The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib

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Introduction

Microparticles are submicron vesicles released from the plasma membrane in response to activation or apoptosis. The microparticle surface possess approximately 50- to 100-fold higher procoagulant activity than the platelet surface (1), primarily through the exposure of tissue factor and phosphatidylserine on their outer membrane (2–4). Microparticles are commonly detected using flow cytometry to assess their antigenic characteristics and capacity to bind annexin V, a calcium-dependent, phosphatidylserine-binding protein (5). Platelet-derived microparticles comprise the majority of circulating microparticles in normal plasma detectable by flow cytometry.

A number of recent reports have questioned the use of annexin V for microparticle detection, stating that a subpopulation of microparticles do not demonstrate detectable levels of annexin V binding (6–10). Whilst investigating the antigenic characteristics of endothelial cell-derived microparticles, Jimenez et al. reported that only a small proportion of microparticles bound annexin V (10), and in a later “Letter to the editor”, the same authors advised that assays that solely rely upon annexin V for microparticle detection may lead to “grossly misleading results” (6). A number of investigators have since considered alternatives to annexin V as a marker of phosphatidylserine exposure (lactadherin, diannexin); however, annexin V binding remains the most frequently-used marker for microparticle detection. There may be a number of explanations for failure to detect annexin V binding to microparticles, including inadequate experimental conditions to permit maximal annexin V binding or the presence of inhibitors to annexin V binding. Alternatively, decreased or absent annexin V binding may simply result from insufficient phosphatidylserine exposure on the membrane surface to allow detectable annexin V bind-

Summary

It has been widely accepted that microparticles expose phosphatidylserine which in turn binds annexin V. It was the objective of this study to compare the antigenic characteristics and phospholipid-dependent procoagulant activity of annexin V positive and -negative subpopulations of platelet-derived microparticles. Annexin V positive and -negative microparticles were identified and characterised using flow cytometry and procoagulant activity was measured by a phospholipid-dependent assay (XACT). In unstimulated platelet-poor plasma, 80% of platelet-derived microparticles failed to bind annexin V. Varying the assay constituents (buffer, calcium and annexin V concentration) did not alter annexin V binding. The proportion of microparticles that bound annexin V was dependent upon the agonist, with physiological agonists such as collagen resulting in fewer annexin V binding microparticles than non-physiological agonists such as ionophore. CD42b (glycoprotein Ib) expression was significantly decreased and CD62p and CD63 expression were significantly increased in annexin V positive compared to annexin V negative subpopulations. There was no significant difference in CD41, CD61, CD42a and CD40L expression between annexin V positive and -negative subpopulations. A significant correlation between annexin V binding and XACT was found (p=0.033). Annexin V inhibited greater than 95% of phospholipid activity, suggesting that annexin V binding was a true reflection of procoagulant activity. The majority of platelet-derived microparticles in unstimulated plasma failed to bind annexin V and showed significantly increased levels of CD42b compared to annexin V positive events. Phospholipid-dependent procoagulant activity is limited to the annexin V positive subpopulation and is agonist-dependent. The significance of annexin V negative microparticles is unclear, however, it is possible that they possess other activities aside from procoagulant phospholipid activity.

Keywords

Annexin V, flow cytometry, microparticles, phosphatidylserine, XACT

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ing, thereby representing true annexin V negative microparticles. Finally, it is possible that these events may not represent microparticles at all, but instead platelet-derived exosomes (resulting from platelet activation), small platelets or the remnants of activated platelets, possessing scattering properties of similar magnitude to microparticles. Whether these annexin V negative events possess procoagulant phospholipid activity is also unclear from previous studies.

We have recently reported an assay (XACT) for the detection of both the procoagulant activity of cellular microparticles and circulating procoagulant phospholipids (11, 12). We demonstrated a strong correlation between the number of annexin V binding microparticles and XACT times; however, in these studies, we did not attempt to quantify the effects of annexin V negative microparticles.

The aims of the current study were to quantify the proportion of microparticles that bind annexin V and determine if experimental conditions may play a role in this. We also investigated the effects of platelet agonists on phosphatidylserine exposure and assessed whether annexin V binding is a true reflection of the procoagulant activity of exposed phosphatidylserine. Furthermore, in order to ensure that the annexin V negative events detected in this study were platelet-derived microparticles and not platelet-derived exosomes, the antigenic characteristics of both annexin V positive and annexin V negative microparticles were assessed and compared.

Materials and methods

Sample collection

Blood samples were collected from normal donors (following informed consent) using the Vacutainer system into 4.5 ml tubes in which 0.105 M sodium citrate was the anticoagulant (unless otherwise specified) using a 21-Gauge winged infusion set.

Sample processing

Platelet-rich plasma (PRP) was generated by centrifugation of whole blood at 150 g for 10 minutes (min). Platelet-poor plasma (PPP) was generated by centrifugation of whole blood at 2,750 g for 15 min.

Platelet agonist stimulation

Where appropriate, platelets were stimulated using physiological and non-physiological agonists including collagen (Paton Scientific, Victor Harbour, SA, Australia), Thrombin Receptor Activating Peptide (TRAP) (Sigma-Aldrich, St Louis, USA) and Iono-phore (Sigma-Aldrich). The effects of two detergent sclerosant agents, Sodium Tetradeyl Sulphate (STS) (Australian Medical and Scientific Limited, Artarmon, NSW, Australia) and Polidocanol (POL) (Smith and Nephew, Mount Waverley, VIC, Australia), which have previously been demonstrated to induce marked in vitro microparticle formation (13) were also studied. Briefly, 80 μl of PRP was added to 20 μl of buffer containing agonist and incubated for 30 min at room temperature.

Procoagulant phospholipid activity testing by XACT

XACT testing was performed as previously described (14). Briefly, 25 μl of test sample was mixed with 25 μl of phospholipid-depleted porcine plasma and incubated for 2 min at 37ºC. One hundred μl of a Factor Xa reagent was added and the time taken to clot measured using an ST4 instrument (Diagnostica Stago, Asnieres, France). Using serial dilutions of a phosphatidylserine/phosphatidylcholine mixture, a standard curve was generated such that a procoagulant phospholipid (PPL) concentration could be made.

Flow cytometry

Flow cytometer tubes, antibodies and annexin V were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Fifty μl of test sample was diluted with 200 μl of HEPES buffered saline solution (45 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM HEPES, pH 7.4). Ten μl of this suspension was then added to flow cytometry tubes containing antibody (5 μl per antibody), 2 μl of annexin V (final concentration 20 μg/ml) and the total volume made up to 50 μl using HEPES-buffered saline solution. To assess annexin V binding, the control sample contained 2.5 mM K2EDTA, whilst the test samples contained 2.5 mM CaCl2. This was incubated for 30 min at room temperature before the addition of 1 ml HEPES containing...
2.5 mM CaCl₂ (Test) or 2.5 mM K₂EDTA (Control). Flow cytometry was performed using an LSR-II flow cytometer and data collected using FACS-Diva software. Counting was performed using TRUCount™ tubes (Becton Dickinson). Flow cytometer settings were checked using Rainbow Calibration (Spherotech, Chicago, IL, USA) beads prior to each experiment. Flow cytometer forward scatter settings were determined using standardised latex beads (Sigma-Aldrich) resuspended in 1 ml of HEPES buffer. A minimum of 10,000 single beads were counted in for each bead tube. In order to minimise the contribution of small platelets to microparticle analysis, the mean forward scatter of 0.82 μm beads was used to define the upper forward scatter limit of the microparticle analysis region, with platelet-derived microparticles detected using an antibody to either CD61 or CD41. These antibodies were used interchangeably as platelet-specific markers since previous work had demonstrated no difference in their expression (data not shown).

**Standardisation of forward scatter detection using latex beads**

In order to standardise forward scatter settings on the flow cytometer to allow an estimation of the size of microparticle populations, the forward scatter characteristics of latex beads of mean diameter 1.09 μm, 1.00 μm, 0.82 μm, 0.60 μm, 0.46 μm and 0.32 μm were analysed (Fig. 1). A monotonic increase (with respect to the bead diameter) in the mean forward scatter of these beads was observed.

**Effect of experimental conditions on annexin V binding**

In order to ensure that the assay conditions were sufficient to allow for optimal annexin V binding to exposed phosphatidylserine, variations to the typical assay constituents (HEPES buffered saline solution, 2.5 mM calcium, 20 μg/ml annexin V) were assessed in PPP samples from normal donors.

Several buffer types were tested, including: phosphate-buffered saline (PBS) solution, Hank’s saline solution and 0.9% sodium chloride. The calcium concentration of all buffers was adjusted to 2.5 mM. The percentage of platelet-derived microparticles that were annexin V positive using each buffer type was compared to HEPES-buffered saline solution (n=5, Fig. 2a). There was no significant difference in the percentage of annexin V positive microparticles between HEPES-buffered saline solution and any buffer type used, with the exception of the phosphate-buffered saline solution, which demonstrated significantly decreased annexin V binding (p=0.0436). The decreased annexin V binding demonstrated in PBS was presumably due to the formation of an insoluble calcium phosphate precipitate. In order to minimise the effects of precipitate formation, a PBS solution with a low phosphate concentration (1.4 mM), as proposed by Biro et al. (15), was also...
assessed. There was no significant difference in the percentage of annexin V positive microparticles between this low concentration phosphate buffer and HEPES buffered saline solution (p=0.8482).

The effects of calcium concentration on annexin V binding in PPP samples (n=6) was then assessed using calcium concentrations of 0 mM, 0.5 mM, 1.25 mM and 5 mM (Fig. 2b). The percentage of annexin V positive microparticles for each concentration was compared to the standard calcium concentration of 2.5 mM. The percentage of annexin V binding microparticles was significantly decreased at calcium concentrations of 0 mM and 0.5 mM; however, there was no significant difference in annexin V binding between the standard calcium concentration and calcium concentrations of 1.25 mM and 5 mM.

Finally, the effect of annexin V concentration on the percentage of annexin V positive microparticles was investigated in PPP samples (n=6, Fig. 2c). There was no significant difference in the percentages of annexin V binding microparticles between the addition of 20 μg/ml of annexin V and the addition of other concentrations of annexin V.

Statistical analysis

Statistical analysis was performed using PRISM (v3.02) software. Correlations were determined using a Spearman-Rank test. Paired analyses were performed using a paired t-test.

Results

The detection of annexin V positive and negative microparticles by flow cytometry

Annexin V binding by platelet derived microparticles was assessed in unstimulated PPP samples obtained from normal donors (n=5). Mean annexin V negative microparticle counts were significantly higher than mean annexin V positive microparticle counts (1,835 ± 422 vs. 360 ± 48 microparticles/μl, respectively, p=0.0346) (Fig. 3). When expressed as a percentage of total platelet-derived microparticles, the annexin V negative population represented 80.5 ± 5.4% of microparticles, whereas the annexin V positive population represented 19.5 ± 5.4% of microparticles.

The effect of platelet agonist stimulation on annexin V binding.

The effect of platelet agonist stimulation on annexin V binding to microparticles was investigated. PPP samples obtained from normal donors were incubated in the presence of either HEPES saline (unstimulated), collagen (20 μg/ml), TRAP (80 μM), ADP (20 μM), Ionophore (3 μM + 2.5 μM CaCl₂), Sodium Tetradecyl Sulphate (STS) (0.1%) or Polidocanol (POL) (0.1%) for 30 min (Fig. 4). The percentage of annexin V positive events was dependent upon the agonist used, with POL, STS and Ionophore stimulation resulting in the highest percentage of annexin V positive microparticles.

Figure 3: Analysis of annexin V binding in platelet-poor plasma (PPP) samples. Counting of annexin V positive (+) and annexin V negative (−) CD61+ microparticles was performed in PPP samples from normal donors (n=5). Data shown are the mean microparticle counts, while error bars represent the standard error of the mean (SEM).

Figure 4: The effect of agonist stimulation on annexin V binding. Platelet-rich plasma samples (n=5) were incubated with either HEPES saline (unstimulated), 20 μg/ml collagen, 80 μM TRAP, 20 μM ADP, 3 μM Ionophore (+2.5 mM calcium), 0.1% STS or 0.1% POL for 30 min, before the percentage of annexin V positive binding was assessed by flow cytometry. The total number of platelet-derived microparticles are shown in brackets. Data shown are the mean values while error bars represent the standard error of the mean (SEM). * represents p<0.05 vs. unstimulated samples, ** represents p<0.01 vs. unstimulated samples.
Table 1: The antigenic characteristics of annexin V negative (-) and annexin V positive (+) microparticles in platelet-rich plasma (PRP). Microparticles from PRP samples obtained from normal donors (n=5) were analysed for annexin V binding. The antigenic characteristics (CD31, CD41a, CD42a, CD42b, CD61a, CD62p, CD63 and CD154 expression) and forward and side scatters were assessed for CD41a positive microparticles. Data for forward scatter (FSC) and side scatter (SSC) is given as the mean scatter. Data above the dotted line are given as the mean fluorescence intensity, whereas data below the dotted line are given as the percentage of CD41a positive microparticles.

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<th>Annexin V +</th>
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<td>1481 ±242</td>
<td>1261 ± 499</td>
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Antigenic characteristics of annexin V positive and negative microparticles

The antigenic characteristics of annexin V positive and annexin V negative platelet-derived microparticles were assessed in samples obtained from normal donors (Table 1). CD41a positive microparticles were gated and the mean fluorescence intensity of antibodies to CD31, CD41a, CD42a, CD42b and CD61 were assessed. The expression of CD31, CD41a, CD42a and CD61 was slightly increased on annexin V positive microparticles when compared to annexin V negative microparticles; however, this reached statistical significance for CD31 expression only. Of particular note, the expression of CD42b was significantly decreased on annexin V positive microparticles when compared to annexin V negative microparticles (mean fluorescence intensity 1,380 ± 354 vs. 5,738 ± 705, respectively; p=0.0010). There was no significant difference between the mean forward or side scatters of annexin V negative and annexin V positive microparticles.

The expression of the platelet activation markers CD62p, CD63 and CD154 (CD40L) were also studied and assessed as a percentage of CD41a positive events (Table 1). Annexin V positive microparticles expressed significantly higher levels of CD62p and CD63 than annexin V negative microparticles. There was no significant difference in the expression of CD154 between annexin V negative and positive microparticles.

The procoagulant activity of annexin V positive and negative microparticles

The relationship between annexin V binding and phospholipid-dependent procoagulant was assessed using two strategies. Firstly, the ability of annexin V to inhibit microparticle procoagulant activity was measured. Microparticles from an unstimulated PPP sample were concentrated by three sequential centrifugations at 16,060 g (Fig. 5). The microparticles were then resuspended in HEPES buffered saline solution. This microparticle rich suspension was incubated in the presence of calcium (2.5 mM) and either annexin V (20 μg/ml) or HEPES-buffered saline (Control) for 30 min, following which XACT times were assessed. The mean XACT time of the microparticle increased from 16.1 seconds in the control.
trol sample to 72.1 seconds in the annexin V incubated sample. When converted to a procoagulant phospholipid concentration using a standard curve, annexin V inhibited greater than 95% of total procoagulant phospholipid activity.

In a second strategy, the procoagulant activity of a fixed number of microparticles with different proportions of annexin V positive and negative events was assessed. PRP was obtained and stimulated using either collagen, TRAP, calcium ionophore or STS for 30 min. In addition, a PRP sample that had been subjected to two repeat cycles of freeze-thawing and a platelet concentrate stored at room temperature for four days were also used. The platelets were removed from all samples by centrifugation at 2,750 g for 15 min. Finally, the plasma was removed by centrifugation at 16,060 g for 30 min, and the microparticles resuspended in HEPES-buffered saline. The percentage of CD41a positive microparticles that bound annexin V positive was then determined (Fig. 6).

The percentage of events that were annexin V positive was dependent upon the agonist used, with STS and freeze-thawing the best agonists to obtain maximal annexin V binding. Following microparticle counting, the remainder of each aliquot was diluted using HEPES buffer so that the concentration of CD41a positive microparticles in each fraction was 4,000 microparticles/μl. XACT

Figure 6: The correlation between XACT times and the percentage of annexin V positive microparticles. Platelet-rich plasma (PRP) was subjected to agonist stimulation before platelets were removed by centrifugation. The number of CD41a positive microparticles was counted by flow cytometry. a) Using the flow cytometric count, samples were diluted such that a known count of CD41a positive microparticles (4,000 microparticles/μl) was tested by XACT. There was a significant correlation between XACT times and the percentage and number of annexin V positive microparticles (p=0.033) as determined by flow cytometry for the agonists; b) Collagen (20 μg/ml); c) TRAP (80 μM); d) Ionomophore (3 μM +2.5 μM CaCl2); e) Aged PRP; f) Freeze-thawed PRP; g) STS (0.1%).
testing was then performed on each aliquot (Fig. 6b). There was a significant correlation between the XACT times and the percentage of annexin V positive microparticles (p=0.033).

## Discussion

Platelet-derived microparticles are believed to elicit their main procoagulant activity through the exposure of the negatively charged phospholipid phosphatidylserine on the outer surface of their membrane (2, 16). Phosphatidylserine is an essential component in the coagulation cascade, acting as the catalytic site for the binding of activated factors X and V and resulting in the conversion of prothrombin to thrombin (5, 17). Microparticles derived from other cell types (namely monocytes) may also express procoagulant activity through the exposure of tissue factor, which forms a complex with factor VII to activate factor X. The activity of this tissue factor / factor VII complex is greatly increased 16 million-fold in the presence of phosphatidylserine (18).

Flow cytometric methods that assess microparticle phosphatidylserine exposure typically rely upon the binding of fluorescently labelled annexin V (5, 19–21). Recent reports have however, questioned the capacity of platelet-derived microparticles to bind detectable levels of annexin V (6–8, 10, 22). Little is known about the procoagulant properties or antigenic characteristics of these annexin V negative microparticles. This study aimed to quantify the proportion of microparticles that bind annexin V and determine if experimental conditions may play a role in this. The antigenic characteristics and procoagulant phospholipid activity of annexin V negative and positive subpopulations were also investigated.

In defining microparticles, size is considered an important discriminator to differentiate them from whole, intact platelets. However despite the importance of microparticle size in their discrimination, there is little consensus concerning the maximum diameter of a microparticle. Using flow cytometry, the forward scatter parameter is generally regarded as the best indicator of cell/particle size. The forward scatter parameter should be utilised with caution when estimating particle size, as the relationship between forward scatter and cell/particle size cannot necessarily be assumed to be monotonic. This has been previously demonstrated in experiments where the forward scatter parameter was used to discriminate between fluorescent beads of diameter 3, 4, 5, 6, 7 or 8 μm (23, 24). This work, performed on four different flow cytometers (FACSCalibur, FACSStar, LSR and XL), showed that the forward scatter measurements of 3 and 4 μm beads were incorrectly aligned in respect to bead diameter on three of the four flow cytometers. As the majority of studies rely upon the forward scatter parameter to resolve microparticle size, an important initial calibration stage is to ensure monotonicity of forward scatter measurements, with respect to events size (25, 26).

In our study, we tested monotonicity by analysing the forward and side scatter characteristics of latex beads of known mean diameter (0.32, 0.46, 0.60, 0.82, 1.00 and 1.09 μm) on the LSR-II flow cytometer. A monotonic increase in forward scatter was detected with respect to bead diameter, confirming forward scatter as an accurate representation of particle size.

Given these findings, a flow cytometric protocol for the detection of microparticles was established using a combination of size and annexin V positivity. Microparticle counting was performed on PPP samples from normal donors. The presence of annexin V negative events derived from platelets was confirmed, and these accounted for approximately 80% of the total number of platelet-derived microparticles.

In an effort to ensure optimisation of the annexin V binding protocol, variations to the assay constituents (buffer type, calcium concentration and annexin V concentration) were evaluated; however, we found no benefit in adjusting the assay constituents. As expected, the addition of 2.5 mM calcium to PBS solution lead to the formation of an insoluble calcium phosphate precipitate that adversely affected annexin V binding, which was remedied by the use of a low phosphate concentration PBS, as recommended by Biro et al. (15). In our study, there was no evidence of increased annexin V binding using this buffer.

Recent reports from the 2007 International Society on Thrombosis and Haemostasis Congress suggested that calcium concentrations of 10 mM may be required to demonstrate complete annexin V binding. It should be noted, however, that the maintenance of phospholipid asymmetry on cellular membranes is an active process that is dependent upon the activity of aminophospholipid translocases (17, 27) and that these processes are inhibited by elevated cytosolic calcium concentrations. It is possible that high calcium concentrations may therefore lead to artefactual increases in phosphatidylserine exposure. Studies by Andre et al. have however shown that calcium concentrations greater than 2 mM are required to allow sufficient annexin V binding to inhibit phosphatidylserine activity (28).

It was interesting to note that the percentage of total events that bound annexin V was dependent upon the type of agonist used.
Physiological agonists such as collagen and ADP produced relatively low percentages of annexin V binding when compared to non-physiological agonists such as calcium ionophore and STS which generated microparticles that displayed greater than 85% positivity for annexin V binding.

The expression (Mean Fluorescence Intensity) of CD31 (PECAM), CD41a (GpIIb), CD42a (GpIX) and CD61 (GpIIa) was slightly higher in annexin V positive microparticles when compared to annexin V negative microparticles, however this only reached statistical significance for CD31. Annexin V positive microparticles did, however, possess a significantly decreased expression of CD42b (GpIb), suggesting that the exposure of phosphatidylserine was accompanied by the selective shedding of platelet glycoprotein Ib.

Other studies have also demonstrated a decrease in GpIb expression on platelet-derived microparticles. Using fluorescently labelled annexin V and antibodies to the platelet glycoprotein complex GpIb/IIa to identify platelet-derived microparticles, Zdebfska et al. demonstrated that only 50% of circulating annexin V positive platelet-derived microparticles expressed detectable levels of GpIb (29). Furthermore, in a recent study using a FACSDiva flow cytometer, Perez-Pujol demonstrated that the expression of GpIb was slightly decreased on annexin V positive microparticles, however all annexin V positive platelet-derived microparticles retained a detectable level of GpIb expression (22).

We found that expression (percentage of CD41+ microparticles) of platelet activation markers CD62p and CD63 was significantly decreased in annexin V negative microparticles, compared to annexin V positive microparticles, suggesting that annexin V positive microparticles are associated with a greater degree of platelet activation.

Heijnen et al., have previously described small particles named exosomes present in samples following platelet activation and degranulation (30). These exosomes expressed few platelet membrane glycoproteins such as GpIb, GpIIb, GpIIa or PECAM-1. Since the annexin V negative events that we describe expressed these antigens, we are confident that these events do not originate from the exosome population that Heijnen et al. describe. Furthermore, exosomes are also typically smaller than microparticles, having a diameter ranging from 40 to 100 nm and are therefore too small to be detected by current flow cytometry methods.

The procoagulant activity of annexin V negative microparticles was assessed using the XACT assay. The incubation of microparticle with annexin V inhibited greater than 95% of the sample procoagulant activity. The XACT times were significantly correlated to the percentage of annexin V binding, suggesting that annexin V binding is a true reflection of the procoagulant activity.

In conclusion, this study demonstrates that the majority of platelet-derived microparticles in unstimulated plasma failed to bind annexin V and showed significantly higher levels of CD42b compared to annexin V positive events. Phospholipid-dependent procoagulant activity is limited to the annexin V positive subpopulation and is agonist dependent. The significance of annexin V negative microparticles is unclear and warrants further investigation.

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