05$ with respect to 15 s monocyte–platelet heteroaggregation. In selected experiments, platelets, pretreated for 10 min at 37 °C with thrombin (2 U/ml) along with the fibrin polymerization inhibitor GPRP-NH_2 (2 mM), were combined with PKH-Red-stained monocytes and Vybrant-DiD-stained neutrophils, and sheared at the indicated levels of shear for 15 s, or 60 s. In another set of runs, monocytes and neutrophils were stimulated using chemotactic peptide fMLP (1 μM) 1 s prior to being sheared with platelets. Graphs show the percentage of monocytes (B) and neutrophils (C) bound to platelets. *$p < 0.05$ with respect to no-treatment controls. (D) Extent of three-species aggregates (containing monocytes, neutrophils, and platelets) based on the total monocyte number. (E) Adhesion efficiencies of platelet binding to monocytes and neutrophils in the absence (monocytes, × and neutrophils, ○) and presence of chemical stimulation with fMLP (monocytes, ◊ and neutrophils □). *$p < 0.05$ with respect to adhesion efficiency for resting monocyte-platelet heteroaggregation. §$p < 0.05$ with respect to neutrophil–platelet adhesion efficiency. Data represent means ± SEM of 3–5 experiments.

chemical stimuli. Maximal adhesion efficiencies in the absence of any exogenously added chemical agonist were observed at the lowest shear rate ($100\ s^{-1}$), at which ~7 of 1000 collisions led to stable monocyte–platelet aggregate formation, whereas ~3 of 1000 collisions led to stable neutrophil–platelet aggregate formation. Monocyte–platelet and neutrophil–platelet adhesion efficiency varied modestly with increasing shear. It is noteworthy that the pure homotypic platelet–platelet, monocyte–monocyte, and neutrophil–neu-

trophil adhesion efficiencies were found to be minimal at all shear rates (data not shown). fMLP-stimulation resulted in enhanced adhesion efficiency of platelet binding to monocytes (~16 of 1000 collisions resulting in stable aggregate formation at 100 s^{-1}) at all shear rates, whereas no significant change was recorded for the adhesion efficiency between fMLP-stimulated neutrophils and platelets.

Role of P-selectin, PSGL-1, β2 integrins, and divalent cations in monocyte–platelet heteroaggregation

Ensuing experiments focused on the elucidation of the molecular constituents mediating platelet binding to monocytes under dynamic shear conditions. As a first step, the roles of P-selectin and PSGL-1 were examined in the absence of exogenous stimuli at both low (100 s^{-1}) and high (800 s^{-1}) shear after 15 and 60 s of shear exposure. The results indicate that incubation of platelets with a function-blocking anti-P-selectin mAb inhibited the extent of monocyte adhesive interactions with platelets to the baseline level for both short (15 s) and long (60 s) shear exposure times (Table 1). Similarly, an anti-PSGL-1 function-blocking mAb was equally effective in reducing monocyte–platelet aggregate formation to basal levels in the absence of an exogenously added agonist (Table 1). The potential contribution of monocyte CD18-integrins was investigated using an anti-CD18 function-blocking mAb. Addition of receptor antagonists directed against CD18-integrins failed to suppress monocyte–platelet adhesive interactions under shear flow (Table 1). Similarly, inclusion of an $\alpha_{IIb}\beta_3$ antagonist failed to reduce the extent of monocyte–platelet heteroaggregation under shear (data not shown). In light of a recent report suggesting that activated platelets upregulate VLA-4 ($\alpha_4\beta_1$) integrin capacity [25], we examined the potential contribution of β_1 integrins in monocyte–platelet binding. However, blocking β_1 integrins failed to inhibit the extent of heteroaggregation (data not shown). Cumulatively, these data suggest that platelet attachment to monocytes, in the absence of exogenous stimuli, is strictly P-selectin/PSGL-1-dependent, whereas neither monocyte CD18 or β_1 integrins nor platelet $\alpha_{IIb}\beta_3$ integrins are involved in the heteroaggregation process both in the low (100 s^{-1}) and high (800 s^{-1}) regimes of shear.

Shear experiments were also performed in the presence of EDTA to assess the requirement of divalent cations in monocyte–platelet adhesive interactions. Previous work has established that these ions are necessary for selectin-mediated adhesion [9]. When EDTA (5 mM) was added to the suspension medium, the extent of platelet binding to monocytes decreased to background level ~5% at both 100 and 800 s^{-1} after 60 s of shear exposure.

Characterization of molecular mechanisms mediating monocyte–platelet heteroaggregation in the presence of exogenously added stimuli

We next examined the molecular requirements of monocyte–platelet aggregation in sheared suspensions in the presence of either exogenously added thrombin/GPRP-NH_2 or fMLP. Blocking either P-selectin or PSGL-1 was equally effective in essentially eliminating the recruitment of thrombin-activated platelets by untreated monocytes at both 100 and 800 s^{-1} (Table 1). These observations support the notion that PSGL-1 on the monocyte surface acts as the primary ligand for platelet P-selectin, and this molecular interaction is necessary for optimal monocyte–platelet aggregation under conditions of hydrodynamic shear. In contrast, blocking CD18-integrins did not significantly alter monocyte–platelet adhesion (Table 1). Moreover, the platelet $\alpha_{IIb}\beta_3$ specific antagonist XV454 failed to reduce the percentage of monocytes with bound platelets in the presence of exogenously added thrombin/GPRP.

In the presence of the exogenously added chemotactic peptide fMLP, blocking either platelet P-selectin or monocyte PSGL-1 reduced monocyte–platelet aggrega-

Table 1
Role of P-selectin, PSGL-1, and CD18 in monocyte–platelet heteroaggregation at low (100 s^{-1}) and high (800 s^{-1}) shear for shear exposure times of 60 and 120 s

Stimulation	100 s^{-1}					800 s^{-1}				
	−	+	CD62P	CD162	CD18	−	+	CD62P	CD162	CD18
—	14.4 ± 7.3	/	7.3 ± 1.0	8.4 ± 0.3	17.8 ± 1.8	20.7 ± 1.8	/	7.1 ± 0.7	8.2 ± 0.9	31.0 ± 3.1
+Th/GPRP	19.4 ± 2.8	74.3 ± 8.7	8.3 ± 2.2	8.6 ± 1.1	71.8 ± 4.0	36.9 ± 5.3	85.2 ± 4.2	9.0 ± 4.2	7.8 ± 1.2	78.0 ± 3.9
+fMLP (60 s)	16.7 ± 1.6	22.3 ± 2.0	11.0 ± 2.4	7.3 ± 0.9	17.4 ± 1.4	29.6 ± 3.9	43.4 ± 3.0	11.3 ± 1.9	6.3 ± 0.7	31.6 ± 0.8
+fMLP (120 s)	27.2 ± 0.3	31 ± 0.6	n/d	n/d	27.6 ± 1.2	38.9 ± 1.3	45.7 ± 1.9	n/d	n/d	40.9 ± 0.6

PKH red-stained monocytes and platelets were subjected to uniform levels of shear for either 60 or 120 s. In select runs, monocytes were treated with a mAb specific against either PSGL1 or CD-18 integrins for 10 min at 37 °C prior to shearing with platelets. In another set of experiments, platelets were incubated with an anti-P-selectin mAb for 10 min at RT prior to the application of shear. All experimental runs were also performed in the presence of 1 μM fMLP to stimulate monocytes. In other set of experiments, platelets were incubated with thrombin (2 U/ml) and GPRP-NH_2 for 10 min at 37 °C; static = 8.8 ± 0.7; n/d, not done. -, in the absence of chemical stimulus. +, in the presence of chemical stimulus.

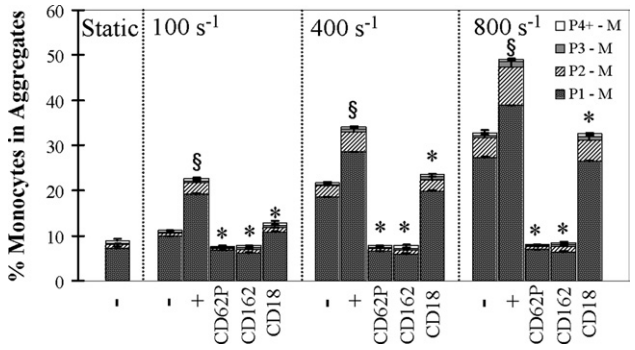

Fig. 5. Effects of platelet P-selectin (CD62P), monocyte PSGL-1 (CD162), and CD18-integrin antagonists on monocyte–platelet heteroaggregation at low (100 s^{-1}), moderate (400 s^{-1}), and high (800 s^{-1}) shear for a shear exposure time of 15 s. Platelets treated for 10 min at RT with an agent specific for P-selectin (AK4) were combined with fMLP-stimulated PKH-Red-stained monocytes in a cone-and-plate rheometer and subjected to 100, 400, or 800 s^{-1} for 15 s. Alternatively, monocytes were preincubated with blocking mAbs specific for PSGL-1 (KPL-1) or CD18-integrins (6.7) for 10 min at 37 °C followed by fMLP-stimulation 1 s prior to shearing with untreated platelets. Data represent means ± SEM of three experiments. $^{\S}p < 0.05$ with respect to monocyte-platelet heteroaggregation in the absence of exogenous stimulation. $^{*}p < 0.05$ with respect to monocyte–platelet heteroaggregation upon fMLP-stimulation of monocytes.

tion to basal levels at both 100 and 800 s^{-1} after both 15 (Fig. 5) and 60 s (Table 1) of shear exposure. Moreover, in marked contrast to the results obtained in the absence of any exogenously added stimuli, an anti-CD18 mAb significantly inhibited the enhanced levels of heteroaggregation conferred upon fMLP-stimulation at all rates after 15 s of shear exposure (Fig. 5). However, it is to be noted that the extent of potentiation of monocyte–platelet heterotypic aggregation was maximal after 15 s of shear exposure, whereas subtle differences were noted at longer shear exposure times. Along these lines, progressively smaller inhibitory effects were detected upon use of an anti-CD18 mAb at longer shear exposure times (Table 1).

Discussion

The major findings of this study are: (1) hydrodynamic shear-induced collisions preferentially augment the ability of platelets to recruit monocytes over neutrophils, (2) the extent of heterotypic adhesion is significantly potentiated upon platelet activation by thrombin/GPRP-NH_2, whereas stimulation of monocytes and neutrophils with fMLP moderately enhances the extent of monocyte–platelet, but not neutrophil–platelet, heteroaggregation levels; (3) platelet P-selectin binding to monocyte PSGL-1 is necessary and sufficient for maximal platelet–monocyte heteroaggregation in the absence of any exogenously added chemical agonist under all shear rates examined in this work; (4) the enhanced platelet binding to fMLP-treated monocytes involves a sequential two-step process wherein P-selectin-PSGL-1 interactions are stabilized by CD18 integrins. Cumulatively, these data provide strong evidence that hydrodynamic shear and the state of cell activation affect the binding kinetics and molecular constituents mediating platelet binding to monocytes and neutrophils.

Untreated platelets aggregate preferentially with monocytes relative to neutrophils when subjected to well-defined hydrodynamic shear in the absence of any exogenous chemical stimulation. Monocyte binding to platelets increases with increasing shear rate and shear exposure time. The adhesion efficiency of heteroaggregation, defined as the probability with which colliding cells form stable aggregates, varies modestly with increasing levels of hydrodynamic shear, from ~7 to ~3 of 1000 collisions leading to aggregate formation at 100 and 800 s^{-1}, respectively. Under these experimental conditions, the molecular interaction between platelet P-selectin and monocyte PSGL1 is necessary and sufficient for optimal monocyte–platelet binding. The absolute requirement of PSGL-1 in the heteroaggregation process between monocytes (or neutrophils) and platelets coupled with the markedly elevated PSGL-1 density on monocytes relative to neutrophils (47,200 ± 9900 vs 26,500 ± 4500 copies of PSGL-1 on their surfaces, respectively [26]) may help explain the preferential recruitment of platelets by monocytes.

Pre-treatment of platelets with thrombin-GPRP-NH_2, known to upregulate platelet surface P-selectin expression [18], significantly increased the extent of monocyte–platelet binding. This finding is in contrast to recently published data indicating that application of shear stress diminishes the formation of platelet–leukocyte complexes induced by ADP [15]. However, this discrepancy can be reconciled by the presence of homotypic platelet aggregation [15] which might interfere with the formation of leukocyte–platelet complexes. Furthermore, upon platelet activation with thrombin/GPRP, we observed the formation of aggregates containing all three cell types in shear flow. The formation of these aggregates was minimal under all other experimental conditions listed here. Taken together, these observations suggest that potent activation of platelets with thrombin induces the formation of leukocyte aggregates consisting of one monocyte, one neutrophil, and multiple platelets which may play an essential role in bridging monocytes and neutrophils, as evidenced by confocal microscopy images (Fig. 2). This observation is in good accord with previous studies which showed that platelets are capable of bridging two leukocytes to form heterotypic aggregates [9].

Stimulation of leukocytes with fMLP has been reported to result in the upregulation of CD11b/CD18-integrin surface expression levels as well as CD18 integrin affinity, though the latter decays with time [27]. At the

15 s time point, the CD18 avidity is high, hence irrespective of the shear rate, aggregation levels are potentiated by fMLP-stimulation of monocytes. This is further supported by the fact that use of an anti-CD18 mAb results in aggregation levels similar to those in the case of unstimulated monocytes. On the other hand, at a shear exposure time of 120 s, we speculate that the monocyte CD18 affinity is markedly reduced, as recently shown for neutrophils [27], so as to prevent a substantial increase in the aggregation levels upon fMLP-stimulation. Furthermore, anti-CD18 mAbs have lesser of an effect on aggregation levels at this time point. Thus, our data suggest that a temporal CD18-integrin-activation on the monocyte surface may occur upon fMLP-stimulation, similar to that observed in the case of neutrophils and homotypic neutrophil activation. Alternatively, at longer shear exposure times, the elevated levels of P-selectin on the platelet surface may partially mask the contribution of CD18 integrins in the heteroaggregation process.

Taken altogether, this work demonstrates the preferential recruitment of platelets by monocytes relative to neutrophils in shear flow. Moreover, it shows that the fluid mechanical environment of the circulatory system in conjunction with the state of platelet and monocyte activation affects the kinetics and molecular constituents mediating the recruitment of platelets by monocytes, critical to the pathogenesis and/or progression of acute coronary syndromes.

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References

[1] A.S. Weyrich, M.R. Elstad, R.P. McEver, T.M. McIntyre, K.L. Moore, J.H. Morrissey, S.M. Prescott, G.A. Zimmerman, Activated platelets signal chemokine synthesis by human monocytes, J. Clin. Invest. 97 (1996) 1525–1534.

[2] F.J. Neumann, N. Marx, M. Gawaz, K. Brand, I. Ott, C. Rokitta, C. Sticherling, C. Meinl, A. May, A. Schomig, Induction of cytokine expression in leukocytes by binding of thrombin-stimulated platelets, Circulation 95 (1997) 2387–2394.

[3] K. Konstantopoulos, J.C. Grotta, C. Sills, K.K. Wu, J.D. Hellums, Shear-induced platelet aggregation in normal subjects and stroke patients, Thromb. Haemost. 74 (1995) 1329–1334.

[4] J.K. Mickelson, N.M. Lakkis, G. Villarreal-Levy, B.J. Hughes, C.W. Smith, Leukocyte activation with platelet adhesion after coronary angioplasty: a mechanism for recurrent disease? J. Am. Coll. Cardiol. 28 (1996) 345–353.

[5] A. Kumar, M.P. Villani, U.K. Patel, J.C. Keith Jr., R.G. Schaub, Recombinant soluble form of PSGL-1 accelerates thrombolysis and prevents reocclusion in a porcine model, Circulation 99 (1999) 1363–1369.

[6] J.G. Bienvenu, J.F. Tanguay, J.F. Theoret, A. Kumar, R.G. Schaub, Y. Merhi, Recombinant soluble p-selectin glycoprotein ligand-1-Ig reduces restenosis through inhibition of platelet-neutrophil adhesion after double angioplasty in swine, Circulation 103 (2001) 1128–1134.

[7] T.G. Diacovo, S.J. Roth, J.M. Buccola, D.F. Bainton, T.A. Springer, Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18, Blood 88 (1996) 146–157.

[8] V. Evangelista, S. Manarini, S. Rotondo, N. Martelli, R. Polischuk, J.L. McGregor, G. de Gaetano, C. Cerletti, Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the beta 2 integrin CD11b/CD18, Blood 88 (1996) 4183–4194.

[9] K. Konstantopoulos, S. Neelamegham, A.R. Burns, E. Hentzen, G.S. Kansas, K.R. Snapp, E.L. Berg, J.D. Hellums, C.W. Smith, L.V. McIntire, S.I. Simon, Venous levels of shear support neutrophil–platelet adhesion and neutrophil aggregation in blood via P-selectin and beta2-integrin, Circulation 98 (1998) 873–882.

[10] S. Sheikh, G.B. Nash, Continuous activation and deactivation of integrin CD11b/CD18 during de novo expression enables rolling neutrophils to immobilize on platelets, Blood 87 (1996) 5040–5050.

[11] C. Weber, T.A. Springer, Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to alphaIIbbeta3 and stimulated by platelet-activating factor, J. Clin. Invest. 100 (1997) 2085–2093.

[12] A.D. Michelson, M.R. Barnard, L.A. Krueger, C.R. Valeri, M.I. Furman, Circulating monocyte–platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin: studies in baboons, human coronary intervention, and human acute myocardial infarction, Circulation 104 (2001) 1533–1537.

[13] M.I. Furman, M.R. Barnard, L.A. Krueger, M.L. Fox, E.A. Shilale, D.M. Lessard, P. Marchese, A.L. Frelinger, 3rd, R.J. Goldberg, A.D. Michelson, Circulating monocyte–platelet aggregates are an early marker of acute myocardial infarction, J. Am. Coll. Cardiol. 38 (2001) 1002–1006.

[14] P.H. Kuijper, H.I. Gallardo Torres, L.A. Houben, J.W. Lammers, J.J. Zwaginga, L. Koenderman, P-selectin and MAC-1 mediate monocyte rolling and adhesion to ECM-bound platelets under flow conditions, J. Leukoc. Biol. 64 (1998) 467–473.

[15] H. Hu, D. Varon, P. Hjemdahl, N. Savion, S. Schulman, N. Li, Platelet–leukocyte aggregation under shear stress: Differential involvement of selectins and integrins, Thromb. Haemost. 90 (2003) 679–687.

[16] I.J. Laurenzi, S.L. Diamond, Monte Carlo simulation of the heterotypic aggregation kinetics of platelets and neutrophils, Biophys. J. 77 (1999) 1733–1746.

[17] S. Jadhav, K. Konstantopoulos, Fluid shear- and time-dependent modulation of molecular interactions between PMNs and colon carcinomas, Am. J. Physiol. Cell Physiol. 283 (2002) C1133–1143.

[18] O.J. McCarty, S. Jadhav, M.M. Burdick, W.R. Bell, K. Konstantopoulos, Fluid shear regulates the kinetics and molecular mechanisms of activation-dependent platelet binding to colon carcinoma cells, Biophys. J. 83 (2002) 836–848.

[19] J.P. Abulencia, N. Tien, O.J. McCarty, D. Plymire, S.A. Mousa, K. Konstantopoulos, Comparative antiplatelet efficacy of a novel, nonpeptide GPIIb/IIIa antagonist (XV454) and abciximab (c7E3) in flow models of thrombosis, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 149–156.

[20] B.S. Bochner, S.A. Sterbinsky, C.A. Bickel, S. Werfel, M. Wein, W. Newman, Differences between human eosinophils and neutrophils in the function and expression of sialic acid-containing counterligands for E-selectin, J. Immunol. 152 (1994) 774–782.

[21] S. Kukreti, K. Konstantopoulos, C.W. Smith, L.V. McIntire, Molecular mechanisms of monocyte adhesion to interleukin-

1beta-stimulated endothelial cells under physiologic flow conditions, Blood 89 (1997) 4104–4111.
[22] M.M. Burdick, K. Konstantopoulos, Platelet-induced enhancement of LS174T colon carcinoma and THP-1 monocytoid cell adhesion to vascular endothelium under flow, Am. J. Physiol. Cell Physiol. 287 (2004) C539–547.
[23] P. Pawar, P.K. Shin, S.A. Mousa, J.M. Ross, K. Konstantopoulos, Fluid shear regulates the kinetics and receptor specificity of *Staphylococcus aureus* binding to activated platelets, J. Immunol. 173 (2004) 1258–1265.
[24] J.A. Nelder, R. Mead, A simplex method for function minimization, Comput. J. 7 (1965) 308–313.
[25] Y. Huo, A. Schober, S.B. Forlow, D.F. Smith, M.C. Hyman, S. Jung, D.R. Littman, C. Weber, K. Ley, Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E, Nat. Med. 9 (2003) 61–67.
[26] J. Kappelmayer, A. Kiss, E. Karaszi, A. Veszpremi, J. Jako, C. Kiss, Identification of P-selectin glycoprotein ligand-1 as a useful marker in acute myeloid leukaemias, Br. J. Haematol. 115 (2001) 903–909.
[27] S. Neelamegham, A.D. Taylor, H. Shankaran, C.W. Smith, S.I. Simon, Shear and time-dependent changes in Mac-1, LFA-1, and ICAM-3 binding regulate neutrophil homotypic adhesion, J. Immunol. 164 (2000) 3798–3805.