Quantitative immunofluorescence mapping reveals little functional co-clustering of proteins within platelet α-granules

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Platelets are small anucleate blood cells that aggregate to seal leaks at sites of vascular injury and are important in the pathology of atherosclerosis, acute coronary syndromes, rheumatoid arthritis, cancer and the regulation of angiogenesis. In all cases, platelet aggregation requires release of stored proteins from α-granules. However, how proteins with potentially antagonistic functions are packaged within α-granules is controversial. One possibility is the packaging of functional agonists and antagonists into different α-granule populations. By quantitative immunofluorescence co-localization, we found that pairwise comparisons of 15 angiogenic-relevant α-granule proteins displayed little, if any, pattern of functional co-clustering. Rather the data suggested a Gaussian distribution indicative of stochastic protein delivery to individual granules. The apparent physiological paradox raised by these data may be explained through alternate mechanisms such as differential content release through incomplete granule fusion or dampened and balanced regulatory networks brought about by the co-release of antagonistic factors.
INTRODUCTION

Alpha-granules, the major storage and secretory organelle of human platelets\(^1\), contain hundreds of proteins\(^2\). The apparently conflicting functions of the proteins stored in \(\alpha\)-granules raises the important question of how release of opposing activities is physiologically balanced. An attractive proposal resolving this paradox is that functionally antagonistic (e.g., pro- and antiangiogenic) factors are packaged into different \(\alpha\)-granule populations\(^3\)\(^4\)\(^5\)\(^6\) which are preferentially released in response to different stimuli. Electron tomography of thick plastic and cryosections provides evidence for both morphological and molecular heterogeneity in the \(\alpha\)-granule population\(^7\). The major \(\alpha\)-granule class by tomography consists of a large ovoid granule containing an electron dense core and eccentrically localized von Willebrand factor (VWF). VWF can be recognized morphologically as long, tubular strands with a characteristic diameter in electron tomograms\(^7\). Morphologically, long, tubular \(\alpha\)-granules are also present\(^7\). These are reported to be a separate class of \(\alpha\)-granules comprising about 16% of the total. Remarkably, these are found in only about half the platelet population. In no case is the section examined sufficiently thick to encompass the entire organelle\(^7\) and hence the morphological record to date is based upon inferences from organelle fragments. In sum, there is qualitative data but little-to-no quantitative data on which to assess the co-clustering of \(\alpha\)-granule proteins into possible distinct granule populations in whole platelets.

Here, we applied the quantitative, whole platelet approach of protein co-distribution mapping based on 3-dimensional, confocal fluorescence microscopy data sets to test for the presence of distinct \(\alpha\)-granule populations in human platelets\(^6\)\(^8\). Our expectation was that quantitative pairwise comparisons of 15 \(\alpha\)-granule stored proteins would identify sets of \(\alpha\)-granule proteins exhibiting high co-localization indices, i.e., a strong indication of distinct \(\alpha\)-granule populations, and would reveal preferential clustering of physiologically synergistic, \(\alpha\)-granule proteins. Instead, little, if any, patterns of co-clustering were apparent by quantitative fluorescence microscopy. These results raise the possibility that there are either many individual \(\alpha\)-granule populations or a major population of large granules in which proteins are differentially segregated in a zonal manner. We favor the later interpretation as the simplest explanation of the data.
METHODS

Purification of Resting State Platelets
Freshly drawn, citrated human blood was fixed immediately with room temperature paraformaldehyde and platelets then purified by centrifugation. All experiments were approved by the University of Arkansas for Medical Sciences institutional review board.

Antibodies Used, Immunostaining, Spinning Disk Confocal Microscopy, 3-dimensional Structured Illumination Microscopy and Image Processing (See Legend to Supplementary Figure 1)
RESULTS and DISCUSSION

Based on visual, non-quantitative fluorescence microscopy, pro- and antiangiogenic α-granule proteins appear to distribute to separate α-granule populations in human platelets\(^6,8\). However, to test definitively this hypothesis, quantitative data are required.

Quantitative Co-localization Analysis of α-Granule Proteins Shows Little Evidence for Co-Clustering into Functionally Distinct α-Granule Populations

To test quantitatively protein co-clustering, we used antibodies to 15 different human α-granule proteins and quantified 28 different pair-wise comparisons (Figures 1,2). Proteins were stratified for pro- versus anti-angiogenic properties (Figure 1) or megakaryocyte synthesized versus endocytized (Supplementary Figure 1) based on the literature with pro- and anti-angiogenic functionalities color-coded as green and red, respectively. All results were corrected for a small pixel shift and normalized against positive and negative controls.

As shown in Figure 1A-L, visually by confocal microscopy, α-granule content proteins showed considerable variation in their co-distribution patterns whether viewed in XY (columns 1-3) or XZ projections (column 4) with thrombospondin (TSP) and platelet factor 4 (PF4) showing extensive co-distribution (high), fibrinogen and TSP showing less (medium), and fibrinogen and plasminogen showing little co-distribution (low). The number of structures labeled appeared constant from pairing to pairing. When examined qualitatively at the 8-fold higher voxel resolution of 3-dimensional structured illumination microscopy (3D-SIM)\(^9\), a resolution ~2x better than that of an α-granule, platelets stained for fibrinogen and VWF showed, as expected, little overlap (Figure 1M, 2x higher magnification, Figure 1M’). However, strikingly, we did observe the occurrence within apparently continuous structures of adjacent immunofluorescence zones that were either red and yellow or green and red (Figure 1M’, arrows), suggestive of distinct zones of protein packaging within an individual α-granule. When compared quantitatively by confocal microscopy (Figure 1N-P), there was no obvious clustering of the 13 α-granule content proteins based on pro- versus anti-angiogenic function (green versus red labeling of the pairs). Indeed, there was no obvious co-clustering across the entire set of pair-wise comparisons (Figure 1N). Rather the data suggested a Gaussian distribution indicative of stochastic protein delivery to α-granules of proteins present in limited amounts (Figure 1O). Consistent with previous studies, VWF appeared to show little intermixing with other α-granule content proteins in multiple pair-wise comparisons. VWF is know to self-polymerize and would be expected to show limited co-distribution due to its location, by thin section immunoelectron microscopy, in an eccentric zone within α-granules\(^10\). Visually, the range of staining intensities observed in the
gray scale images presented in Figure 1 suggested that there was considerable variation in the concentration per unit volume of any given protein within an α-granule. Finally, to control for the possibility of donor-to-donor variation, we prepared platelets from 5 donors and determined the extent of co-distribution of a fibrinogen and endostatin pairing. In all cases, the quantitative result was ~25% co-distribution with no statistically significant variation being noted (Figure 1P).

We expected at the resolution of confocal microscopy that every α-granule content protein would be associated with membrane proteins known to localize to α-granules. As shown in Figure 2 (A-L), this frequently was not the case. Visual pair-wise comparisons of VEGF with CD40L (aka CD154), fibrinogen with p-selectin and fibrinogen with VAMP8 (a v-SNARE protein involved in membrane fusion) showed that many content positive staining areas were membrane protein marker negative. Because α-granule membrane proteins are also associated with the membranes of other organelles, i.e., dense granules, to obtain a quantitative assessment for co-localization, we chose to score for the frequency of α-granule content proteins associated with the marker membrane protein rather than making a pixel by pixel comparison. Quantitatively, we found that ~50% of the content stained areas were also positive for the membrane protein. By electron tomography, we find that the frequency of dense granules in human platelets relative to α-granules is low, <10% (Storrie, Kamykowski and Gilpin, unpublished). Hence, we consider that membrane protein signal is predominantly from α-granules. Likely there is significant clustering of these proteins within the granule membrane.

In conclusion, we propose that the simplest interpretation of our quantitative data is that α-granule content proteins are packaged into distinct zones, perhaps, in a manner similar to VWF within large, relatively homogeneous α-granules in humans. Electron tomography points to such a population being the major α-granule population in humans. Furthermore, we suggest that examples from other systems may give models to resolve the apparent physiological paradox raised by co-packaging within α-granules of proteins with conflicting function. One possibly instructive example is the chromaffin granule in which the proteins, co-packaged within the same granule, can be differentially released by kiss-and-run exocytosis. A second and alternative rationale comes from C. elegans where the co-presence of agonists and antagonists are important physiological regulators of the differentiation process by dampening and balancing activated signaling responses. By analogy, dampening and balancing of angiogenic responses may be a significant regulatory mechanism of platelets.
AUTHORSHIP
J.K. drew blood, performed experiments, analyzed data, and prepared figures. P.C. together with B.S. collected 3D-SIM micrographs. P.C. aligned the two color channels in these images. S.S. did p-selectin experiments and prepared figures. B.S. designed experiments, analyzed data and wrote and edited the manuscript. Marie Chow and Jerry Ware, University of Arkansas for Medical Sciences, commented on drafts.

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CONFLICTS OF INTEREST
None
REFERENCES


FIGURE LEGENDS

Figure 1. Quantitative co-distribution data indicate a stochastic pattern to the intermixing of \(\alpha\)-granule proteins in resting human platelets. Resting human platelets were isolated and the distribution of 13 different platelet \(\alpha\)-granule content proteins determined by pair wise immunolabeling followed by Z-series spinning disk confocal microscopy. (A-L): qualitative distribution of representative high, medium and low co-distribution pairings (A-D, thrombospondin and PF4; E-H, fibrinogen and thrombospondin; I-L, fibrinogen and plasminogen), (A-L): images are maximum intensity projections of confocal image stacks. Note that the XZ images are stretched in the Z dimension because of the ~3-fold lower resolution of confocal microscopy in the Z than XY dimension. (M, M'): 3D-SIM of human platelets stained for fibrinogen (Fibr, green channel) and VWF (red channel). M', 2x higher magnification, arrows point to examples of zoned fluorescence within an apparently continuous structure (red versus yellow, green versus red). Single plane XZ image slices are shown. (N-P): The quantitative confocal microscopy outcomes are tabulated in the right-hand column and then plotted in N. N, quantitative co-distribution of the respective content protein pairings color-coded for pro- (green) and anti- (red) angiogenic properties. There is little, if any, co-distribution trend based on physiological function. O, quantitative co-distribution of the 23 pairings plotted as a histogram displaying a near Gaussian pattern. P, Quantitative co-distribution data indicate that there is little, if any, variation in the extent of fibrinogen and endostatin co-localization between individuals. All pairings are quantified for a minimum of 30 individual platelets, averaged and presented as the mean plus and minus the standard error of the mean.

Figure 2. Granule membrane proteins display a limited co-distribution with \(\alpha\)-granule content proteins. (A-L): qualitative comparisons of the distributions of VEGF versus CD40L (membrane protein, A-D), fibrinogen versus p-selectin (membrane protein, E-H), and fibrinogen versus VAMP8 (membrane protein, I-L). Images are maximum intensity projections of confocal image stacks. (M): quantification of the limited co-distribution. All pairings are quantified for a minimum of 30 individual platelets, averaged and presented as the mean plus and minus the standard error of the mean.
Figure 1

Protein Pairs Scored
- TSP+PF4 (86% co-distribution)
- IGF+CD40L (70%)
- Fibr+TSP (51%)
- Fibr+SgIII (48%)
- PDGF+PF4 (42%)
- VEGF+Endo (41%)
- TSP+TGF-β (39%)
- Fibr+VEGF (38%)
- TSP+Endo (37%)
- VEGF+CD40L (34%)
- IGF+FGF (32%)
- vWF+SgIII (28%)
- Plasm+SgIII (28%)
- FGF+TSP (27%)
- vWF+CD40L (27%)
- Fibr+Endo (24%)
- VEGF+vWF (23%)
- vWF+Plasm (21%)
- VEGF+PDGF (19%)
- FGF+vWF (19%)
- Fibr+vWF (18%)
- Fibr+Plasm(18%)
- VEGF+FGF (12%)

Co-Distribution
- High
  - TSP
  - PF4
- Medium
  - Fibr
  - TSP
- Low
  - Fibr
  - Plasm

3D SIM, XZ
- Fibr+VWF

N
- Co-Distribution (% Co-Localization)

O
- Incidence (Percentage Class)

P
- Co-Distribution (% Co-Localization)

Comparison of Fibrinogen and Endostatin Across Five Individuals
Figure 2

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Graph M:
- Co-Localized Structures (%)
- VAMP8, CD40L, P-Selectin on the x-axis
- Fibrinogen (Fibr), IGF, vWF on the y-axis
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