

ORIGINAL ARTICLE

Platelet–adenovirus vs. inert particles interaction: Effect on aggregation and the role of platelet membrane receptors

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Abstract

Platelets are involved in host defense via clearance of bacteria from the circulation, interaction with virus particles, and uptake of various size particulates. There is a growing interest in micro- and nanoparticles for drug delivery and there is evidence that the properties of these particles critically influence their interaction and uptake by various tissues and cells including platelets. Virus mediated gene therapy applications are still challenged by the resultant thrombocytopenia and the mechanism(s) of platelet–foreign particles interaction remains unclear. We studied the specifics of platelet interaction with an active biological agent (adenovirus) and inert latex microspheres (MS) and investigated the role of platelet proteins in this interaction. We show that activated and not resting platelets internalize MS, without influencing platelet aggregation. In contrast, adenovirus induces and potentiates ADP-induced platelet aggregation and results in rapid expression of P-selectin. Platelets then internalize adenovirus and viral particles appear inside the open canalicular system. Inhibition of platelet α IIb β 3, GPIIb α , and P-selectin decreases both platelet aggregation and internalization of MS. Inhibition of α IIb β 3 and α V β 3 does not abolish adenovirus platelet internalization and adenovirus-induced platelet activation is maintained. Our study demonstrates that platelets react differentially with foreign particles and that α IIb β 3 is a key player in platelet engulfing of foreign particles but not in mediating adenovirus internalization. Other platelet candidate molecules remain to be investigated as potential targets for management of adenovirus-induced thrombocytopenia.

Keywords: Adenovirus, thrombocytopenia, platelet integrins, covercytes, microspheres, CAR

Introduction

Platelets play a crucial role in hemostasis and thrombosis. However, their participation in inflammation, tissue regeneration, and immune response is also documented and was recently reviewed [1]. Platelets can engulf different particulates, including bacteria and viruses, and so take part in human host defense. Platelets' interaction with virus particles and their uptake of various size particulates have been the focus of many laboratories for several years [2–6]. Platelet ultrastructure studies have shed light on the mechanisms that explain some of these events [2, 4], but many facets remain to be investigated.

There is a growing interest in micro- and nanoparticles for drug delivery and targeting of therapeutics. The properties of these different particles critically influence their interaction and uptake by various tissues, organs, and cells, including platelets. Much interest in this is focused on platelet activation and thrombus formation via assessment of platelet aggregation. Platelets' interaction, internalization, and subsequent clearance of these particles can limit their utility due to reduction of their number, bioavailability, and thus therapeutic concentration [7].

There is ample evidence that platelet–virus interaction occurs via platelet receptors. For example, human platelets express the complement (C3d) receptor type II (CR2) on their

surface, which is also a receptor for Epstein Barr virus [8]. Hepatitis C virus can bind platelet membrane GPVI, which then promotes the virus transport and persistence [9]. Platelets engulf human immunodeficiency virus (HIV) [10], and CLEC-2 and DC-SIGN are platelet receptors that mediate the capture and transfer of HIV-1 virus [11]. For many viruses such interactions can be mediated by platelet integrins. Thus, rotavirus VP4-mediated cell entry involves the α IIb β 1 integrin, whereas VP7 appears to interact with α X β 2 and α IV β 1 integrins [12]; hantaviruses bind β 3 integrins [13], and β 1 integrins mediate the internalization of mammalian retroviruses to cultured cells [14].

Others and we have shown that adenovirus mediates thrombocytopenia in the first 24 hours following virus administration in mice [15–18]. We have also shown that adenovirus mediates platelet activation and platelet leukocyte aggregate formation that result in elevated levels of ultrahigh molecular weight VWF multimers in mice [15]. Others have shown that platelets are the pre-dominant adenovirus (Ad) binding blood cell type in mice and have documented viral particles inside platelets [19]. We have identified coxsackie adenovirus receptor (CAR), the adenovirus attachment receptor, on human platelets. [15] Although this was contradicted in another study [20] and despite the absence of studies demonstrating the effect of CAR blocking on platelet virus

internalization, CAR appears to be the most logic explanation of initiating adenovirus platelet internalization. In a separate study, we have recently confirmed the presence of CAR on human platelets with particular localization at the sites of intercellular interaction and have provided the explanation for some of the reported discrepancies in the literature [21].

Following CAR binding, cell surface integrins (mainly $\alpha V\beta 3$) recognize and bind to the arginine–glycine–aspartic acid (RGD) sequence in adenovirus' penton and facilitate its internalization. Other surface integrins involved in this process are, to a lesser extent, $\alpha V\beta 5$, $\alpha V\beta 1$, and $\alpha 5\beta 1$ [22]. Human platelets also express on their surface $\alpha 5\beta 1$ and $\alpha V\beta 3$, as well as $\alpha IIb\beta 3$ (GPIIb/IIIa) and GPIb (a part of the GPIb-IX-V complex). Both $\alpha IIb\beta 3$ and GPIb play an important role in platelet aggregation, interaction with other cells including bacteria and their subsequent internalization [18, 23]. Another major platelet receptor is P-selectin, which is located on the membrane of α -granules and appears rapidly on the platelet surface following platelet activation [24]. Our previous observation that adenovirus–platelet interaction leads to P-selectin expression in mice [15] indicates that P-selectin may be involved in platelet microbial interaction.

Understanding the role of platelet receptors in engulfing micro/nanoparticles is important in targeting therapeutics using these otherwise inert particles. Understanding platelet adenovirus interaction can help prevent adenovirus-mediated thrombocytopenia and improve adenovirus gene therapy applications. To date, data regarding the role of $\alpha IIb\beta 3$ in platelet interaction with adenovirus as well as with microparticles are still lacking. In addition, the effect of adenovirus on platelet aggregation remains unclear.

The aim of this study was to investigate the specifics of platelets' ability to internalize adenovirus and foreign particles, the influence of this process on platelet activation and aggregation, and the role of the different platelet receptors in these events.

Materials and methods

Reagents and antibodies

Adenoviral vector: E1/E3 deleted, a replication deficient adenovirus derived from human adenovirus group C, serotype 5. The vector was propagated in 293 cells and purified on CsCl density gradient centrifugation as previously described [15]. The vector was dialyzed against Tris buffer (10 mM Tris, pH 7.4, 2 mM MgCl₂, 150 mM NaCl, 3% sucrose, and 2% glycerol). Vector titer was determined by spectrophotometric measurement of the optical density at 260 nm and reported as virus particles per milliliter (vp/ml). In all adenovirus–platelet experiments we used 1×10^{10} vp/ml; a dose shown previously to mediate platelet activation [15]. For confocal experiments, adenovirus was conjugated with CyTM₃ Ab labeling kit, Amersham, Buckinghamshire, UK according to manufacturer's instructions. Kistrin is a member of a family of low-molecular-mass RGD-containing, cysteine-rich proteins, named disintegrins, with a dual inhibitory effect on integrins $\alpha IIb\beta 3$ and $\alpha V\beta 3$ [25, 26]. For platelet glycoprotein $\alpha IIb\beta 3$ inhibition experiments, we used Kistrin itself (0.5 μ M) (Chemicon International Inc., Temecula, CA), F(ab')₂ fragment of anti- $\alpha IIb\beta 3$ monoclonal antibody CRC64 (0.2 mg/ml)

kindly provided by Dr Mazurov [27], nonpeptide inhibitor of $\alpha IIb\beta 3$ tirofiban, AGGRASTAT[®] (0.25 mg/ml, Medicure Pfarma, Inc., Somerset, NJ), RGD fragment contained peptide inhibitor eptifibatide, INTEGRILIN[®] (2 mg/ml, Schering–Plough Corporation, Kenilworth, NJ), peptide inhibitor with RGDS fragment kindly provided by Mikhail Ovchinnikov (2 mg/ml laboratory of peptide synthesis Russian Cardiology Research Complex), mouse monoclonal antibodies against human platelet glycoprotein Ib α (AK2; 2 mg/ml) [28], mouse monoclonal antibody against human P-selectin (AK4; 50 μ g/ml) [29], FITC-stained microspheres (MS) 0.2 μ m (Fluoresbrite YG Microspheres Polysciences, Inc., Warrington, PA) and Adenosine 5-diphosphate (ADP, Sigma Chemical).

Human platelets: Preparation, activation, and staining

Human platelets used in this study were obtained from blood of healthy volunteers (2 weeks medications and aspirin free prior to blood withdrawal) following their informed consent and approval according to the institution's guidelines. Blood was collected from the antecubital vein using a 21-G needle into citrated tubes and gently mixed. Platelet rich plasma (PRP) was prepared by centrifugation at 170 g for 17 min at room temperature (RT) and platelet count was adjusted to $2\text{--}3 \times 10^6$ platelets/ μ l. For platelet activation and confocal studies, platelets from concentrates were pelleted by centrifugation at 800 g for 5 min and then washed in buffer A (140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, and pH 6). Platelets were activated by thrombin 0.3 U/ml for 2 min or ADP 5.0 μ M for 5 min at RT. Platelets suspended in phosphate buffered saline (PBS) were used as a negative control representing the resting platelets. In adenovirus experiments, we used TRIS buffer (10 mM Tris, pH 7.4, 2 mM MgCl₂, 150 mM NaCl, 3% sucrose, and 2% glycerol) as a control. In confocal experiments, platelet actin cytoskeleton was stained using Texas Red[®]-X phalloidin from Molecular Probes (Invitrogen). Fibrinogen binding was tested using fluorescently labeled AlexaFluor488 human fibrinogen conjugates (Molecular Probes, Illinois). In-flow cytometry experiments, platelets were fixed using 2% paraformaldehyde (PFA) in PBS prior to analysis. Platelet activation was assessed using monoclonal antihuman P-selectin (CD62P)-Fluorescein (R&D system, Minneapolis, MN) via calculation of the index of platelet activation (IPA⁺) (percentage of positive cells \times mean channel fluorescence).

Platelet aggregation

Platelet aggregation was assessed in PRP using the Biola 230LA aggregometer (Biola, Russia). This apparatus measures simultaneously platelet aggregation and mean aggregate size. The former is based on a conventional turbidometric method measuring changes in light transmission (LT) and expressed in percentage [30]. The latter is based on the analysis of LT fluctuations produced by the changes in the number of platelets in the optical channel [31]. The relative value of these fluctuations is proportional to the mean aggregate size (expressed in relative units, RU). The high sensitivity of this method makes it ideal to study spontaneous platelet aggregation and aggregation induced by low concentrations of

agonists, which cannot be evaluated by the standard LT method. Spontaneous and 0.1 μM ADP-induced ability to form small aggregates (3–100 cells) was estimated using this technique.

Platelets were incubated with MS in a ratio 1/100 for 1 hour at RT and aggregation was assessed. As a control, we used platelet incubated with PBS (same buffer in which MS are suspended). In adenovirus aggregation experiments, platelets were incubated with 6 μl adenovirus of 1×10^{10} vp/ml in a ratio 1/200 for 1 hour at RT and aggregation was performed as described above. As a control, we used platelets incubated with TRIS buffer (same buffer in which adenovirus was suspended). For platelet membrane receptor inhibition experiments, aggregation was assessed after incubation of PRP (10 min, RT) with each of the following inhibitors: 10 μl of anti-GPIb AK2 antibodies at 1:50 dilution, 10 μl of anti-P-selectin AK4 antibodies at 1:20 dilution, 10 μl of anti- $\alpha\text{IIb}\beta 3$ RGD peptide fragments and F(ab')₂ fragment of anti- $\alpha\text{IIb}\beta 3$ monoclonal antibody (CRC64) (each at 1:50 dilution). Spontaneous, 0.1 and 5 μM ADP-induced aggregation was recorded after 2 min incubation at 37°C. Aggregation experiments were performed at a stirring speed of 800 rpm and were repeated three times.

Fluorescence microscopy

Platelets (PRP) were incubated with adenovirus, MS, and platelet receptor inhibitors for aggregation studies with or without prior (as a control) pre-activation with 5 μM ADP (5 min, RT). Samples were then fixed in 4% PFA for 10 min, RT. The resulting pellet was washed three times in PBS before applying to a drop of glycerol–gelatine on a glass slide and analyzed with fluorescent microscopy (Leica DM5000B). For quantitative analysis, 10 random fields were selected and the number of platelets coupled with MS was counted relative to the total number of platelets and expressed as a percentage (the total number of platelets counted was no less than 1000 in any experiment).

Electron microscopy

Platelets (with and without incubation with MS), were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 in ratio 1:30 for 1.5 hours, at RT, then were placed on polycarbonate membranes (Isopore Membrane Filters, Millipore) with 0.40 μm diameter holes, dehydrated in a dilution series of ethanol, and finally stored in 100% ethanol. The samples were dried (HCP-1 critical-point drying apparatus; Hitachi, Japan), coated with carbon-gold and examined with a scanning electron microscope (SEM) (PHILLIPS PSEM 550x, Netherlands) with magnification $\times 5000$. For Kistrin experiments, platelets were incubated with adenovirus for 30 min with and without Kistrin and platelet pellets from the two tubes as well as untreated platelets (no virus) were fixed with 2.5% glutaraldehyde and processed for electron microscopy (EM) as above.

Confocal microscopy

Coverslips (24 \times 40 mm²; VWR Scientific, West Chester, PA) were coated with human collagen type I (50 $\mu\text{g}/\text{ml}$ in 0.02 N acetic acid) for 2 hours at 37°C. Platelets (2×10^7) with and

without Kistrin (15 min at RT) were adhered to collagen coated coverslips and incubated for 2.5 hours at 37°C. Coverslips were washed with PBS to remove unbound platelets and then stained for actin with phalloidin-Texas Red. Control experiments with actin staining only were included. Coverslips were mounted with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA) and examined using 100 \times aperture oil objective with the Leica TCS SP2 confocal microscope using an excitation laser of 543 nm. A quantification analysis was performed using ImagePro Plus (Media Cybernetics, Silver Spring, MD).

Statistics

All values are presented as the mean \pm SEM. Statistical significance was calculated using the Student *t*-test. *P*-value < 0.05 was considered statistically significant.

Results

Activated but not resting platelets internalize latex MS without influencing platelet aggregation

MS were visualized inside platelets from healthy subjects after 1 hour incubation with these inert particles. This was, however, only after platelets were pre-activated with 5.0 μM of the common platelet agonist ADP. Such pre-activation led to platelet shape change, pseudopodia formation and MS incorporation (Figure 1E and F). Without pre-activation, platelets did not internalize MS and they remained free in suspension (Figure 1C–D). We also found that only $6.4 \pm 3.4\%$ of platelets were able to uptake MS ($n = 10$, $p < 0.05$) (Figure 1E). The low dose of the ADP (0.1 μM), induced platelet aggregation without an induction of the platelet ability to internalize the MS. Activation of platelets with 5 μM ADP for 5 min was sufficient to induce this ability. Further increase in the concentration of ADP or the duration of incubation did not enhance the internalization of MS. These data collectively indicate that MS themselves do not activate platelets and for their internalization, platelets require prior activation.

Regarding platelet aggregation, Figure 2(A) shows there is no difference in the patterns and magnitude of aggregation (percentage of LT) or aggregate size (RU) between control platelets in PRP, incubated with PBS and those incubated with MS.

Adenovirus activates human platelets, induces rapid expression of P-selectin, and potentiates ADP-induced platelet aggregation

Although pre-activated platelets uptake MS, these inert particles did not influence platelet aggregation in response to different doses of ADP. We therefore compared the ability of platelet to engulf MS to that of a biologically active particle (adenovirus) and studied the effect on platelet function. We found that the addition of adenovirus induced and potentiated platelet aggregation and aggregate size (Figure 2B). Since we used adenovirus that was dialyzed in Tris buffer, we tested the effect of Tris buffer alone on platelet aggregation and consistent with a previous report [32], we found that Tris reduces platelet aggregation.

Similar to what was previously shown in murine platelets [15], we found that adenovirus induces rapid and significant

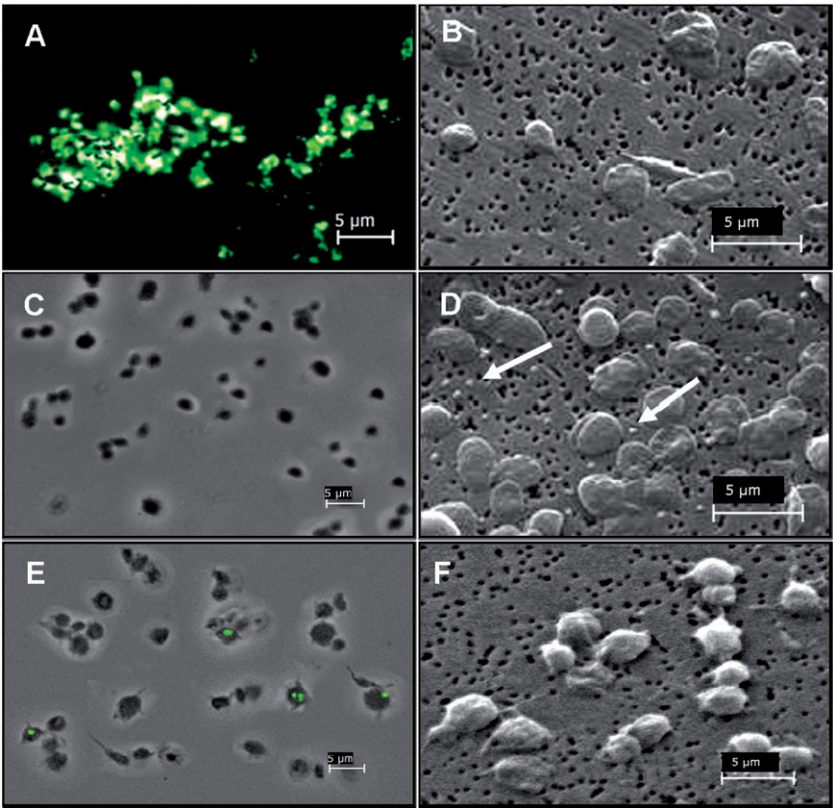


Figure 1. Activated and not resting platelets internalize latex MS. Activated (ADP 5 μ M at 37°C for 5 min) or resting platelets were incubated with MS for 1 hour. Left panel: fluorescent microscopy, magnification $\times 1000$. Right panel: SEM, magnification $\times 5000$. (A) MS control; (B) platelets control (no ADP); (C) platelets incubated with MS without pre-activation with ADP – there is no internalized MS or free MS in the sample because of thorough washing; (D) the same procedure as in C (platelets incubated with MS without ADP pre-activation) prepared for SEM – free MS remained free in suspension on the filter surface during the sample preparation (see “Methods” section). The arrows show that MS remain free in suspension despite thorough washing; and (E) and (F) activated platelets with spherules and pseudopodia formation. MS were visualized inside platelets after 1 hour incubation with these inert particles and are seen inside activated but not resting platelets.

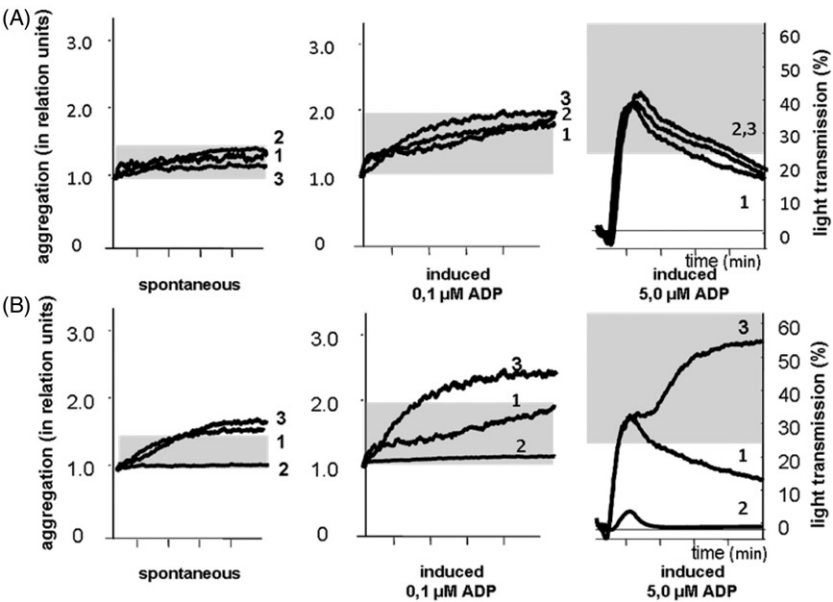


Figure 2. Influence of MS vs. adenoviruses (Ad) on platelet aggregation. Platelet aggregation was measured simultaneously using traditional turbidometric method and aggregate size formation. Small aggregates are seen with spontaneous and with 0.1 μ M ADP-induced aggregation (left axis, measured in RU), large aggregates were seen and the percentage of LT increases with high dose (5 μ M ADP) of inductor (right axis). (A) Aggregation in response to MS: 1, control platelets in PRP; 2, following incubation with PBS (1 hour); and 3, following incubation with MS in PBS (1 hour); (B) aggregation in response to Ad: 1, control platelets in PRP; 2, following incubation with 15 μ l 10 mM Tris (1 hour); 3, following incubation with Ad in Tris (1 hour). Normal ranges are shaded in gray.

P-selectin expression on human platelets. There was a significant fold increase in the IPA⁺ (percentage of positive events \times mean fluorescence) for platelets incubated with adenovirus (2.8-fold increase) and thrombin activated platelets (positive control; 3.3-fold increase) ($n = 3$, $p < 0.05$) compared to resting platelets (data not shown). These results indicate that while platelets can engulf both inert and biological particles, the dynamics and effects on platelet functions vary considerably.

Inhibition of platelet α IIB β 3, GPIb α , and P-selectin inhibits internalization of MS and platelet aggregation

Using the Latex MS model, we performed a series of experiments using different inhibitors to platelet surface receptors: GPIb α and P-selectin. We wanted to test the influence of the inhibition of these proteins on platelet aggregation following incubation with MS and also on their ability to internalize MS. We found that platelet aggregation and aggregate size are reduced with all three inhibitors (Figure 3A). The inhibition of α IIB β 3 almost abolished platelet ability to internalize MS (only $0.38 \pm 0.14\%$ of platelets internalized MS ($n = 10$, $p = 0.0015$)). The inhibition of GPIb α significantly reduced the percentage of platelets engulfing MS ($1.68 \pm 0.7\%$ of tested platelets internalized MS ($p = 0.014$)). The inhibition of P-selectin slightly reduced this percentage ($4.76 \pm 1.53\%$ of platelets internalized MS) and this was not statistically significant ($p = 0.13$) (Figure 3B). These data suggest that α IIB β 3 plays a key role in the mechanism of MS uptake and to less extent GPIb α with a possible role for P-selectin facilitating this process.

Inhibition of α IIB β 3 using various peptides inhibits internalization of MS, platelet aggregation, and platelet aggregates

We also compared the properties of different α IIB β 3 inhibitors. In these series of experiments we used monoclonal

antibody CRC64 and a variety of α IIB β 3 peptide and nonpeptide inhibitors: eptifibatide, nonpeptide inhibitor tirofiban, and peptide inhibitor with RGDS fragment. As shown in Figure 4(A), all four inhibitors reduced the platelet aggregation and the aggregate size following incubation with MS in response to spontaneous, low dose and high dose ADP when compared to control platelets. In addition, Figure 4(B) shows that platelet internalization of MS was inhibited significantly by all four inhibitors. The percentage of platelets with engulfed MS was $0.86 \pm 0.32\%$, $1.32 \pm 0.89\%$, $0.63 \pm 0.18\%$, and $0.38 \pm 0.14\%$ with the use of anti- α IIB β 3 MAb CRC64, peptide inhibitor with RGD fragment eptifibatide, synthesized inhibitor with RGD fragment, RDGS peptide, and the nonpeptide inhibitor tirofiban ($n = 10$; $p = 0.009$, 0.022 , 0.002 , and 0.014), respectively.

Dual inhibition of α IIB β 3 and α V β 3 does not prevent adenovirus internalization and maintains virus-induced platelet activation

We next investigated whether α IIB β 3 was also essential for the platelet interaction/coupling or engulfing adenovirus. We used Kistrin, a known inhibitor to both α IIB β 3 and α V β 3 integrins [25, 26] which are expressed on platelet surface. This series of experiments tested the influence of these integrins on: (a) virus-induced platelet activation (via P-selectin expression), (b) platelet ability to internalize the virus.

We first verified the specific inhibition of Kistrin to α IIB β 3 in the context of platelet fibrinogen binding. There was a significantly lower fold increase in IPA⁺ for thrombin activated platelets after treatment with Kistrin (two-fold increase) compared with thrombin activated platelets without Kistrin inhibition (4.4-fold increase; $n = 3$, $p < 0.05$) (data not shown). We did not test in this study the specific inhibition of Kistrin to α V β 3. We then assessed P-selectin expression and found that adenovirus mediated P-selectin expression is not

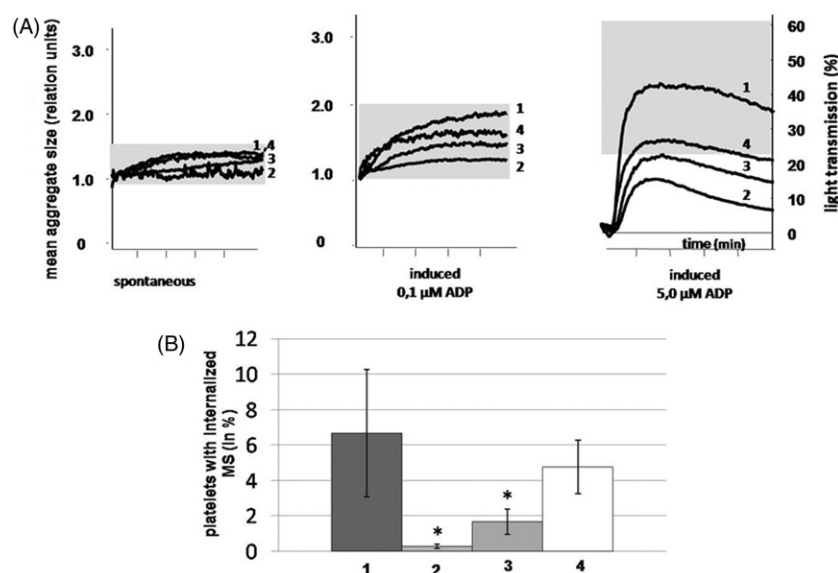


Figure 3. Effect of inhibition of membrane receptors on platelet functions and ability to internalize MS. (A) Platelet aggregation and aggregate size in response to spontaneous, 0.1 and 5.0 μ M ADP; (B) ability to internalize MS: fixed samples were analyzed with fluorescence microscopy and the percentage of platelet/MS couples were evaluated in 10 randomly selected fields by direct counting.

Note: * p -value < 0.05 . 1, control curve; 2, anti- α IIB β 3 MAb CRC64; 3, anti-GPIb MAb AK2; 4, anti-P-selectin MAb (AK4).

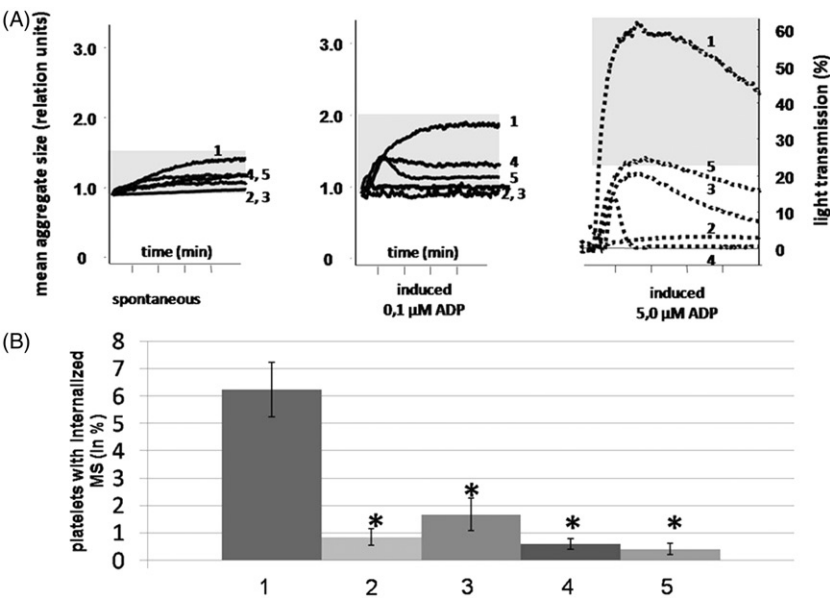


Figure 4. Effect of various α IIb β 3 inhibitors on platelet function and ability to internalize MS. (A) Platelet aggregation in response to spontaneous, 0.1 and 5.0 μ M ADP following incubation with different antibodies. Normal ranges are shaded in gray. 1, control curve; 2, anti- α IIb β 3 MAb CRC64; 3, anti- α IIb β 3 eptifibatide peptide inhibitor; 4, anti- α IIb β 3 tirofiban nonpeptide inhibitor; 5, anti- α IIb β 3 peptide inhibitor with RGD fragment. (B) Platelet internalization of MS. Note: * p -value < 0.05.

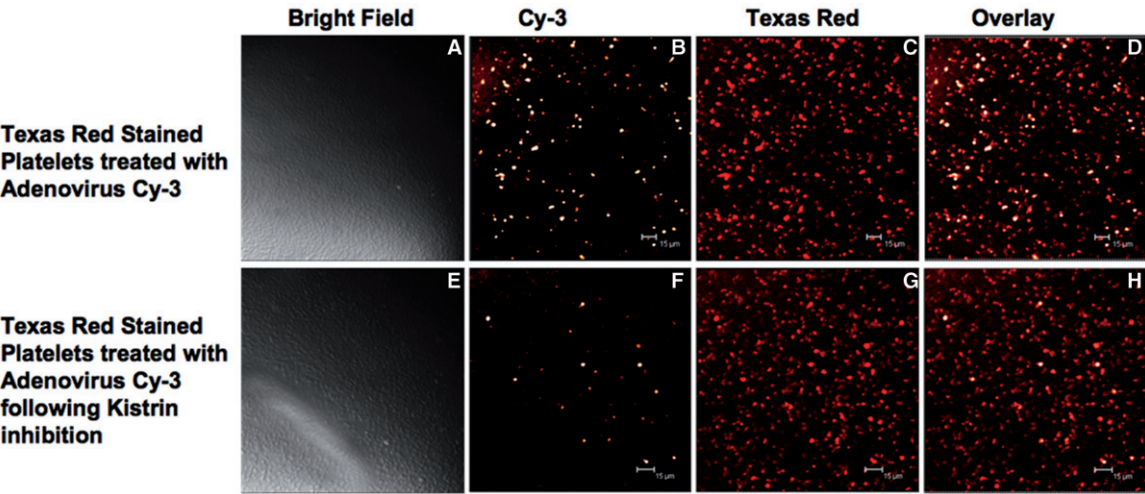


Figure 5. Confocal microscopy studies of platelet adenovirus interaction with or without Kistrin treatment. (A) and (E) Bright field views; (B) and (F) adenovirus; (C) and (G) platelet staining; and (D) and (H) overlay showing platelet Ad couples. Couples are still seen in H indicating that Kistrin does not prevent platelet/adenovirus coupling. Cy-3 is pseudocolored in white.

affected by Kistrin. The change in IPA⁺ representing P-selectin expression for Kistrin treated vs. nonKistrin treated showed 1.9- and 1.7-fold increase) ($n = 3$, $p > 0.05$) (data not shown).

Kistrin does not prevent adenovirus platelet coupling as visualized by confocal microscopy. Actin-stained platelets bound to collagen-coated slides were seen in conjunction with fluorescently conjugated Cy3-adenovirus in both Kistrin-treated and untreated platelets. Although the latter showed less coupling with the Cy3-signals (Figure 5), quantitative analysis of five randomly selected fields in three independent experiments, did not demonstrate significance difference ($n = 5$; $p = 0.18$, 0.12 , and 0.43). However, we were unable

to conclude based on this analysis whether the Cy3-adenovirus particles were only bound to the platelet surface or also found inside platelet cytoplasm.

Kistrin does not prevent adenovirus internalization by platelets as visualized by EM. Due to the limitation of the confocal microscopy, we examined platelet adenovirus coupling by EM in experiments independent of the confocal microscopy. Consistent with a previous report [19], the virus was seen inside platelets. Virus particles were found in association with the cell surface and are localized to the open canalicular system and were much more evident in the adenovirus-platelet compared to Kistrin adenovirus treated preparation (Figure 6A and B). These data collectively

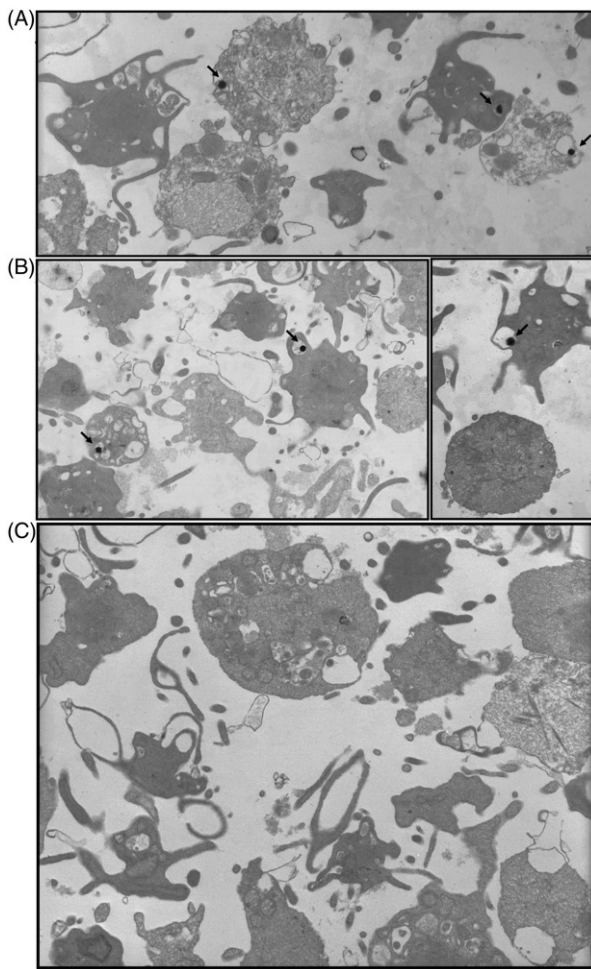


Figure 6. EM studies showing the effect of Kistrin on platelet adenovirus interaction. (A) Platelets incubated with Ad only (B) Kistrin treated platelets/Ad mix. Adenovirus is seen inside platelets in both A and B as indicated by small black arrows. While Kistrin may inhibit, it does not prevent adenovirus internalization. (C) Platelet control (without Ad or Kistrin). (Magnification at 7000 \times).

indicate that platelet surface molecules other than α IIb β 3 and α V β 3 may be involved in this process of adenovirus internalization.

Discussion

Our study shows that platelets do not engulf inert MS without prior activation or with low doses of ADP. Also, although high doses of ADP stimulate platelets to internalize MS, only 6% of platelet populations possess this ability. MS uptake is accompanied by platelet shape change (from discs to spheres) and pseudopodia formation but has no effect on platelet aggregation. The platelet–MS interaction depends mainly on α IIb β 3 since the inhibition of this protein using monoclonal antibody and a variety of RGD peptide and nonpeptide inhibitors significantly reduced the percentage of platelets engulfing MS. GPIb α was also found to mediate such interaction together with platelet P-selectin but to a lesser degree.

Studies of platelet MS interactions helps evaluate safety related to systemic administration of biodegradable micro- and nanoparticles for diagnosis or drug delivery since these may come into contact with platelets in circulation resulting in

thrombotic events. According to Ramtoola et al., 2011, biodegradable particles 2–9 μ m do not have any influence on platelet activation or aggregation, irrespective of particles' composition, size, and surface morphology and were thus considered safe. Considering the size of platelets (2–4 μ m), it is highly unlikely that these particles are engulfed by platelets. In our study we tested particles that are 10 times smaller than platelets, yet internalization occurred only under high dose of platelet agonists and only in a small population of platelets. This indicates that smaller MS particles may also be utilized and may therefore warrant further studies.

Compared to MS, adenovirus itself induces platelet aggregation and unlike previously reported [33], our study shows it also potentiates ADP-induced platelet aggregation. This is a novel finding which indicates that platelets react differently to foreign particles: inert vs. biological particles. This can also be illustrated by the fact that MS–platelet interaction is almost P-selectin independent compared to platelet interaction with adenovirus [15] or bacteria [3] which is associated with upregulation of platelet P-selectin. Perhaps the host defense role requires platelets to be activated in order to remove microbes, since activated platelets are cleared from circulation by the reticuloendothelial system [19, 24]. We can also further speculate that platelets may react differentially to different microbes.

Platelet activation was reported in response to a variety of microbes such as staphylococci [3], chlamydia [34], influenza virus [35], and HIV [36] and thrombocytopenia is associated with several viral infections and viral mediated gene therapy [16, 18]. ITP has also been reported in association with viral infection [37]. Microbe-induced thrombocytopenia can be explained on the basis of the increase in nonspecific platelet destruction due to circulating immune complexes, appearance of specific antiplatelet antibody, decrease in megakaryocytopoiesis or, simply a direct effect or microbe invasion of platelets which results in clearance of loaded platelets by the reticuloendothelial system [2, 3, 38–40].

Adenovirus-induced platelet activation (as shown by P-selectin expression) is independent of α IIb β 3. It is interesting that inhibition of α IIb β 3 almost completely abolished the platelets ability to internalize MS but did not prevent internalization of adenovirus. Our data from inhibition of a wide spectrum of inhibitors from all the α IIb β 3 inhibitors family [RGD containing (Kistrin, INTEGRILIN and RGDS peptide), RGD alone, F(ab')₂ fragment monoclonal antibody, and a nonpeptide inhibitor] showed comparable effects – almost complete inhibition of MS uptake. Regarding adenovirus, Kistrin did not prevent Ad internalization completely which indicates that simple RGD–integrins binding might not be sufficient for Ad entry into platelets and perhaps other molecules need to be investigated. Despite the limitation of not using the same α IIb β 3 blockers in MS and Ad, these data indicate that the mechanism of engulfing foreign particles may vary according to the nature of the particle: inert vs. biologically active, and possibly also the type of microbe itself.

It is known that virus internalization in cells is mediated by α V β 3 integrins [22] and that α IIb β 3 is involved in bacteria internalization. However, in this study, based on the confocal

and EM data, we found that platelet adenovirus interaction and internalization continued in the presence of Kistrin, the simultaneous peptide inhibitor of both $\alpha V\beta 3$ and $\alpha IIb\beta 3$. This is in agreement with Shimony et al.'s specific $\alpha IIb\beta 3$ monoclonal antibody blocking studies [20]. It must be noted that the virus was associated mainly with the platelet surface and surface connected canalicular system rather than true internalization. This finding is supported by Stone et al. [19]. Interestingly, platelet activation (as shown by increased P-selectin expression) also continued in the presence of Kistrin. This together with our aggregation data in which we showed that adenovirus potentiates ADP-induced platelet aggregation indicate that platelet activation and virus uptake are two different processes.

Understanding of the role of platelet receptors in platelet interactions with other cells can help prevent adenovirus mediated thrombocytopenia and improve gene therapy applications. However, based on this study, we can speculate that the inhibition of platelets' $\alpha V\beta 3$ and $\alpha IIb\beta 3$ is insufficient to prevent adenovirus-induced thrombocytopenia and that other platelet integrins may be involved and need to be studied. In addition, we believe that the modification of adenovirus [41] may also be helpful in this regard.

In summary, this study demonstrates that platelets interact with foreign particles by mechanisms that vary according to the nature of these particles. Adenovirus activates platelets and enhances their aggregation. While platelet $\alpha IIb\beta 3$ is the key protein involved in platelet engulfing of inert particles, it is not critical for mediating adenovirus internalization. Other platelet candidate molecules remain to be investigated as potential targets for management of virus-induced thrombocytopenia.

Acknowledgments

This study was supported by a research grant from the Canadian Haemophilia Society. We thank Dr David Lillicrap for providing laboratory facilities for the generation of adenovirus. We thank Dr Alexey V. Mazurov and Dr Mikhail Ovchinnikov for kindly providing the antagonists to platelet membrane receptors.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

This part of the study was supported by a Russian Foundation for Basic Research (RFBR) Grant 09-04-01329.

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