Angiogenesis is regulated by a novel mechanism: Pro- and anti-angiogenic proteins are organized into separate platelet α-granules and differentially released

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Abstract:

Platelets, in addition to their function in hemostasis, play an important role in wound healing and tumor growth. Because platelets contain both angiogenesis stimulators and inhibitors, the mechanisms by which platelets regulate angiogenesis remain unclear. As platelets adhere to activated endothelium, their action can enhance or inhibit local angiogenesis. We therefore suspected a higher organization of angiogenesis regulators in platelets. Using double immunofluorescence and immunoelectron microscopy we show that pro- and antiangiogenic proteins are separated in distinct subpopulations of α-granules in both platelets and megakaryocytes. Double immunofluorescence labeling of VEGF (an angiogenesis stimulator) and endostatin (an angiogenesis inhibitor), or for thrombospondin-1 and basic FGF, confirms the segregation of stimulators and inhibitors into separate and distinct α-granules. These observations motivated the hypothesis that distinct populations of α-granules could undergo selective release. The treatment of human platelets with a selective PAR-4 agonist (AYPGKF-NH₂) resulted in release of endostatin-containing granules, but not VEGF-containing granules, while the selective PAR-1 agonist (TFLLR-NH₂) liberated VEGF, but not endostatin-containing granules. We conclude that the separate packaging of angiogenesis regulators into pharmacologically and morphologically distinct populations of α-granules in megakaryocytes and platelets may provide a mechanism by which platelets can locally stimulate or inhibit angiogenesis.
Introduction

Angiogenesis, the process of new vessel development, plays an essential role in embryogenesis, but postnatal angiogenesis is limited to sites of abnormal vascular surface. An activated vascular endothelium can be induced by tissue injury or wound healing, by hormonal cycling such as in pregnancy and ovulation, or by tumor-induced vessel growth. In all of these circumstances, platelets act as the initial responder to vascular change, and provide a flexible delivery system for angiogenesis related molecules\(^1\)-\(^4\). The process of postnatal angiogenesis is regulated by a continuous interplay of stimulators and inhibitors of angiogenesis, and their imbalance contributes to numerous inflammatory, malignant, ischemic, and immune disorders\(^5\). There is a revived interest in the overlap between angiogenesis and platelets\(^6\) because a number of clinical trials have now shown that anticoagulation can improve cancer survival\(^7,8\) beyond the benefit derived from the treatment of deep vein thrombosis alone.

It is known that platelets stimulate endothelial cells in culture and can promote the assembly of capillary-like structures in vitro\(^9,10\). Platelets may modulate angiogenesis by releasing promoters such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), and matrix metalloproteinases (MMPs)\(^1,6,11\)-\(^18\). The repertoire of angiogenesis inhibitors contained within platelets includes endostatin, platelet factor-4, thrombospondin-1, alpha-2-macroglobulin, plasminogen activator inhibitor-1, and angiostatin\(^19,20\). Although platelets contain three types of secretory granules (\(\alpha\)-granules, dense granules, and lysosomes), most angiogenic regulatory proteins have been localized to \(\alpha\)-granules. \(\alpha\)-granules are 200-500 nm in size, and contain proteins that enhance the
adhesive process, promote cell-cell interactions, and stimulate vascular repair. By adhering to the endothelium of injured organs and tissues, and then secreting the contents of their \( \alpha \)-granules, platelets may be capable of depositing high concentrations of angiogenesis regulatory proteins in a localized manner.

A body of experimental data and clinical investigations suggests that platelets are major regulators of angiogenesis\(^{21} \). However, because platelets contain both pro- and antiangiogenic regulatory proteins and because it has been assumed that the contents of \( \alpha \)-granules are homogeneous, it has been unclear how platelets could either stimulate or inhibit angiogenesis. We provide new details about the organization of angiogenesis regulatory proteins in the \( \alpha \)-granules of platelets and address the mechanism of how the selective release of these granules leads to the regulation of angiogenesis. Here we report the novel finding that angiogenic and antiangiogenic proteins are segregated into different sets of \( \alpha \)-granules in platelets. We provide a mechanism for the differential release of these \( \alpha \)-granules, and show that these distinct populations of \( \alpha \)-granules may be regulated by differential G-protein-mediated signaling pathways.

**Materials and methods**

Approval was obtained from the Partners Human Research Committee institutional review board, Boston, MA, for these studies. Informed consent was provided according to the Declaration of Helsinki.

**Preparation of Resting Platelets**

Human blood from healthy volunteers, drawn into 0.1 volume of Aster-Jandl anticoagulant, was centrifuged at 110 g for 10 min. All volunteers had not ingested
aspirin or other nonsteroidal anti-inflammatory drugs for at least 10 days prior to blood collection. The platelet-rich plasma was gel-filtered through a Sepharose 2B column equilibrated with a solution containing 145 mM NaCl, 10 mM Hepes, 10 mM glucose, 0.5 mM Na₂HPO₄, 5 mM KCl, 2 mM MgCl₂, and 0.3% BSA, pH 7.4. The number of platelets was counted by fluorescence activated cell sorting and adjusted to 2 X 10⁸/ml. The isolated platelet suspension was incubated at 37° C for up to 1 hr. The resting state of the platelets was routinely confirmed by PAC1 antibody and anti-tubulin immunofluorescence staining.

**Activation of platelets**

Release of α-granules was examined in vitro in response to 10 µm AYPGK-NH₂, a selective PAR4-activating peptide, or 8 µm TFLLR-NH₂, a PAR1-activating peptide. Peptides were prepared by solid-phase synthesis at the Peptide Synthesis Facility of Synbiocsi (Livermore, CA). Isolated platelets were exposed to PAR activating peptide or vehicle for 10 minutes, fixed with 4% formaldehyde for 20 minutes, attached to polylysine-coated coverslips, and then processed for immunofluorescence microscopy.

**Megakaryocyte Cultures**

Livers were recovered from mouse fetuses and single cell suspensions were generated using methods described previously.²² Between the fourth and sixth day of megakaryocyte culture, cells were placed on a 1.5-3% albumin step gradient and sedimented to obtain enriched populations of megakaryocytes.
**Immunofluorescence Microscopy**

Rabbit anti-VEGF antibody (Ab-1) and mouse anti-VEGF (Ab-7) were obtained from Lab Visions (Fremont, CA). Rabbit anti-endostatin antibody (Ab-1) was obtained from Lab Visions. Mouse anti-thrombospondin antibody (Ab-4, 6.1) was obtained from Lab Visions. Rabbit polyclonal anti-fibroblast growth factor basic was obtained from Abcam. Mouse anti-fibrinogen was obtained from BD Biosciences (Franklin Lakes, NJ) and Rabbit anti-fibrinogen was obtained from Santa Cruz Biotechnologies. Rabbit anti-von Willebrand factor was obtained from Chemicon and Dako. Alexa 568 anti-mouse, Alexa 488 anti-rabbit, Alexa 568 anti-rabbit, and Alexa 488 anti-mouse secondary antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA). Actin filament integrity was assayed by fluorescence microscopy of fixed specimens stained with 1 mM phalloidin-Alexa 488 (Molecular Probes, Eugene, OR) for 30 min and washed 4 times with blocking buffer. Resting platelets were fixed for 20 min in suspension by the addition of 1 vol of 8% formaldehyde. Solutions of fixed platelets in suspension were placed in wells of a 24-well microliter plate, each containing a polylysine-coated coverslip, and the plate was centrifuged at 250 g for 5 min to attach the cells to the coverslip. Megakaryocytes were fixed with 4% formaldehyde in Hank’s balanced salt solution (GIBCO BRL) for 20 min, centrifuged at 500 g for 4 min onto coverslips previously coated with poly-L-lysine, and permeabilized with 0.5% Triton X-100 in Hanks’. Specimens were blocked overnight in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA), incubated in primary antibody for 2-3 hours, washed, and treated with appropriate secondary antibody for 1 hr, and then washed extensively. Primary antibodies were used at 1 µg/ml in PBS containing 1% BSA and
secondary antibodies at 1:500 dilution in the same buffer. Controls were processed identically except for omission of the primary antibody. Controls consisted of either incubating cells with one or both primary antibodies without fluorescently labeled secondary antibodies, or cells incubated with one or both fluorescently labeled secondary antibodies in the absence of primary antibodies. Preparations were mounted in Aqua polymount from Polysciences (Warrington, PA) and analyzed at room temperature on a Nikon TE 2000 Eclipse microscope equipped with a Nikon100X objective (numerical aperture, 1.4), and a 100-W mercury lamp. Images were acquired with a Hamamatsu (Bridgewater, NJ) Orca IIER CCD camera. Electronic shutters and image acquisition were under the control of Molecular Devices Metamorph software (Downington, PA). Images were acquired by fluorescence microscopy with an image capture time of 200-500 milliseconds.

**Immunogold-electron microscopy**

For preparation of cryosections, isolated human platelets were fixed with 4% paraformaldehyde in 0.1M Na Phosphate buffer, pH 7.4. After 2 hours of fixation at room temperature the cell pellets were washed with PBS containing 0.2M glycine to quench free aldehyde groups from the fixative. Prior to freezing in liquid nitrogen cell pellets were infiltrated with 2.3M sucrose in PBS for 15 minutes. Frozen samples were sectioned at -120° C, the sections were transferred to formvar-carbon coated copper grids and floated on PBS until the immunogold labeling was carried out. The gold labeling was carried out at room temperature on a piece of parafilm. All antibodies and protein A gold were diluted with 1% BSA. The diluted antibody solution was centrifuged for 1
minute at 14,000 rpm prior to labeling to avoid possible aggregates. All antibodies were used at a concentration of 1 µg/ml. Grids were floated on drops of 1% BSA for 10 minutes to block for nonspecific labeling, transferred to 5µl drops of primary antibody and incubated for 30 minutes. The grids were then washed in 4 drops of PBS for a total of 15 minutes, transferred to 5µl drops of Protein-A gold for 20 minutes, washed in 4 drops of PBS for 15 minutes and 6 drops of double distilled water. For double labeling, after the first Protein A gold incubation, grids were washed in 4 drops of PBS for a total of 15 minutes then transferred to a drop of 1% glutaraldehyde in PBS for 5 minutes, and washed in 4 drops of PBS/0.15M glycine. The second primary antibody was then applied, followed by PBS washing and treatment with different size Protein-A gold as above. Contrasting/embedding of the labeled grids was carried out on ice in 0.3% uranyl acetete in 2% methyl cellulose for 10 minutes. Grids were picked up with metal loops, leaving a thin coat of methyl cellulose. The grids were examined in a Tecnai G² Spirit BioTWIN transmission electron microscope and images were recorded with an AMT 2k CCD camera.

**Preparation of photomicrographs**

The digital images produced in Metamorph were assembled into composite images by using Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA).

**Results**

The localization of angiogenesis regulatory proteins within the platelet is important for understanding how platelets contribute to new blood vessel formation. The
capacity of platelets to regulate angiogenesis could result from segregation of pro-
angiogenic and antiangiogenic regulators into separate granules. To test this possibility,
we compared the localization of the most well-characterized pro-angiogenic protein
VEGF, and the established antiangiogenic regulator endostatin, in resting platelets by
immunofluorescence microscopy. The majority of \( \alpha \)-granules stained for either VEGF
(labeled green) or endostatin (labeled red), and little evidence of co-localization as would
be indicated by yellow in the merged image was observed (Figure 1a-c). Similarly,
double immunofluorescence microscopy comparing the localization of the endogenous
angiogenesis inhibitor thrombospondin-1 and basic fibroblast growth factor, another
angiogenesis stimulator, also showed segregation of these proteins into separate, distinct
granules (Figure 1d-f). To establish whether the segregation of proteins into distinct \( \alpha \)-
granules was specific to angiogenesis regulatory proteins, we examined the localization
of von Willebrand factor (vWF) and fibrinogen. To evaluate the degree of overlap of
proteins, we investigated the combination of fibrinogen and vWF. Surprisingly, fibrinogen
and vWF also segregated into separate and distinct \( \alpha \)-granules (Figure 1g-i).
Immunofluorescence microscopy further revealed that vWF co-localized with endostatin
(Figure 1j-l) and fibrinogen predominantly with the VEGF-containing \( \alpha \)-granules (Figure
1m-o).

The organization of angiogenesis regulators into distinct \( \alpha \)-granules is not
exclusive to platelets. Megakaryocytes have been shown to generate platelets by
remodeling their cytoplasm into long proplatelet extensions that transport individual \( \alpha \)-
granules on their microtubule tracks.\textsuperscript{24} To address whether inhibitors and stimulators of
angiogenesis are packaged into distinct populations of \( \alpha \)-granules in the precursor cells of
platelets, we analyzed the distribution of angiogenic regulatory proteins in proplatelet-producing mouse megakaryocytes. As observed in platelets, VEGF and endostatin were localized to separate α-granules in the proplatelet extensions (Figure 2a-c). A similar segregated staining pattern was also observed for thrombospondin-1 and basic FGF (Figure 2d-f) as well as fibrinogen and vWF (Figure 2g-i). Most vWF co-localized with the endostatin-containing α-granules (Figure 2j-l). We confirmed the presence of distinct populations of α-granules in human platelets at the ultra-structural level using immunoelectron microscopy (Figure 3). As expected, anti-VEGF (Figure 3a) and anti-endostatin antibodies (Figure 3b) label only a subpopulation of α-granules. Double immunogold microscopy confirmed that the majority of VEGF and endostatin are localized to separate and distinct granules in platelets (Figure 3c). Single immunogold studies revealed that anti-fibrinogen (Figure 3d) and anti-vWF antibodies (Figure 3e) label only a subpopulation of α-granules. Double immunogold microscopy confirmed that the majority of fibrinogen and vWF are localized to separate and distinct granules in resting platelets (Figure 3g). Anti-P-selectin antibodies specifically labeled almost all α-granules and the plasma membrane of resting platelets (Figure 3g). Quantitative analysis of gold labeling in serial sections revealed that antibodies to VEGF, endostatin, vWF and fibrinogen each stain approximately 50% of the granule population (Figure 3h). In contrast, anti-P-selectin antibodies label the membrane of all α-granules as well as the surface of the resting platelet. Less than 10% of granules contained gold labeling for both endostatin and VEGF or vWF and fibrinogen together (Figure 3h).

The packaging of VEGF and endostatin into separate α-granules suggested that distinct granule populations may undergo selective release. We tested this hypothesis by
stimulating platelets with either PAR4-activating peptide or PAR1-activating peptide. Selective granule release was assessed by immunofluorescence microscopy (Figure 4). Phalloidin staining demonstrated that exposure of platelets to either ligand resulted in aggregation and extension of lamellipodia and filopodia, leading to activation (Figure 4b,d,f,h). Immunofluorescence microscopy revealed that PAR4 treatment resulted in loss of the endostatin labeling, suggesting that most of the endostatin-containing granules were released from the platelets ligated with PAR4-activating peptide (Figure 4c). However, numerous VEGF-containing granules were retained in the cytoplasm of PAR4-treated platelets (Figure 4a). In contrast, immunofluorescence microscopy revealed that ligation of PAR1 resulted in the release of VEGF-containing (green) granules, suggesting that release of VEGF-containing granules was elicited by the PAR1 agonist (Figure 4e). However, a large number of endostatin-containing granules were still retained in the cytoplasm of PAR1-treated platelets. (Figure 4g). To confirm the phenomenon of differential granule release, we analyzed the agonist-mediated release of α-granules at higher resolution using immunoelectron microscopy. Stimulation of platelets with PAR4 agonist resulted in the release of almost all endostatin-containing granules; the majority (84%) of granules remaining in the activated platelets were positive for VEGF (Figure 4i). Treatment of platelets with PAR1 agonist induced the release of the majority of VEGF-containing granules; the majority (88%) of granules remaining in the PAR1-activated platelets were positive for endostatin (Figure 4j).

DISCUSSION
Angiogenesis is a critical element of many physiological processes such as wound healing, as well as pathological processes such as tumor growth. In both situations, new blood vessel development is driven locally, by the release of pro-angiogenic factors such as VEGF, bFGF, and PDGF. However, angiogenesis can also be inhibited by local release of antiangiogenic factors such as endostatin and thrombospondin. The proximity to and interaction with the endothelium allow platelets to strongly influence tumor development and wound healing. Platelets have been presumed to contribute to these angiogenesis-dependent processes by providing many pro- and antiangiogenic proteins, but their regulatory role is incompletely understood. In this study, we have shown that platelets contain distinct populations of \( \alpha \)-granules that can undergo differential release in vitro. This study suggests that at least two populations of \( \alpha \)-granules containing endogenous angiogenic regulatory proteins are present in platelets and raises the possibility that platelets contain multiple types of \( \alpha \)-granules. Platelets contain a large number of angiogenic regulatory proteins, whose localization will need to be thoroughly established to understand the complexity of \( \alpha \)-granule organization within resting platelets. Yet, it can be inferred that this subcellular organization has a physiological purpose in facilitating the differential release of these proteins in response to tissue stimuli. Our findings of differential granule release also support and provide a mechanistic explanation for earlier studies examining the secretion reaction of platelets. Two independent groups have documented the differential release of \( \alpha \)-granule proteins from platelets\(^{25,26}\). In addition, morphometric evaluation of the platelet release reaction during thrombogenesis has demonstrated that platelets do not release all of their granules when they are incorporated into a thrombus\(^{27}\). The above results also raise the question...
of whether other cell types containing secretory granules segregate angiogenic regulatory proteins to regulate differential release. For example, Weibel Palade bodies, the specific secretory organelles of endothelial cells, contain a number of angiogenesis regulators and have been recently shown to differentially package and release P-selectin and vWF through protease activated receptors\textsuperscript{28}.

What molecular mechanisms regulate the differential packaging of specific proteins into α-granules? α-granules contain a mixture of proteins synthesized by the megakaryocyte as well as proteins endocytosed from the circulation by both megakaryocytes and the platelets. While the formation of α-granules is poorly understood\textsuperscript{29,30}, it appears that α-granules develop from budding vesicles in the Golgi complex within megakaryocytes, where they transform into multivesicular bodies, which also fuse with endocytic vesicles. Coated pits and vesicles have been observed in platelets and function to take up proteins, such as fibrinogen, by receptor mediated endocytosis. These endocytic vesicles fuse with the multivesicular bodies. Multivesicular bodies, which are prevalent in early megakaryocytes, are believed to be a common precursor of both α- and dense granules. However, the mechanisms by which α- and dense granules develop into distinct entities are unknown. It is tempting to speculate that a similar process may be employed to segregate proteins into distinct subsets of α-granules, and that genetic defects which affect the α-granule segregation or differential release may provide an explanation of the wide range of angiogenic responses manifested by different individuals\textsuperscript{31}. Several angiogenesis-dependent processes may be explained by the sequential release of angiogenesis regulators. For example, in early endothelial injury, an unstable platelet clot is formed and the high-affinity thrombin receptor (PAR-1) signals to
release by majority pro-angiogenic proteins such as VEGF. In the late stage of tissue reconstruction, a high thrombin state occurs when the majority of the clot is crosslinked by factor XIII, and the low affinity protease-activated receptor (PAR-4) is engaged and mainly inhibitors of angiogenesis are released.

Recognizing that activated platelets release growth factors, investigators have begun to enhance tissue regeneration by applying platelets and their derivatives into sites of injuries or surgical intervention. The concept of platelet and tissue interaction and the resulting release of angiogenesis regulators has already been used in the empirical application of platelet preparations to chronic diabetic ulcers, chronic cutaneous ulcers, dehiscent wounds and tissue regeneration. Although the majority of evidence indicates that platelets and their derivatives (gels, releasates, and lysates) are promising therapeutic agents for regenerative medicine, little is known about the specific mechanisms underlying platelet-accelerated tissue repair. The ability to generate selective platelet releasates by manipulating protease activated receptors may provide new opportunities for research and applications of tissue engineering and may aid in therapeutic strategies to promote or inhibit angiogenesis.

Our findings of distinct populations of α-granules that can be differentially released suggest implications and potential for a substantial role in anti-angiogenic therapy. It is now well accepted that the growth of a tumor beyond ~1 mm is dependent on the development of a neovasculature. One possibility is that tumors also hijack the angiogenic properties of platelets to promote new blood vessel growth by manipulating the protease activated receptors on platelets and triggering the selective release of predominantly pro-angiogenic factors. The protease-activated receptors on platelets and
endothelial cells are likely to play an important role in the sequential and highly selective contribution of angiogenesis regulators to tissues. If confirmed, then it may be possible to develop drugs that instruct platelets which interact with tumors to release predominantly anti-angiogenic proteins.

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**Authorship**

Contribution: JEI designed and performed experiments and data analysis, interpreted results, provided guidance for the group, and drafted the manuscript. JLR, SPH, EB, AZ, and SS performed experiments and data analysis, interpreted results. SR and JF designed experiments, interpreted results, formulated discussions and assisted in manuscript preparation and editing. GLK, corresponding author, designed experiments, provided guidance for the group, interpreted results, formulated discussions and assisted in manuscript preparation and editing.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


Figure Legends

Figure 1. Pro- and anti-angiogenic regulators organize into separate, distinct α-granules in resting platelets. a, b, c, Double immunofluorescence microscopy of resting platelets using antibodies against VEGF (a) and endostatin (b) and an overlay (c). d, e, f, Double immunofluorescence microscopy of resting platelets using antibodies against bFGF (d) and TSP-1 (e) and an overlay (f). g, h, i, Double immunofluorescence microscopy of resting platelets using antibodies against fibrinogen (g) and von Willebrand factor (h) and an overlay (i). j, k, l, Double immunofluorescence microscopy of resting platelets using antibodies against von Willebrand factor (j) and endostatin (k) and an overlay (l). m, n, o, Double immunofluorescence microscopy of resting platelets using antibodies against VEGF (m) and fibrinogen (n) and an overlay (o).

Figure 2. Pro- and anti-angiogenic regulatory proteins are segregated into separate, distinct α-granules in megakaryocyte proplatelets. a, b, c, Double immunofluorescence microscopy of proplatelets using antibodies against VEGF (a) and endostatin (b) and an overlay (c). d, e, f, Double immunofluorescence microscopy of proplatelets using antibodies against bFGF (d) and TSP-1 (e) and an overlay (f). g, h, i, Double immunofluorescence microscopy of proplatelets using antibodies against fibrinogen (g) and von Willebrand factor (h) and an overlay (i). j, k, l, Double
immunofluorescence microscopy of proplatelets against VEGF (j) and fibrinogen (k) and an overlay (l).

**Figure 3.** Localization of proteins in resting, human platelets using immunoelectron microscopy of ultrathin cryosections. Single immunogold labeling on ultrathin platelet sections was performed with anti-VEGF (a) and anti-endostatin (b) antibodies. Double immunogold labeling on platelet sections was performed with the use of anti-VEGF antibody and anti-endostatin antibodies. Large gold particles representing anti-VEGF staining (15 nm, arrows) are evident on one population of α-granules and small gold particles (5nm) representing endostatin staining are abundantly present on a different population of α-granules (arrowheads) (c). Single immunogold labeling on ultrathin platelet sections was performed with anti-fibrinogen (d) and anti-vWF (e) antibodies. Double immunogold labeling on platelet sections was performed with the use of anti-fibrinogen antibody, which was revealed with a 15–nm, gold-conjugate (arrows) and then with an antibody to vWF, which was revealed with a 5-nm, gold-conjugate (arrowheads) (f). Single immunogold labeling on ultrathin platelet sections was performed with anti-P-selectin antibody (g). Gold particles representing P-selectin staining are abundantly present on the α-granules as well as the cell-surface membrane.

Bar, 300 nm. The bar graph shows the quantitation of the percent of α-granules positive (via immunogold staining) for specific factors. The data represents three separate experiments. Over 100 granules were scored for each study.
Figure 4. Activation of specific protease activated receptors stimulates the selective release of α-granules containing either endostatin or VEGF. Platelets were treated with platelet buffer in the presence of agonists for 10 minutes with PAR4-activating peptide (a,b,c,d), and PAR1-activating peptide (e,f,g,h) and then fixed and processed for immunofluorescence microscopy. Cells were stained with either anti-VEGF antibodies (Alexa 488 green labeling; a,e) or anti-endostatin antibodies (Alexa 568 red labeling; c,g,) to assay for granule retention or release. All micrographs were taken at the same exposure time. Corresponding staining with Alexa-phalloidin (b,d,f,h) in the lower panels highlights the morphology of the platelets. Negative controls consisting of incubation with both secondary fluorescently labeled antibodies only or incubation with only primary antibodies failed to show appreciable fluorescence (data not shown). Images are representative of at least 10 high power fields for each experiment, and each experiment was performed 3 times. (i,j) Representative images of immunoelectron microscopy of platelets treated with either PAR4-AP (i) or PAR1-AP (j). Double immunogold labeling on platelet sections was performed with the use of anti-VEGF antibody and anti-endostatin antibodies. In the PAR4-treated samples (i), large gold particles representing anti-endostatin staining (15 nm) are evident on one α-granule (arrow) and small gold particles (5nm) representing VEGF staining are abundantly present on separate population of multiple α-granules. In the PAR1-treated samples (j), large gold particles representing anti-VEGF staining (15 nm, arrow) are evident on one α-granule (arrow) and small gold particles (5nm) representing endostatin staining are abundantly present on separate population of multiple α-granules. (k) A model illustrating the mechanism of differential granule release from platelets. A simplified
summary of the pathway is shown. Resting platelets contain both pro-angiogenic (green) and anti-angiogenic (red) granules. Selective activation of the PAR1 receptor causes release of granules containing pro-angiogenic factors, whereas selective activation of the PAR4 receptor causes release of granules containing anti-angiogenic factors.
Figure 1
Figure 2
Figure 4
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