PP-70

Novel Approaches in the Detection of Binding of Cancer Cell Microparticles to Activated Platelets

Bradford RL, Chantrathammachart P, Mooberry M, Mazepa M, Wang JG, Glover SL*, Gabriel DA*, Mackman N, Key NS

Hematology/Oncology Division, University of North Carolina School of Medicine, Chapel Hill, NC, USA

*Invitrox Inc, Research Triangle Park, NC, USA

Abstract

Previous reports have demonstrated that microparticles (MPs) derived from monocytes and cancer cell lines such as THP-1 cells can bind to (activated) platelets in a P-selectin/PSGL1 dependent manner. The transfer of tumor-derived RNA via this interaction may facilitate tumor metastasis, but may also represent a novel tumor biomarker. The transfer of cancer cell-derived MP-borne tissue factor (TF) to platelets may also promote thrombosis. In this study, we used two strategies to characterize the binding of tumor cell MPs to platelets, namely flow cytometry and electrophoretic quasi-elastic light scattering (EQELS; Invitrox, Inc). MPs were generated from THP-1 cells by the addition of LPS (1µg/mL x 12 hours at 37°C), and were then isolated from the supernatant by high-speed centrifugation. MPs were similarly harvested from the supernatant of cultured pancreatic cell lines HPAC and HPAF-II. Normal platelets were isolated by gel filtration in the presence of PGE_1. Activated platelets were prepared by the addition of 50µM TRAP-6 and 2mM Ca^{2+}. MPs were added to resting and activated platelet samples and incubated at 37°C for 45 mins before centrifugal isolation and washing of the platelets x2. By flow cytometry, activated platelets were defined by their expression P-Selectin (CD62P). The binding of THP-1 MPs was defined by the appearance of integrin β2 (CD18) expression on platelets. On average, 6.8% ± 3.3% of activated platelets became positive for CD18 versus 1.0% ± .34% of resting platelets when using 10^6 THP cells to generate MPs. When 10^7 THP-1 cells were used to generate MP's, CD18 expression was present in ≈2% of resting and ≈25.0% of activated platelets. Only $CD62P^+$ platelets expressed CD18. MP binding was inhibited by pre-incubation of platelets with rhPSGL-1, and was progressively diminished by serial dilution of the number of added MPs. Binding of THP-1 derived MPs was confirmed by EQELS, where the addition of MPs to activated platelets resulted in a change in platelet electrophoretic mobility (from -1.4 µmcm/Vs to -0.1 µmcm/Vs). In contrast, no change in surface charge/mobility was seen when MPs were added to resting platelets. HPAC and HPAF-II cells express MUC-1 antigen (CD227), which was similarly used to track binding of derived MPs to platelets by flow cytometry. However, only ≈5% of activated and <1% of resting platelets demonstrated CD227 positivity following incubation with these MPs, and the interaction was not inhibited by rhPSGL-1. We are currently evaluating other potential mechanisms of binding of pancreatic cancer cell-derived MPs to activated platelets using this approach.

Conclusions

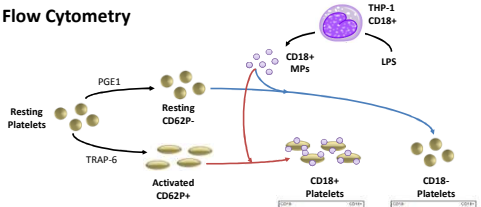
1. The binding of MPs derived from both THP-1 (monocytic leukemia cells) and HPAC and HPAF-II (pancreatic cancer cells) to activated platelets can be shown using flow cytometry and electrophoretic quasi-elastic light scattering (EQELS)[1]
2. By either approach, no binding of MPs to unactivated platelets is apparent
3. Using either approach, rPSGL-1 inhibits approximately 50% of THP-1 MP binding to activated platelets, consistent with previous demonstrations of interaction between P-selectin and PSGL-1 on monocyte MPs [2, 3]
4. These findings may have implications regarding the metastatic spread of tumors via platelet-bound MPs, as well as the previous demonstration of TF transfer to platelets via MPs. [2, 3]

Methods

Platelet Isolation

- Blood drawn with 21G needle and light tourniquet into a syringe containing Na Citrate
- PGE_1 immediately added to a concentration of 1ug/mL
- Blood layered over equal volume of GE Ficoll-Paque Plus, 1500rpm x 30min
- Buffy Coat mixed with equal volume of CGS, 250g x 10min
- Supernatant gel filtered through 50mL Sepharose CL-2B
- Filtered platelets quantified and diluted with Tyrode's buffer

Flow Cytometry



- Activated platelets (a-Plt): 50uM TRAP, 37°C for 30 minutes
- Resting platelets (u-Plt): Maintained by the addition of PGE_1 to 1 ug/mL
- MPs generated from LPS treated THP-1, or Serum Starved HPAC/HPAF-II cells
- MPs added to platelets and incubated for 45min, 37°C
- Unbound MPs cleared by pelleting platelets at 2000g for 3 minutes
- Labeled with respective fluorescent Ab for 30 minutes and measured via BD FACSCalibur

Electrophoretic Quasi-Elastic Light Scattering (EQELS)

- Platelet and MP bound platelet samples were mixed in a 5mM HEPES, 5mM NaCl, and 270mM sucrose solution and measured

Monocyte MP Binding

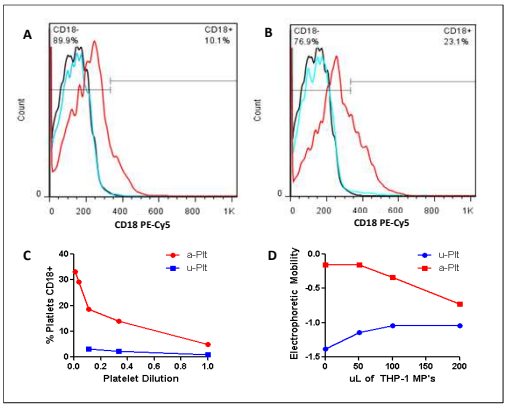


Figure 1: Measurement of THP-1 MP Binding to Platelets

THP-1 MPs prepared by the addition of 1ug/mL of LPS to 10^6 **(A)** or 10^7 **(B)** THP-1 cells, were incubated with resting (blue) and TRAP-activated (red) platelets at a concentration of 20,000 plts/uL and labeled with PE-Cy5 conjugated antibody for integrin beta-2(CD18). Following removal of free MPs by washing, resting and activated platelets showed CD18 positivity at an average of 6.8 ± 3.3% and 1.0 ± .34% (respectively) for 10^6 THP-1 cells, and 24.0 ± 1.3% and 2.0 ± .4% for 10^7 THP-1 cells. Using MPs from $2x10^6$ THP-1 cells, the proportion of CD18 positive platelets increases as the platelet concentration is diluted from 20,000plts/uL to 200plts/uL **(C)**. Measured by EQELS **(D)**, the addition of increasing numbers of THP-1 MP's to activated platelets impacts the electrokinetic fingerprint (EKF) of the platelets. In the case of resting platelets, the change in EKF is due to contamination by activated platelets, which is confirmed by CD62P expression(data not shown).

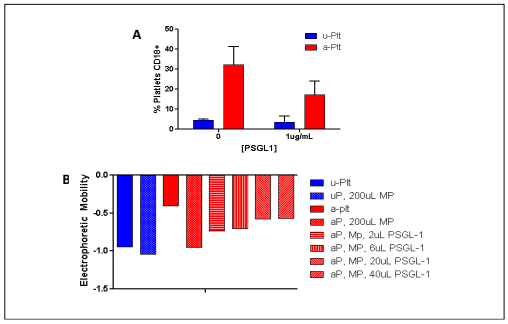


Figure 2: THP-1 MP Binding to Platelets is Blocked by PSGL-1

Using MPs generated from 10^7 THP-1 cells, recombinant PSGL-1 was added to both activated and resting platelet samples exposed to MPs. CD18-PECy5 fluorescence was measured by flow to identify the binding of THP-1 MPs to platelets(**A**). At a concentration of 1ug/mL, rPSGL-1 reduced the portion of platelets positive for CD18 by 53%. By EQELS, resting platelets have a more negative surface charge density, or electrokinetic fingerprint (EKT)(**B**). Platelets become less negative upon activation. When MPs bind to activated platelets, the complex becomes more negative. With increasing concentrations of rPSGL-1 the EKT of activated platelets approaches their MP-free EKT level.

Pancreatic MP Binding

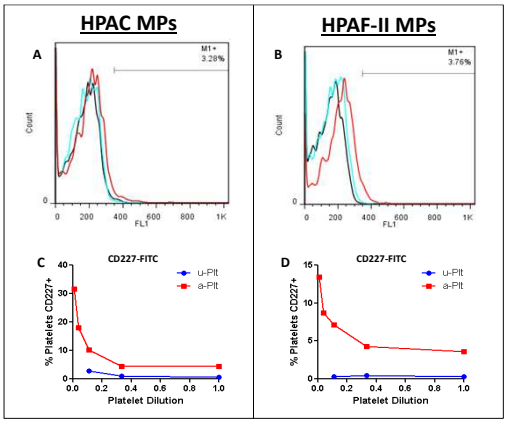


Figure 3: Measurement of Pancreatic Cell Line Derived MP Binding to Platelets

Cell lines(HPAC and HPAF-II) were used to evaluate the binding of pancreatic cell derived MPs to platelets. To generate MPs, 2x 10^6 HPAC and HPAF-II cells were serum starved for 24 hours. In the same manner as THP-1 MPs, pancreatic MPs were incubated with resting or activated platelets and labeled with a FITC-conjugated antibody to MUC-1(CD227) (**A, B**). Dilution of the platelet concentration increases the percent of platelets positive for CD227 (**C,D**).

References

1. Gabriel DA, Giordano K (2010). "Microparticle sizing and counting using light scattering methods." Seminars in Thrombosis and Hemostasis. 36(8): 824-32.
2. Furie B, Furie BC (2004). "Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation." Trends in Molecular Medicine. 10(4): 171-8.
3. Conde I, Shrimpton C, et.al (2005). "Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation." Blood. 106(5):1604-11.

Acknowledgements

Supported by NIH grants HL095096 (to NSK and NM) and T32 HL007149 (to NSK)