Journal of Thrombosis and Haemostasis, **3**: 2545–2553

ORIGINAL ARTICLE

Two subpopulations of thrombin-activated platelets differ in their binding of the components of the intrinsic factor X-activating complex

M. A. PANTELEEV,*†[1] N. M. ANANYEVA,†‡[1] N. J. GRECO,§ F. I. ATAULLAKHANOV*¶** and E. L. SAENKO†‡

**National Research Center for Hematology, Russian Academy of Medical Sciences, Moscow, Russia; †Holland Laboratory, American Red Cross, Rockville, Maryland, USA; ‡Department of Biochemistry & Molecular Biology, University of Maryland School of Medicine, Rockville, Maryland, USA; §Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio, USA; ¶Faculty of Physics, Moscow State University, Leninskie Gory, Moscow, Russia; and **Institute of Theoretical and Experimental Biophysics, Pushchino, Moscow Region, Russia*

To cite this article: Panteleev MA, Ananyeva NM, Greco NJ, Ataullakhanov FI, Saenko EL. Two subpopulations of thrombin-activated platelets differ in their binding of the components of the intrinsic factor X-activating complex. *J Thromb Haemost* 2005; **3**: 2545–53.

Summary. Binding of fluorescein-labeled coagulation factors IXa, VIII, X, and allophycocyanin-labeled annexin V to thrombin-activated platelets was studied using flow cytometry. Upon activation, two platelet subpopulations were detected, which differed by 1–2 orders of magnitude in the binding of the coagulation factors and by 2–3 orders of magnitude in the binding of annexin V. The percentage of the high-binding platelets increased dose dependently of thrombin concentration. At 100 nM of thrombin, platelets with elevated binding capability constituted ~4% of total platelets and were responsible for the binding of ~50% of the total bound factor. Binding of factors to the high-binding subpopulation was calcium-dependent and specific as evidenced by experiments in the presence of excess unlabeled factor. The percentage of the high-binding platelets was not affected by echistatin, a potent aggregation inhibitor, confirming that the high-binding platelets were not platelet aggregates. Despite the difference in the coagulation factors binding, the subpopulations were indistinguishable by the expression of general platelet marker CD42b and activation markers PAC1 (an epitope of glycoprotein IIb/IIIa) and CD62P (P-selectin). Dual-labeling binding studies involving coagulation factors (IXa, VIII, or X) and annexin V demonstrated that the high-binding platelet subpopulation was identical for all coagulation factors and for annexin V. The high-binding subpopulation had lower mean forward and side scatters compared with the low-binding subpopulation (~80% and ~60%, respectively). In its turn, the high-binding subpopulation was not homogeneous and included two subpopulations with different scatter values. We conclude that activation by thrombin induces the formation of two distinct subpopulations of platelets different in their binding of the components of the intrinsic fX-activating complex, which may have certain physiological or pathological significance.

Keywords: echistatin, factor X, flow cytometry, intrinsic tenase, platelet activation, platelets.

Correspondence: Mikhail A. Panteleev, National Research Center for Hematology, Russian Academy of Medical Sciences, Novozykovskii pr. 4a, Moscow, 125167, Russia.
Tel.: +7 095 212 35 22; fax: +7 095 212 88 70; e-mail: mapanteleev@yandex.ru

[1]The first two authors contributed equally to this work.

Received 20 March 2005, accepted 27 July 2005

Introduction

Major reactions of blood coagulation do not proceed efficiently in solution and require negatively charged phospholipid membranes, which provide optimal mutual orientation and redirect interactions among enzymes, substrates and cofactors from a three-dimensional space to a two-dimensional space [1]. Among membrane-dependent reactions are activation of coagulation factor X (fX) by intrinsic [2] and extrinsic [3] tenases, activation of prothrombin by prothrombinase [4], activation of fV, fVII and fVIII by fXa [5–7], etc. In the presence of phospholipids, rates of these reactions are increased by three-to-five orders of magnitude [8]. Although plasma lipoproteins and various cells of blood and vasculature have been shown to support coagulation reactions under certain conditions [9–13], activated blood platelets are considered the principal physiological source of procoagulant membranes [14–16]. Platelets can be activated by a variety of agonists resulting in shape change, adhesion and aggregation, secretion of pro- and anticoagulant substances, expression of procoagulant membranes and shedding of microparticles [14]. The degree of the platelet response depends on the potency of the activator. While ADP, thromboxane A_2 and epinephrine stimulate platelet functions only partially, thrombin and

© 2005 International Society on Thrombosis and Haemostasis

collagen generate all responses, including expression of a procoagulant surface able to bind coagulation factors and support membrane-dependent reactions [14,16].

The binding of coagulation factors to platelets is the first step in all membrane-dependent reactions and its understanding is a prerequisite for the analysis of their mechanisms. The binding of the intrinsic fX-activating complex components to platelets has been a subject of numerous studies using either radiolabeled factors [17,18] or flow cytometric analysis of platelets pre-incubated with factors and fluorescently labeled antibodies to these factors [19]. However, inconsistency remains between results of these experiments, and the heterogeneity of platelets with respect to the binding of coagulation factors is still unclear. Studies with radiolabeled ligands provided only mean binding parameters generated by measuring properties of the entire platelet population. Some flow cytometry studies showed that platelets from a single individual display distinct subpopulations with respect to the binding of fIXa, fX, fXa, and fV [20–25]. In other studies such subpopulations were not observed, in particular, no subpopulations were reported for the fIXa binding to thrombin-stimulated platelets [15,19]. Also, there is no agreement whether one or several activating agents are required to induce/reveal platelet heterogeneity: some reports state that activation by thrombin alone is sufficient [22], whereas other reports conclude that platelet heterogeneity is induced only upon combined activation by thrombin and convulxin [23–25].

We addressed the problem of platelet heterogeneity by studying the binding of all components of the intrinsic fX-activating complex to thrombin-activated platelets in a wide range of thrombin concentrations. To avoid possible loss of the subpopulations phenomenon because of instability of platelet-binding properties [26], we directly studied the binding of fluorescein-labeled coagulation factors to activated platelets. We demonstrated that upon platelet activation by thrombin, two subpopulations are generated and their ratio is determined by thrombin concentration. The subpopulations differ by several orders of magnitude in their binding of fIXa, fVIII, fX, and annexin V, and platelets within the high-binding subpopulation are heterogeneous.

Experimental procedures

Reagents

Human α-thrombin, BSA, PPACK, apyrase grade VII from potato, and echistatin from *Echis carinatus* were from Sigma (St Louis, MO, USA). Prostaglandin E_1 was from MP Biochemicals (Irvine, CA, USA). All other reagents were of analytical quality.

Proteins

Human fX was from Enzyme Research Laboratories (South Bend, IN, USA). Human fIXa-EGR was from Haematologic Technologies (Essex Junction, VT, USA). Human plasma-derived fVIII was purified as described previously [27]. For binding experiments, fIXa-EGR, fVIII, and fX were labeled with fluorescein using FluoReporter Fluorescein-EX Protein Labeling Kit (Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions. The degree of labeling (molecules of dye per molecule of protein) was determined by absorbance at 280 and 494 nm and constituted 0.41, 4.25, and 2.79 for fIXa-EGR, fVIII, and fX, respectively. Proteins were stored at −80 °C in small aliquots (5–10 μL) and thawed only once before use. APC-labeled annexin V was from Molecular Probes. PE-conjugated antihuman CD42b antibody was from eBioscience (San Diego, CA, USA). FITC-conjugated antihuman CD62P and PAC1 antibodies were from Beckton Dickinson Biosciences (San Jose, CA, USA).

Platelet isolation

Platelets were isolated as described [26] with minor modifications from freshly drawn human blood collected from healthy donors under a protocol approved by the Institutional Review Board (Holland Laboratory, American Red Cross, Rockville, MD, USA). Blood was collected into 3.2% sodium citrate at 9:1 v/v ratio. Prostaglandin E1 (1 μM) and apyrase (0.1 unit mL^{-1}) were added to prevent activation. Whole blood was centrifuged at 180 *g* for 15 min at room temperature to obtain platelet-rich plasma. Platelets were concentrated by centrifugation at 500 *g* for 15 min, resuspended in buffer A (150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 20 mM HEPES, 5 mM glucose, 0.5% BSA) and subjected to gel filtration on a 2.5 × 10 cm Sepharose CL-2B (Sigma) chromatography column (Spectrum Chromatography, Houston, TX, USA) equilibrated with buffer A. Cells were quantitated using a Cell-Dyn 3700 hematology analyzer (Abbott Laboratories, Abbott Park, IL, USA). Platelet preparations contained < 0.01% and 1% of white and red blood cells, respectively. Blood from 19 donors was used for this study.

Binding experiments

Platelets were activated by incubation with thrombin (1–200 nM) at 37 °C in buffer A with 5 mM $CaCl_2$ for 5 min. Antihuman PE-labeled CD42b antibody was added at 4% v/v (the quantities of antibodies and annexin V added are given hereafter in volume fractions of the solutions provided by the manufacturer) to identify platelet events in flow cytometry experiments. Thrombin was blocked by 1 μM PPACK prior to addition of fluorescein-labeled coagulation factors or APC-labeled annexin V. These reagents were incubated with platelets in buffer A in the presence of 2.5 mM $CaCl_2$ at 37 °C for 15 min. Control experiments confirmed that after this period of time, equilibrium was achieved (data not shown). After 15 min, the samples were diluted 10-fold with buffer A containing 2.5 mM $CaCl_2$ and immediately acquired for 10 s in a

© 2005 International Society on Thrombosis and Haemostasis

FACSCalibur flow cytometer (BD Biosciences). Acquired data were analyzed using a WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). Platelets were identified and gated using PE-labeled CD42b fluorescence measured in FL2 channel. Approximately, 10 000 of FL2-positive events were collected in each experiment and only FL2-positive events are displayed in dot plots. Bound fluorescein-labeled factor was determined by measuring mean fluorescence intensity of FL1-positive events. Fluorescence intensity was converted to a mean number of molecules per platelet using the calibration curve prepared with Quantum Fluorescent Microbead Standard for Fluorescein (Sigma) and the known dye/protein ratio. Control experiments confirmed that, during the time of sample dilution and acquisition, $< 5\%$ of coagulation factors and annexin V dissociated from platelets (data not shown). All experiments were performed at least in triplicates unless otherwise specified, and representative experiments are given in the figures. Data from different experiments for each condition were averaged and mean values and SEM were determined.

Results

Two subpopulations of thrombin-activated platelets differ in binding coagulation factors and annexin V

Platelets were activated with increasing concentrations of thrombin (0–100 nM), and their procoagulant surfaces were characterized by studying the binding of fluorescein-labeled fX, fVIII, fIXa (components of the intrinsic fX-activating complex) or APC-labeled annexin V (which binds to expressed phosphatidylserine on platelet membrane) using flow cytometry. Two distinct subpopulations of platelets could be detected in the presence of the coagulation factors (Fig. 1) or annexin V (not shown). In control experiments in the absence of calcium, where EDTA was added at 10 mM, only the low-binding platelet subpopulation was present (data not shown), in agreement with the previous report for fIXa [22]. Two distinct subpopulations with different binding properties were detectable within a wide range of factor concentrations (e.g. 8–2048 nM for fX); the concentrations giving the better presentation are shown in the dot plots.

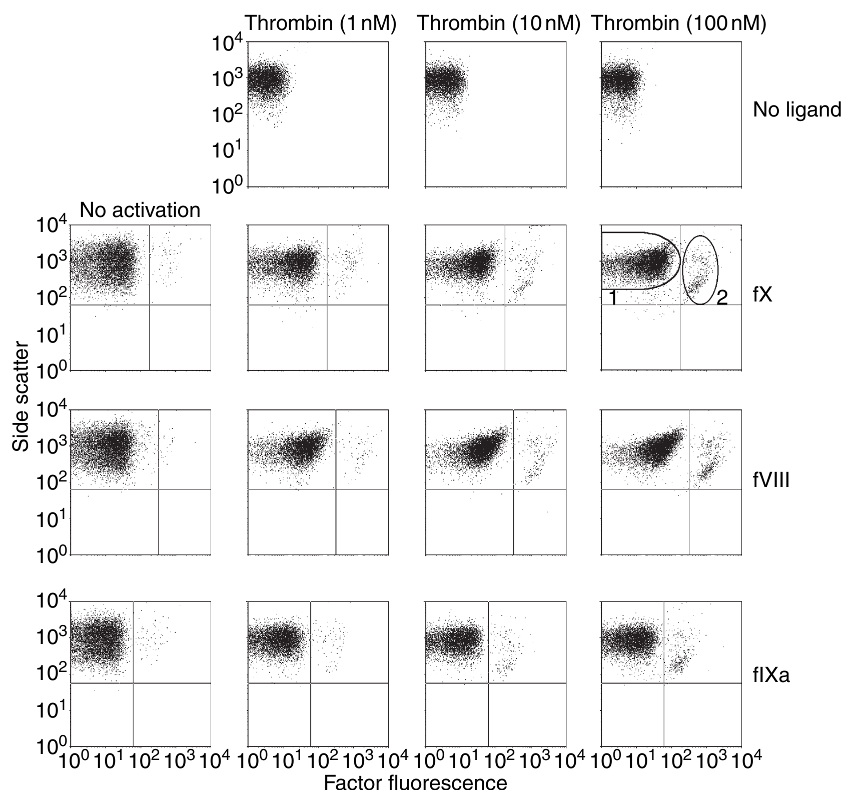

Fig. 1. Binding of fX, fVIII, and fIXa to platelets activated with increasing concentrations of thrombin. Platelets (final concentration 2×10^8 mL^{-1}) were activated by thrombin at the indicated concentrations (columns) and incubated with fluorescein-labeled fX (200 nM), fVIII (50 nM), or fIXa (200 nM) in buffer A with 2.5 mM $CaCl_2$ at 37 °C for 15 min. The samples were immediately analyzed in a FACSCalibur flow cytometer as described under Experimental Procedures. The plot diagrams show platelets displayed as side scatter on the ordinate and the coagulation factor binding (FL1 channel) on the abscissa. Platelets with the low and high binding of coagulation factors were observed in the upper left and right quadrants, respectively (shown as encircled subpopulations 1 and 2 for fX at 100 nM thrombin).

© 2005 International Society on Thrombosis and Haemostasis

Figure 2A shows the percent of platelets with the elevated binding capability for different factors and for annexin V as a function of thrombin concentration. The high-binding platelet subpopulations for each ligand constituted very similar percentages of total platelet population at each thrombin concentration reaching ~4% at 100 nM of thrombin (Fig. 2A) with no apparent saturation. Although the two subpopulations were clearly observed within a wide range of thrombin concentrations, the following experiments were performed at 100–200 nM thrombin to obtain significant numbers (several hundreds) of high-binding events in each flow cytometry acquisition for correct analysis. This thrombin concentration is within the reported range of maximal thrombin concentrations (100–500 nM) achieved in thrombin generation assay in plasma or reconstituted system [15,25,28].

Determination of the number of molecules of each factor bound per platelet yielded 15 000–25 000 molecules per platelet for the high-binding subpopulation and 1500–2000 molecules per platelet for the low-binding subpopulation at 100 nM thrombin (Fig. 2B). The specificity of the factor binding to platelet subpopulations was tested by addition of excess unlabeled factor. Fig. 2C shows histograms of fX binding in the absence (filled histogram) and in the presence (gray line) of excess unlabeled fX. In the latter case, the high-binding subpopulation was not observed, indicating that the labeled factor is displaced by excess unlabeled ligand fX and thus confirming that the binding to this subpopulation is specific. The low-binding subpopulation was observed in both experiments, and its mean fluorescence was not significantly changed.

Dual-labeling of platelets with intrinsic tenase complex components (or platelet markers) and annexin V

The apparent similarity of the percentage and of the parameters of platelets with elevated binding capability for all tested ligands (Fig. 2A,B) suggested that this high-binding subpopulation may be identical for all coagulation factors and annexin V. To test this hypothesis, platelets activated with 100 nM thrombin were co-incubated with both annexin V and different coagulation factors. These dual-labeling studies showed that all annexin V-positive platelets were also positive in their binding of fIXa, fVIII, and fX and vice versa, as can be seen by the presence of this subpopulation in the upper-right quadrant in Fig. 3 (right column except for control).

To gain insight into the nature of the high-binding subpopulation, we performed flow cytometric studies of thrombin-activated platelets evaluating them for the expression of platelet-specific surface antigens: general platelet marker CD42b (representing the GPIb alpha-chain moiety of the von Willebrandt receptor GPIb [29]) and platelet activation markers CD62P (representing platelet (P)-selectin [30]) and PAC1 (representing the activated fibrinogen receptor which becomes exposed on GPIIb/IIIa upon activation [31]). In these experiments, fluorescently-labeled antibodies were used individually or in combination with APC-labeled annexin V. Dual-labeling

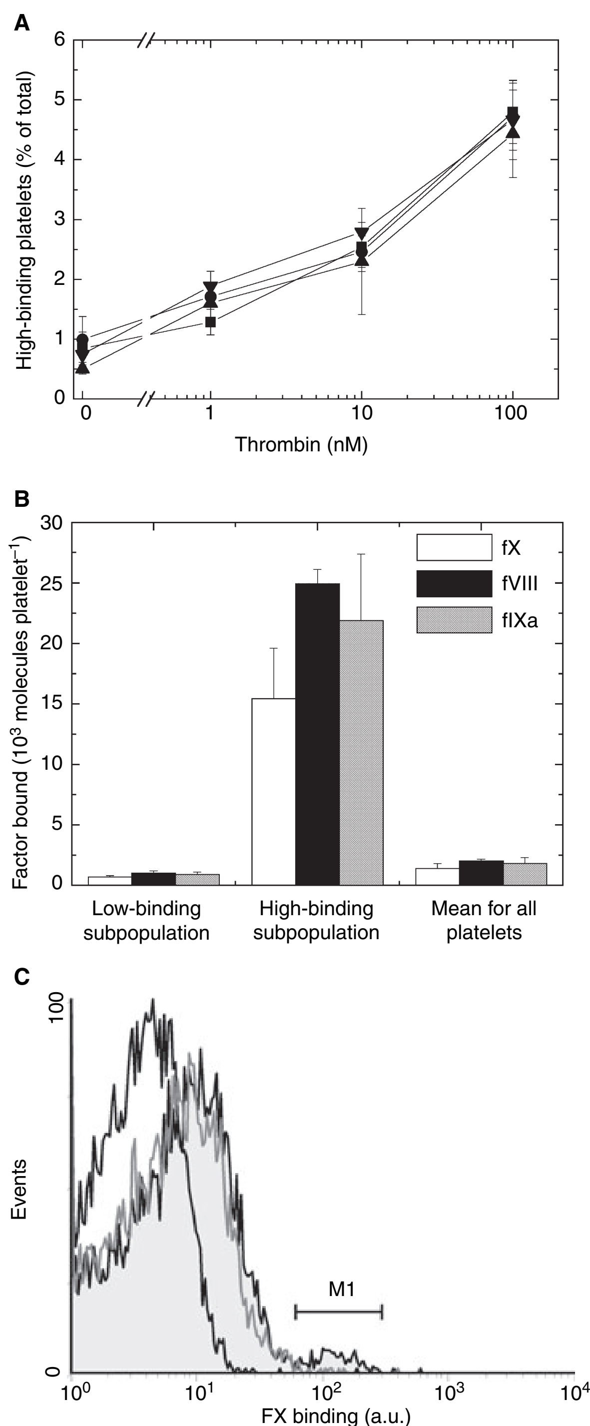

Fig. 2. Characteristics of high-binding platelet subpopulations. Binding of coagulation factors and annexin V (0.4% v/v) to activated platelets was performed as in Fig. 1. (A) The percentages of high-binding platelets for different ligands are plotted vs. thrombin concentration: (■), annexin V; (●), fX; (▲), fVIII; (▼), fIXa. (B) Number of molecules of fX, fVIII, fIXa bound to platelets of each subpopulation following activation with 100 nM thrombin. Results in panels A and B are means of three independent experiments with platelets from different donors ± SEM. (C) Histogram of the fluorescein-labeled fX (32 nM) binding to platelets activated with 100 nM thrombin in the absence (filled histogram) or in the presence of 1250 nM unlabeled fX (gray line histogram). Control (no labeled fX) is shown as black line histogram.

© 2005 International Society on Thrombosis and Haemostasis

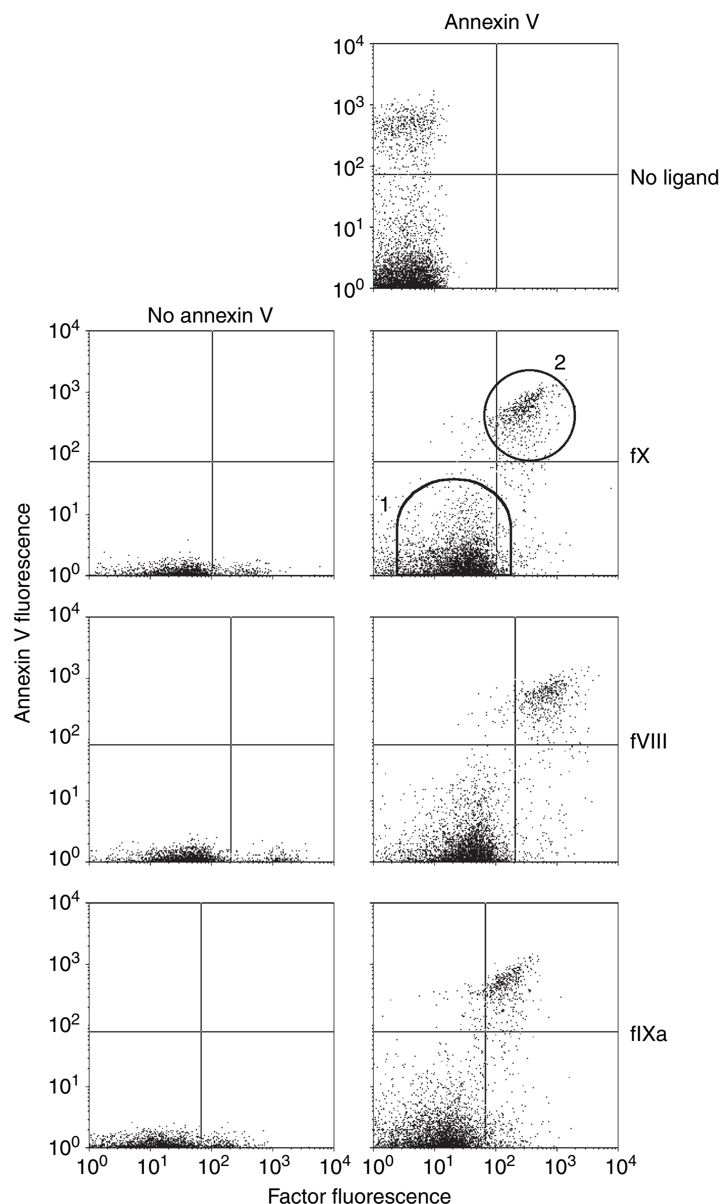

Fig. 3. Dual-binding of annexin V and fX, fVIII or fIXa to activated platelets. Platelets were activated with 100 nM thrombin and incubated with fluorescein-labeled coagulation factors at concentrations indicated in Fig. 1 and with APC-labeled annexin V (0.4% v/v) in buffer A with 2.5 mM $CaCl_2$ at 37 °C for 15 min. The samples were immediately analyzed in a FACSCalibur flow cytometer as described under Experimental Procedures. Dot plots of platelet region data display coagulation factor binding (FL1 channel) on the abscissa and annexin V binding (FL4 channel) on the ordinate. Platelets positive for the binding of factors are seen in the right quadrants and those positive for annexin V binding are seen in the upper quadrants. The double-labeled subpopulations are observed in the upper-right quadrants (shown as encircled subpopulation 2 for fX/annexin V; subpopulation 1 is double-negative).

binding studies using annexin V in parallel with PAC1, CD62P, and CD42b (Fig. 4) demonstrated that the two subpopulations had similar expression of platelet general and activation markers, in contrast to the dramatic difference in their binding capability for coagulation factors and annexin V.

Heterogeneity of the high-binding subpopulation

Accurate analysis of APC-labeled annexin V binding to thrombin-activated platelets revealed heterogeneity of the high-binding platelet subpopulation (Fig. 5A, compare black and gray line histograms). Noteworthy, cells of the high-binding subpopulation can be subdivided into two subpopulations differentiated by side and forward scatter (Fig. 5B,C, black line). The left peak events in the high-binding subpopulation had much lower scatter than the platelets of the low-binding subpopulation (Fig. 5A, gray line), while the right peak events were comparable in scatter to the low-binding subpopulation. To test whether the high-binding events with higher scatter represent aggregates of platelets or platelet-derived

© 2005 International Society on Thrombosis and Haemostasis

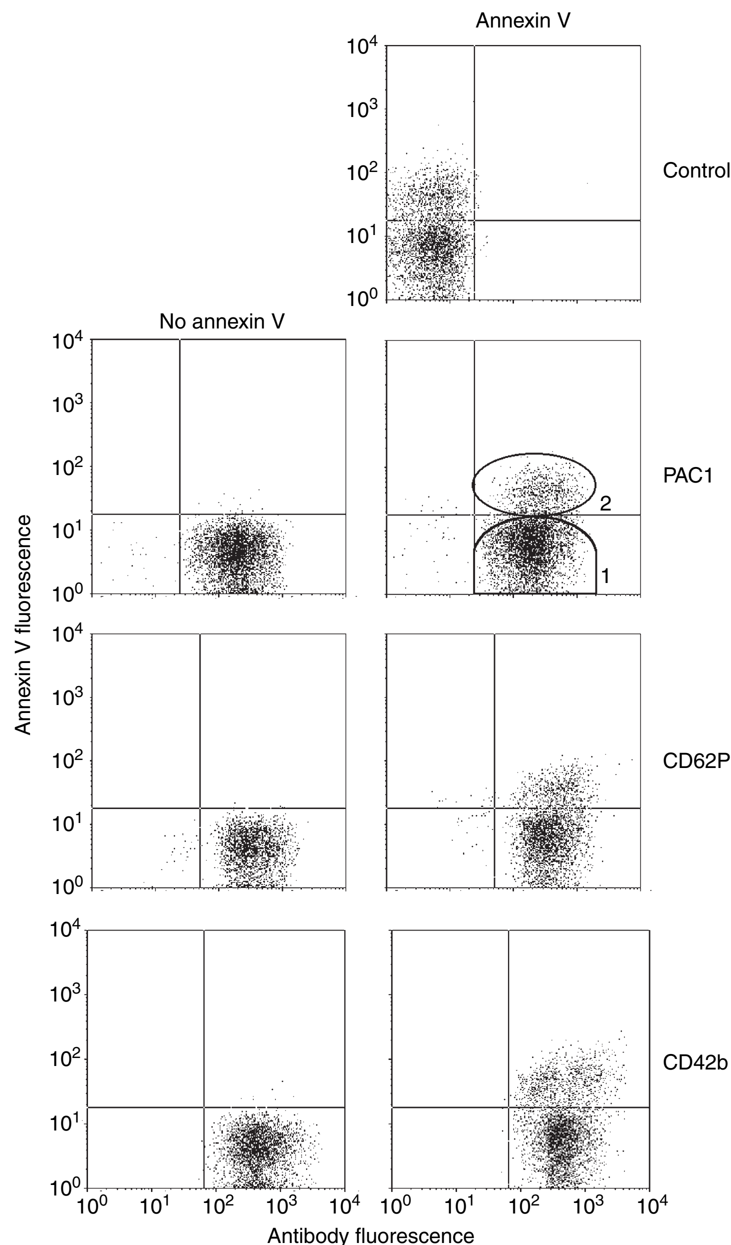

Fig. 4. Dual-binding of annexin V and CD42b, PAC1 or CD62P antibodies to activated platelets. Platelets (final concentration 4×10^8 mL^{-1}) were activated by 200 nM thrombin and incubated with PE-labeled antihuman CD42b (4% v/v) and with fluorescein-labeled antihuman PAC1 or CD62P antibodies (10% v/v) and with APC-labeled annexin V (0.4% v/v) in buffer A with 2.5 mM $CaCl_2$ at 37 °C for 15 min. The samples were immediately analyzed in a FACSCalibur flow cytometer as described under Experimental Procedures. Dot plots of platelet region data display antibody binding (FL1 channel for control, PAC1 and CD62P, FL2 channel for CD42b) on the abscissa and annexin V binding (FL4 channel) on the ordinate. Platelets positive for antibody binding are seen in the right quadrants and those positive for annexin V binding are seen in the upper quadrants. The low- and high-binding subpopulations (shown for PAC1/annexin V as encircled subpopulations 1 and 2, respectively) do not differ by expression of these markers.

microparticles, we performed experiments in the presence of 4000 nM echistatin, one of the most potent antiaggregating agents. In the presence of echistatin, the right peak of the high-binding subpopulation decreased while the left peak increased, suggesting that at least some of the high scatter high-binding events might represent aggregates of low scatter high-binding events (compare black and gray line histograms in Fig. 5B,C). However, the percentage of platelets with elevated binding capability was not reduced confirming that these platelets form a distinct subpopulation and are not aggregates of low-binding events.

Discussion

The present study analyzed the heterogeneity of thrombin-activated platelets with respect to the binding of the components of the intrinsic fX-activating complex. This was carried out using a flow cytometry-based technique, which determined

© 2005 International Society on Thrombosis and Haemostasis

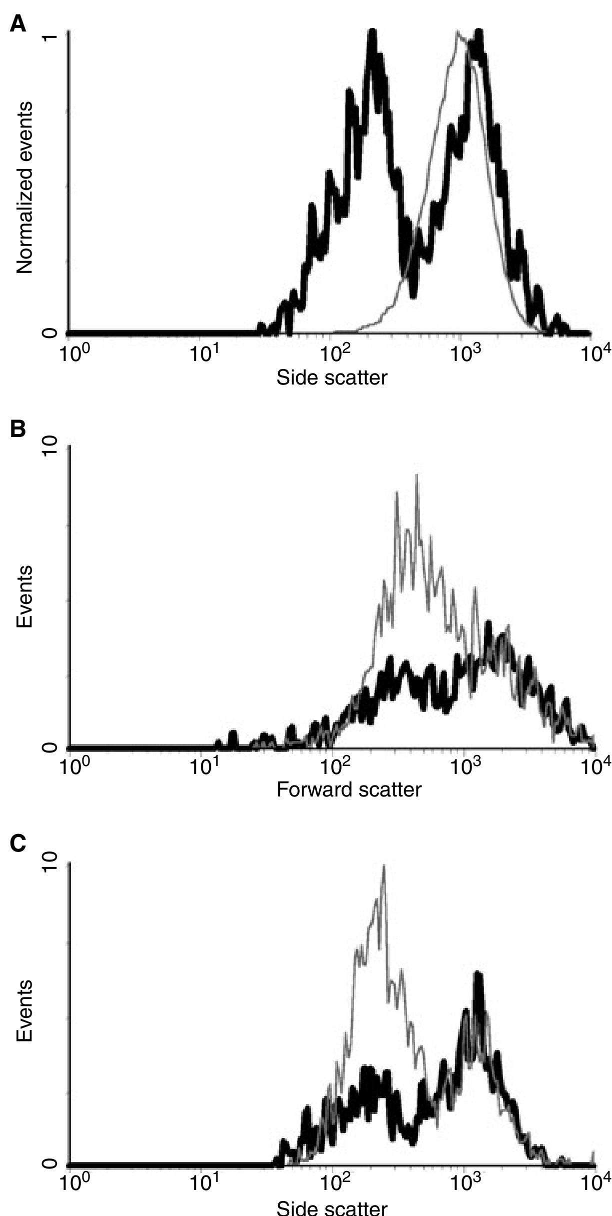

Fig. 5. Heterogeneity of the high-binding subpopulation and the effect of echistatin. Platelets were activated by 100 nM thrombin, incubated with APC-labeled annexin V, and subjected to flow cytometry as described in the legend to Fig. 3. The high- and low-binding subpopulations for annexin V were gated according to the quadrants as in Fig. 3. (A) Histograms of side scatter distribution for the low- (gray curve) and high-binding (black curve) subpopulations in the absence of echistatin (normalized to the maximal value). (B) Forward scatter and (C) side scatter histograms of the high-binding subpopulation in the absence (black curves) and in the presence of 4000 nM echistatin (gray curves).

the binding of coagulation factors and annexin V [26]. The principal findings of this study are the following: (i) platelet activation by thrombin induces two subpopulations of platelets, which differ in their binding of fIXa, fVIII, fX, and annexin V by several orders of magnitude; (ii) the number of platelets in the high-binding subpopulation increases with the increase of thrombin concentration, i.e., with the degree of activation; (iii) the two subpopulations do not differ in the expression of platelet-specific activation markers; (iv) the cells of the high-binding subpopulation are characterized by reduced forward and side light scatter properties.

Initial flow cytometry studies of thrombin-activated platelets described a homogeneous subpopulation and did not report the existence of subpopulations with respect to their ability for fIXa binding [19]. Approximately, 12–20% of events in thrombin-activated cell suspension were platelet-derived microparticles, and fIXa binding to these microparticles was negligible [19]. However, later studies demonstrated the presence of two distinct subpopulations of platelets, which exhibited different fXa/fVa-binding properties [20,21]. Activation of platelets by thrombin plus convulxin was reported to generate a subpopulation of platelets (~30% of total platelets) with high alpha-granule fV expression and fXa binding [24]. Noteworthy, activation by any single agonist was not sufficient to generate this subpopulation in that study. However, a recent study demonstrated that activation of platelets by thrombin or thrombin receptor-activating peptide alone led to the appearance of a subpopulation of platelets with a high fIXa-binding capability [22]. This subpopulation constituted ~6% of the total platelets, was also positive for annexin V binding, suggesting high phosphatidylserine expression, and was indistinguishable from other platelets by GPIX expression. In contrast to the results described in [19], microparticles were not observed when platelets were activated by thrombin (0.2–2 units mL^{-1}) [22]. Another recent study again was not able to identify distinct platelet subpopulations upon activation by thrombin alone, while combination of thrombin and convulxin induced appearance of two subpopulations with different fV, fVIII, fIX, and fX binding [25].

There may be several reasons for the divergent results of previous studies. Firstly, it is known that the ability of activated platelets to bind fVIII is maintained only for a limited time interval and is completely lost after 30 min [26]. Therefore, experiments involving prolonged incubations of platelets with antibodies [22], secondary antibodies [19,25] or even fixation [19,25] may not be able to detect the high-binding subpopulation. Secondly, different results may reflect different degrees of platelet activation. Thirdly, most of contradictory results were obtained in studies with different coagulation factors. To avoid the loss of platelet-binding activity with time, we used fluorescein-labeled coagulation factors instead of labeled antifactor antibodies as this approach allows rapid, direct and quantitative determination of concentrations of bound factor. We used increasing concentrations of thrombin to study the effect of activation on the binding properties of platelets. Finally, we studied the binding of all components of the intrinsic fX-activating complex and of annexin V to ensure that our results do not depend on the specific factor chosen. Instead of using labile active cofactor fVIIIa, its stable precursor fVIII was used.

Our results are consistent with the previous study [22] as they demonstrate that platelet activation by thrombin alone is sufficient to induce a subpopulation with calcium-dependent

© 2005 International Society on Thrombosis and Haemostasis

binding of annexin V and fIXa; in addition, we have shown that the same subpopulation is characterized by a high-degree calcium-dependent binding of fX and fVIII. Thus, the identical subpopulation of phosphatidylserine-expressing platelets preferentially binds all components of the fX-activating complex and annexin V. At 100 nM thrombin, these cells constituted ~4% of total platelets (Fig. 2) and bound ~50% of total bound coagulation factor. Moreover, they were responsible for almost all calcium-dependent and specific binding (Fig. 2C), as excess unlabeled fX displaced the labeled fX thus confirming the specificity of the binding to this subpopulation. Importantly, the high-binding platelet subpopulation was not eliminated by echistatin, a potent inhibitor of aggregation, thus excluding the aggregation effect and confirming the presence of two distinct subpopulations of thrombin-activated platelets.

Previous studies, which reported existence of two subpopulations of thrombin-activated platelets, used thrombin at fixed concentrations. These results were interpreted as *pre-existence* of two initially different types (subpopulations) of platelets, which respond differently to activation. In our study, using increasing concentrations of thrombin within a two-order-of-magnitude range (1–100 nM), we found that the number of cells in the high-binding subpopulation increased with thrombin concentration. The fact that the number of the high-binding platelets continuously changes with the degree of activation suggests that they do not initially belong to a separate platelet subpopulation and heterogeneity *is acquired* during the activation process. In view of these findings, the previously reported difference in the binding of coagulation factors at different thrombin concentrations [32] seems to reflect not different expression of binding sites on the platelet surface but rather different degree of platelet transition from the low- to the high-binding subpopulation with equal potential for binding site expression on each subpopulation. Regulation of the expression of procoagulant surface by activation may have significant physiological or/and pathological implications.

Noteworthy, the study [24], which described a high-binding subpopulation of thrombin/convulxin-activated platelets, revealed a correlation between the proportion of cells in that subpopulation and up-regulation of fXa and thrombin generation. That study also showed that the number of platelets in the high-binding subpopulation depends on convulxin concentration upon simultaneous activation by thrombin and convulxin [24]. It should be noted, however, that platelet heterogeneity following activation with thrombin plus convulxin [23–25] and heterogeneity upon activation by thrombin alone reported in [22] and in the present work are not necessarily the same phenomena. In particular, PAC1 expression in the high-binding subpopulation in our study did not differ from that in the low-binding subpopulation, whereas platelets after dual-agonist activation showed very low levels of PAC1 binding [23] indicating different occupancy of fibrinogen-binding sites on GPIIb/IIIa. Elucidation of the differences between these phenomena requires additional investigation.

A notable finding of the current study is the difference in scatter parameters between the high- and low-binding subpopulations and heterogeneity within the high-binding subpopulation. The mean forward scatter and side scatter of the high-binding platelets were, respectively, ~1.3 and ~1.7-fold below those of the low-binding platelets suggesting, although not proving, significant differences in their size, as the logarithm of forward scatter [33] only partially reflects size. Additional experiments are required to elucidate the specific cause of the observed scatter difference. Moreover, the high-binding subpopulation was found to consist of two subpopulations with different scatter parameters (Fig. 5B,C). As the presence of echistatin, a potent inhibitor of aggregation, reduced the mean scatter parameters of the high-binding subpopulation, the higher scatter peak could represent aggregated small platelets or platelets with adherent platelet-derived microparticles. It seems unlikely that the lower scatter high-binding platelet subpopulation represents microparticles shed from platelets upon activation as these events were not a separate subpopulation on scatter plots as would be expected for microparticles [19,26].

In summary, our study confirms that platelet activation by thrombin induces a distinct subpopulation of platelets with a high-binding capability for coagulation factors, and shows that this phosphatidylserine-expressing subpopulation is the same for all components of the intrinsic fX-activating complex: fIXa, fVIII, and fX. At least half of the molecules of tested coagulation factors bind to this subpopulation in a calcium-dependent manner suggesting its importance for coagulation.

Acknowledgements

This work was supported by grants HL66101 and HL72929 from the National Institutes of Health to E.L.S., by grant from the Russian Foundation for Basic Research (project No. 03-04-48338) to F.I.A., and by NATO Collaborative Linkage Grant No. 979210 to E.L.S. and F.I.A. We thank Drs Gary Moroff (American Red Cross) and Mikhail Ovanesov (John Hopkins University, MD) for helpful discussions. We are indebted to Drs James Kurtz and Shalini Seetharaman (American Red Cross) for their expert counsel and assistance with flow cytometry.

References

1 Zwaal RF, Comfurius P, Bevers EM. Lipid-protein interactions in blood coagulation. *Biochim Biophys Acta* 1998; **1376**: 433–53.

2 Panteleev MA, Saenko EL, Ananyeva NM, Ataullakhanov FI. Kinetics of Factor X activation by the membrane-bound complex of Factor IXa and Factor VIIIa. *Biochem J* 2004; **381**: 779–94.

3 Gentry R, Ye L, Nemerson Y. Surface-mediated enzymatic reactions: simulations of tissue factor activation of factor X on a lipid surface. *Biophys J* 1995; **69**: 362–71.

4 Krishnaswamy S, Nesheim ME, Pryzdial EL, Mann KG. Assembly of prothrombinase complex. *Methods Enzymol* 1993; **222**: 260–80.

5 Neuenschwander P, Jesty J. A comparison of phospholipid and platelets in the activation of human factor VIII by thrombin and factor Xa, and in the activation of factor X. *Blood* 1988; **72**: 1761–70.

6 Butenas S, Mann KG. Kinetics of human factor VII activation. *Biochemistry* 1996; **35**: 1904–10.

© 2005 International Society on Thrombosis and Haemostasis

7 Monkovic DD, Tracy PB. Activation of human factor V by factor Xa and thrombin. *Biochemistry* 1990; **29**: 1118–28.
8 Kalafatis M, Swords NA, Rand MD, Mann KG. Membrane-dependent reactions in blood coagulation: role of the vitamin K-dependent enzyme complexes. *Biochim Biophys Acta* 1994; **1227**: 113–29.
9 Griffin JH, Fernandez JA, Deguchi H. Plasma lipoproteins, hemostasis and thrombosis. *Thromb Haemost* 2001; **86**: 386–94.
10 Andrews DA, Low PS. Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999; **6**: 76–82.
11 Tracy PB, Eide LL, Mann KG. Human prothrombinase complex assembly and function on isolated peripheral blood cell populations. *J Biol Chem* 1985; **260**: 2119–24.
12 Ananyeva NM, Kouiavskaia DV, Shima M, Saenko EL. Intrinsic pathway of blood coagulation contributes to thrombogenicity of atherosclerotic plaque. *Blood* 2002; **99**: 4475–85.
13 Khrenov A, Sarafanov A, Ananyeva N, Kouiavskaia D, Shima M, Schwinn H, Josic D, Saenko E. Molecular basis for different ability of low-density and high-density lipoproteins to support activity of the intrinsic Xase complex. *Thromb Res* 2002; **105**: 87–93.
14 Solum NO. Procoagulant expression in platelets and defects leading to clinical disorders. *Arterioscler Thromb Vasc Biol* 1999; **19**: 2841–6.
15 Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1381–9.
16 Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost* 2002; **88**: 186–93.
17 Ahmad SS, London FS, Walsh PN. Binding studies of the enzyme (factor IXa) with the cofactor (factor VIIIa) in the assembly of factor-X activating complex on the activated platelet surface. *J Thromb Haemost* 2003; **1**: 2348–55.
18 Ahmad SS, London FS, Walsh PN. The assembly of the factor X-activating complex on activated human platelets. *J Thromb Haemost* 2003; **1**: 48–59.
19 Hoffman M, Monroe DM, Roberts HR. Coagulation factor IXa binding to activated platelets and platelet-derived microparticles: a flow cytometric study. *Thromb Haemost* 1992; **68**: 74–8.
20 Bouchard BA, Tracy PB. Platelet regulation of thrombin generation in cardiovascular disease. *Ital Heart J* 2001; **2**: 819–23.
21 Feng P, Tracy PB. Not all platelets are equal procoagulants? *Blood* 1998; **98**: 1441 (abstr.).
22 London FS, Marcinkiewicz M, Walsh PN. A subpopulation of platelets responds to thrombin- or SFLLRN-stimulation with binding sites for factor IXa. *J Biol Chem* 2004; **279**: 19854–9.
23 Dale GL, Friese P, Batar P, Hamilton SF, Reed GL, Jackson KW, Clemetson KJ, Alberio L. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature* 2002; **415**: 175–9.
24 Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood* 2000; **95**: 1694–702.
25 Kempton CL, Hoffman M, Roberts HR, Monroe DM. Platelet heterogeneity. Variation in coagulation complexes on platelet subpopulations. *Arterioscler Thromb Vasc Biol* 2005; **25**: 861–6.
26 Gilbert GE, Sims PJ, Wiedmer T, Furie B, Furie BC, Shattil SJ. Platelet-derived microparticles express high affinity receptors for factor VIII. *J Biol Chem* 1991; **266**: 17261–8.
27 Saenko EL, Shima M, Gilbert GE, Scandella D. Slowed release of thrombin-cleaved factor VIII from von Willebrand factor by a monoclonal and a human antibody is a novel mechanism for factor VIII inhibition. *J Biol Chem* 1996; **271**: 27424–31.
28 Ovanesov MV, Ananyeva NM, Panteleev MA, Ataullakhanov FI, Saenko EL. Initiation and propagation of coagulation from tissue factor bearing cell monolayers to plasma: initiator cells do not regulate spatial growth rate. *J Thromb Haemost* 2005; **3**: 321–31.
29 Ruan C, Tobelem G, McMichael AJ, Drouet L, Legrand Y, Degos L, Kieffer N, Lee H, Caen JP. Monoclonal antibody to human platelet glycoprotein I. II. Effects on human platelet function. *Br J Haematol* 1981; **49**: 511–9.
30 McEver RP. Properties of GMP-140, an inducible granule membrane protein of platelets and endothelium. *Blood Cells* 1990; **16**: 73–80.
31 Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. *J Biol Chem* 1985; **260**: 11107–14.
32 Ahmad SS, Rawala-Sheikh R, Walsh PN. Comparative interactions of factor IX and factor IXa with human platelets. *J Biol Chem* 1989; **264**: 3244–51.
33 Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem* 1988; **263**: 18205–12.

© 2005 International Society on Thrombosis and Haemostasis