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Human Platelet Vesicles Exhibit Distinct Size and Proteome

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Abstract

In the past 50 years, isolated blood platelets have had restricted use in wound healing, cancer therapy, and organ and tissue transplant, to name a few. The major obstacle for its unrestricted use has been, among others, the presence of ultrahigh concentrations of growth factors and the presence of both pro-angiogenic and anti-angiogenic proteins. To overcome this problem requires the isolation and separation of the membrane bound secretory vesicles containing the different factors. In the current study, high-resolution imaging of isolated secretory vesicles from human platelets using atomic force microscopy (AFM) and mass spectrometry enabled characterization of the remaining vesicles size and composition following their immunoseparation. The remaining vesicles obtained following osmotic lysis, when subjected to immunoseparation employing antibody to different vesicle-associated membrane proteins (VAMPs), demonstrate for the first time that VAMP-3-, VAMP-7-, and VAMP-8-specific vesicles each possesses distinct size range and composition. These results provide a window into our understanding of the heterogeneous population of vesicles in human platelets and their stability following both physical manipulation using AFM and osmotic lysis of the platelet. This study further provides a platform for isolation and the detailed characterization of platelet granules, with promise for their future use in therapy. Additionally, results from the study demonstrate that secretory vesicles of different size found in cells reflect their unique and specialized composition and function.

Graphical Abstract

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B.P.J. and D.A.W. developed the idea, designed experiments for the study, and wrote the manuscript with the help of K.T.L. and P.M.S. B.P.J. and D.A.W. isolated the vesicles. S.W., G.M., and K.T.L. performed AFM imaging studies, and P.M.S. performed the mass spectrometry. All authors participated in discussions and proofreading the manuscript.

The authors declare the following competing financial interest(s): B.P.J. and D.A.W. have filed for patent protection on human platelet vesicle isolation technology and its use in therapy.

Keywords

human platelet vesicle proteome; atomic force microscopy; tandem mass spectrometry

1. INTRODUCTION

Platelet-rich plasma therapy has been restrictively practiced primarily due to the presence of ultrahigh concentrations of growth factors and the presence of opposing molecules such as pro-angiogenic and anti-angiogenic factors each present in different secretory vesicles.^{1–6} Human platelets measuring just 2 μm possess an impressive array of secretory vesicles categorized as α -granules, dense granules, T-granules, and lysosomes. In the process of secretion, these granules have been demonstrated to dock and fuse either at the cell plasma membrane or at tube-like fenestrated channels called the open canaliculi system (OCS) that stretch across the cytoplasm to openings at the cell plasma membrane. To be able to understand the molecular mechanism of secretion in human platelets and utilize this knowledge in therapy, a better understanding of the various vesicle pools, of both their size and composition, is essential. Additionally, an understanding of why this heterogeneity in the secretory vesicle population exists in all cells including platelet, and whether this is reflected in the vesicle composition, hence function, needs to be addressed. Previous studies using immunofluorescence microscopy report that in human platelets, vesicles expressing VAMP-3 and VAMP-8 are centrally localized along with the vesicle cargos von Willebrand factor and serotonin, in contrast with VAMP-7 vesicles containing TIMP2 and VEGF that translocate to the periphery of the cell.⁷ In cells, secretion involving membrane fusion is mediated via a specialized set of proteins present in opposing bilayers. Target membrane proteins, SNAP-25 and syntaxin (t-SNAREs), and secretory-vesicle-associated membrane protein, VAMP or v-SNARE, are part of the conserved protein complex involved in fusion of opposing lipid membranes.^{8–10} The specificity of certain secretory vesicles to VAMP-3, VAMP-7, and VAMP-8, therefore suggested possible differences in size, composition, and hence function. In an elastic membrane, the surface free energy is given by the equation: $(1/2)k_a(\Delta A)^2/A_0$, where k_a is the bending modulus, ΔA is the increase in surface area, and A_0 is the initial unstressed area.¹¹ Therefore, an increase in surface area results in an increase in the Gibbs free energy, and the spontaneous fusion between opposing bilayers becomes less probable.^{11–13} Hence, large vesicles are less fusogenic than smaller vesicles. This would explain why neurons, being fast secretory cells, possess small 40–50 nm diameter vesicles for rapid and efficient fusion and release of neurotransmitters,^{14,15} as opposed to a slow secretory cell like pancreatic acinar cells with secretory vesicles

measuring 200–1200 nm in diameter.^{16,17} Therefore, both secretory vesicle size and their chemical composition would critically influence both the rate and composition of content release during cell secretion. Hence, the objective of the current study was to determine size distribution of the various VAMP-specific vesicles that remain following osmotic lysis of human platelets and determine their composition.

2. MATERIALS AND METHODS

Human platelets were isolated according to published procedures and protocols approved by the institutional review board of Wayne State University School of Medicine.¹⁸ Isolated platelets were lysed using the atomic force microscopy (AFM) cantilever tip and to image the vesicles within. Additionally, platelets were osmotically lysed to isolate the entire vesicle pool, from where the VAMP-3, VAMP-7, and VAMP-8 specific vesicles were immunoseparated to be imaged using the AFM, and their composition was determined using mass spectrometry.

2.1. Platelet Isolation

To isolate platelets, human blood was dispersed in acid citrate–dextrose solution (ACD; 0.1 M citric acid, 0.2 M sodium citrate, 0.4 M dextrose, pH 6.8), which functions as an anticoagulant and antistimulant for platelets. Blood samples were imaged using light microscopy. Whole blood with an equal amount of ACD was centrifuged at 100*g* for 15 min, and platelet-rich supernatant was mixed with an equal amount of buffer A (20 mM HEPES-NaOH pH 7.4, 3.3 mM NaH₂PO₄, 2.9 mM KCl, 1 mM MgCl₂, 128 mM NaCl, 5.5 mM D-glucose, pH 7.4). The supernatant was centrifuged at 1000*g* for 10 min to obtain a platelet-enriched fraction. After washing in phosphate-buffered saline (PBS) pH 7.4, the isolated platelets were imaged using electron microscopy to determine their purity. Isolated platelet preparations for electron microscopy were fixed using 2% paraformaldehyde and 1% glutaraldehyde for 1 h at room temperature, followed by washing and resuspension in PBS.

2.2. Platelet Secretory Vesicle Isolation

One mL of isolated platelet preparation was subjected to 3 mL of ice-cold ddH₂O and gently agitated for 2 min, followed by the addition of 0.5 mL of a 10× PBS pH 7.4 and mixed to make the suspension medium isoosmotic. The lysed platelet suspension was centrifuged at 14 000*g* for 6 min, and the resultant suspension was the total remaining vesicle preparation for VAMP-3, VAMP-7, and VAMP-8 immunoisolation, followed by analysis of size and composition.

2.3. Immunoisolation of VAMP-3, VAMP-7, and VAMP-8 Vesicles

The entire vesicle preparation was divided into four parts, with one part exposed to protein A-sepharose beads and the other three fractions subjected to protein A-sepharose-conjugated VAMP-3, VAMP-7, or VAMP-8 (Santa Cruz Biotechnology) and incubated for 2 h on ice with intermittent mixing every 30 min. The incubate was centrifuged at 1000*g* for 1 min, and the pellet containing beads with immunoisolated vesicles was washed three times in 500 μ L of PBS pH 7.4. The beads were eluted using 50 μ L of PBS pH 3 to release the bound vesicles, centrifuged at 1000*g* for 1 min to separate the protein A-sepharose-conjugated

VAMP antibody, and the pH of the resulting suspension was raised to 7.0 in a total volume of 350 μL for both AFM and mass spectrometry.

2.4. Atomic Force Microscopy

AFM was performed according to minor modification of previously published procedures on fixed (2% glutaraldehyde/2% paraformaldehyde) platelet and vesicles on mica.^{17,19–21}

Aldehyde-fixed cells in PBS were placed on mica, air-dried for 1 min, washed using ddH₂O to remove salt crystals, followed by air-drying again for 1 min, and imaged in air using the AFM. Cells were imaged using a Nanoscope IIIa AFM from Digital Instruments. (Santa Barbara, CA). Images were obtained in the “tapping” mode in air, using aluminum-coated silicon tips with a spring constant of 40 N·m⁻¹ and an imaging force of <200 pN. Images were obtained at line frequencies of 1 to 2 Hz, with 256 lines per image and constant image gains. To slice open platelets using the AFM cantilever tip, 30–50 nN force was applied in the contact mode. No vesicles were found to be isolated in the absence of VAMP-specific antibodies. Topographical dimensions of cellular structures were analyzed using the software Nanoscope IIIa4.43r8, supplied by Digital Instruments.

2.5. Tryptic Digestion of Immunoisolated Platelet Vesicles

In studies using in-solution digestion, purified secretory vesicles from human platelets were solubilized in lysis buffer, followed by precipitation in cold methanol, as previously described.²² The resulting protein pellets (25 μg) were solubilized in 0.05% ProteaseMax and 40 mM TRIS, pH 8.0, a potent buffer system that has been effective for tryptic digestion. After reduction with 5 mM dithiothreitol and alkylation with 15 mM iodoacetamide, trypsin (Promega Gold) was added to the diluted sample solution (1:20 w/w) for overnight digestion at 37 °C.

2.6. LC–MS/MS Analysis and Database Search

All analyses were made using an Acclaim PepMap RSLC, 75 $\mu\text{m} \times 25 \text{ cm}$ column with LC–MS/MS performed on an Orbitrap Fusion mass spectrometer. Tandem mass spectra were extracted by Proteome Discoverer version 1.4. Charge-state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, U.K.; version 1.4.1.14), Sequest (Thermo Fisher Scientific; version 1.4.1.14), and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). The Uniprot human complete database (downloaded 2014.06.24, 20 207 entries) was searched assuming the digestion enzyme trypsin. The fragment ion mass tolerance was 0.60 Da, and the parent ion tolerance was 10.0 ppm. Carbamidomethyl of cysteine was specified in Mascot, Sequest, and X! Tandem as a fixed modification. Deamidation of asparagine and glutamine and oxidation of methionine were specified in Mascot and Sequest as variable modifications. Glu $\rightarrow$ pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln $\rightarrow$ pyro-Glu of the n-terminus, deamidation of asparagine and glutamine, and oxidation of methionine were specified in X! Tandem as variable modifications. Scaffold (version 4.7.5, Proteome Software, Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >99.0% probability. Peptide probabilities from Mascot and Sequest were assigned by the Scaffold Local false discovery rate (FDR) algorithm. Peptide Probabilities from X! Tandem were

assigned by the Peptide Prophet algorithm with Scaffold delta-mass correction.²³ Protein identifications were accepted if they could be established at >99.0% probability to achieve an FDR <1.0% and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.²⁴ Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

3. RESULTS AND DISCUSSION

The current study was undertaken to test the hypothesis that human platelets reported to possess VAMP-3-, VAMP-7-, and VAMP-8-specific pools of secretory vesicles containing specialized cargo could exhibit vesicle size specificity for each pool, given the presence of a heterogeneous vesicle population. An additional objective of the study was to determine the proteome of the three vesicle pools and to identify both similarities and differences in the proteome that may exist between them. AFM of the isolated platelets on mica demonstrates them to measure 2 to 2.5 μm (Figure 1A). Teasing open the platelet using the sharp tip of the AFM cantilever to access and image the vesicles within (Figure 1B–E) enabled the size measurement of all remaining unspent or nonlysed vesicles adhering to the cell plasma membrane as well as to the mica surface. Our results demonstrate vesicle size distribution ranging from 20 to 150 nm in diameter, with an average vesicle diameter of ~65 nm (Figure 1F). Large granules typically measuring 400–500 nm were absent, suggesting that those must have undergone secretion following exposure to the fixative or lysis during nanosurgery of the platelet using the AFM cantilever tip.

To determine the size and proteome of VAMP-3-, VAMP-7-, and VAMP-8-specific pools of secretory vesicles, they were immunoisolated (Figure 2) from the remaining vesicle pool obtained after osmotic lysis of the human platelets, as described in the Methods. Large granules measuring 400–500 nm were also absent following osmotic lysis of platelets, again suggesting that those must have undergone secretion following exposure to osmotic shock. The purity of vesicles and their size distribution was assessed using AFM imaging at both low and high magnification (Figure 3A–D). The low-resolution images (Figure 3A,B) demonstrate granule purity, and the absence of any subcellular components or fragments and high-resolution imaging (Figure 3C) enabled measurement of vesicle size (Figure 3D) whose distribution range was estimated to be between 20 and 150 nm, very similar to the vesicle measurements in teased open cells using the AFM cantilever tip (Figure 1F). Interestingly, the average size of the entire isolated vesicle preparation was determined to be 95 nm (Figure 3D) compared with 65 nm (Figure 1F) in cells teased open using the AFM tip. A possible explanation may be the floating away, lysis, or degranulation of some of the larger vesicles following teasing open of the cell by the AFM tip. The entire pool of isolated vesicles from lysed platelets was used as the starting material for immunoisolation of all three pools of VAMP vesicles. As a comparison, isolation of the VAMP-3- and VAMP-8-specific pools of vesicles from the total vesicle pool was imaged using the AFM (Figure 4A–D). The average size of VAMP-3 vesicles was found to be less than half that of the VAMP-8 vesicles, that is, 43 nm as opposed to 97 nm (Figure 4B,D). No vesicles were found to be isolated in the absence of VAMP-specific antibodies.

Determination of the proteome of immunoisolated VAMP-3-, VAMP-7-, and VAMP-8-specific vesicles (Table 1 and Figure 5) demonstrates the presence of certain major proteins with abundant differences between groups ($p < 0.05$). Results from this study demonstrate that the three groups of VAMP-associated vesicles, VAMP-3-, VAMP-7-, and VAMP-8, each possess unique size distribution and composition of the major proteins, reflecting on their specialized cellular function. Proteome heat map (Figure 5), obtained using the log of each signal relative to the mean of the V8 group, shows the VAMP-8 vesicles to have much higher concentration of the majority of proteins compared with the VAMP-7 and VAMP-3 vesicle fractions. In contrast, VAMP-3 has the lowest concentration of those proteins. Interestingly, the gene products for SMTN, ADHX, HV303, PSMD9, SC22B, and AMPD2, are present at greater concentration in the VAMP-7 vesicles compared with VAMP-3. The presence of the vesicle trafficking protein Sec22b (SC22b), for example, in VAMP-7 vesicles, would support previous findings that VAMP-7 vesicles containing TIMP2 and VEGF need to be transported to the periphery of the cell, while VAMP-3 vesicles remain at the center.⁷ Similarly, the presence of AMP deaminase 2 (AMPD2) in VAMP-7 vesicles reflects its important role in energy metabolism. Results from mass spectrometry further demonstrate the presence of IGHG1, VINC, FIBB, PPIA, TPM3, and K22E as the most abundant gene products in all three vesicle fractions (Table 1). It is no surprise, therefore, that the presence of fibrinogen beta chain (FIBB) in all VAMP vesicles reflects an important role of all of these vesicles in facilitating blood coagulation. Additionally, various cleavage products of the FIBB gene regulate cell adhesion and spreading, critical to wound healing. These results therefore reflect on how vesicle size and composition would reflect on the differential transport, timing, and release of secretory vesicles in human platelets.

In summary, this study is the first report on the isolation of vesicles from human platelets and their unique composition and size between the different VAMP groups. Additionally, this study demonstrates that different size groups of secretory vesicles present in cells reflect their unique and specialized cellular function. A further characterization of the lipid composition of platelet vesicles and their competence to fuse is underway, which will further our understanding of the role of the heterogeneous vesicle population in cells.

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Figure 1.

Atomic force microscopy (AFM) of intact and lysed human platelets, demonstrating the size heterogeneity of the intracellular vesicle population. (A) Atomic force micrograph of an intact (top) and partially lysed platelet. (B–E) AFM micrograph of lysed platelet imaged at different resolution, demonstrating the presence of a heterogeneous population of vesicles within the cell. (F) Vesicle size distribution determined using AFM section analysis and a bar graph depicting the average size of associated vesicles following lysis of the platelet. Note that vesicle size varies from 20 to 140 nm in diameter, with an average vesicle size of 65 nm ($n = 200$).

Figure 2. Immunoprecipitation of VAMP-3, VAMP-7, and VAMP-8 vesicles from human platelets following osmotic lysis.

Figure 3. AFM micrograph of purified vesicles at three different resolution demonstrating vesicle purity and size heterogeneity (A–C). (D) Vesicle size distribution determined using AFM section analysis and a bar graph depicting the average vesicle size.

Figure 4. AFM micrograph of immunoisolated VAMP-3 and VAMP-8 vesicles from the entire pool of vesicles obtained from human platelets. (A,B) AFM micrograph of immunoisolated VAMP-3 and (C,D) VAMP-8 vesicles. Note the clear difference in average size and distribution between the VAMP-3 and the VAMP-8 pool of vesicles.

Figure 5.

Proteome of VAMP-3, VAMP-7, and VAMP-8 pools of isolated vesicles from human platelets demonstrates unique chemistry. Proteome heat map in triplicates of VAMP-3 (V3), VAMP-7 (V7), and VAMP-8 (V8) vesicles, obtained using the log of each signal relative to the mean of the V8 group. Note that the VAMP-8 vesicles have much higher concentration of the majority of proteins listed compared with the VAMP-7 and VAMP-3 vesicle fractions. In contrast, VAMP-3 has the lowest concentration of those proteins. Note, however, that the gene products for SMTN, ADHX, HV303, PSMD9SC22B, and AMPD2 are present at greater concentration in the VAMP-7 vesicles compared with either the VAMP-3 or the VAMP-8 vesicles.

MALDI-TOF/TOF Results on the Average Relative Abundance of the Various Proteins Present in the Immunoisolated VAMP-3, VAMP-7, and VAMP-8 Vesicles from Human Platelets

Table 1.

gene symbol	MW (kDa)	protein name	average relative abundance		
			VAMP-3	VAMP-7	VAMP-8
IGHG1	36	Ig gamma-1 chain C region	53.0	63.7	68.7
LIMS1	37	LIM and senescent cell antigen-like-containing domain protein 1	6.3	8.3	8.7
SMTN	99	smoothelin	0.3	1.0	1.3
MYL6	17	myosin light polypeptide 6	14.3	18.0	19.3
FYB	85	FYN-binding protein	2.3	4.3	5.0
PDLI5	64	PDZ and LIM domain protein	0.7	1.0	1.0
VINC	124	vinculin	53.3	67.7	69.0
VASP	40	vasodilator-stimulated phosphoprotein	21.3	26.7	26.7
PSA6	27	proteasome subunit alpha type-6	1.0	1.7	2.3
SEPT2	41	septin-2	1.3	2.0	3.0
SC22B	25	vesicle-trafficking protein SEC22b	0.7	1.7	2.3
TGFI1	50	transforming growth factor beta-1 induced transcript 1 protein	0.7	2.3	2.7
KPCB	77	protein kinase C beta type	0.3	0.3	0.3
ADHX	40	alcohol dehydrogenase class-3	0.0	1.0	1.7
MYLK	211	myosin light chain kinase smooth muscle	0.7	1.0	1.7
PSMD9	25	26S proteasome non-ATPase regulatory subunit 9	0.0	0.3	0.7
CAN1	82	calpain-1 catalytic subunit	7.3	12.3	14.0
FIBB	56	fibrinogen beta chain	29.0	37.0	41.7
DPI3B	74	DCC-interacting protein 13-beta	0.0	0.3	1.3
HV303	13	Ig heavy chain V—III region VH26	0.0	0.7	1.3
STIA1	34	sulfotransferase 1A1	0.7	1.0	1.7
PNPH	32	purine nucleoside phosphorylase	3.0	3.0	2.7
1433F	28	14-3-3 protein eta	2.0	3.0	4.7
PPIA	18	peptidyl-prolyl <i>cis</i> — <i>trans</i> isomerase A	21.3	26.0	26.7
DNM1L	82	dynamitin-1-like protein	2.7	3.0	3.7
GTR3	54	solute carrier family 2 facilitated glucosetransporter member 3	0.7	0.7	0.7

gene symbol	MW (kDa)	protein name	average relative abundance			
			VAMP-3	VAMP-7	VAMP-8	
TPM3	33	tropomyosin alpha-3 chain	38.0	49.0	51.0	
AMPD2	101	AMP deaminase 2	0.3	1.0	1.7	
NEUG	8	neurogranin	3.0	4.3	4.7	
ITA6	127	integrin alpha-6	0.3	0.3	0.7	
PIMT	25	protein-L-isoaspartate(D-aspartate) O-methyltransferase	2.0	3.0	3.3	
K22E	65	keratin, type II cytoskeletal 2 epidermal	22.0	22.3	21.3	