Platelets actively sequester angiogenesis regulators

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Clinical trials with antiangiogenic agents have not been able to validate plasma or serum levels of angiogenesis regulators as reliable markers of cancer presence or therapeutic response. We recently reported that platelets contain numerous proteins that regulate angiogenesis. We now show that accumulation of angiogenesis regulators in platelets of animals bearing malignant tumors exceeds significantly their concentration in plasma or serum, as well as their levels in platelets from non–tumor-bearing animals. This process is selective, as platelets do not take up a proportional amount of other plasma proteins (eg, albumin), even though these may be present at higher concentrations. We also find that VEGF-enriched Matrigel pellets implanted subcutaneously into mice or the minute quantities of VEGF secreted by microscopic subcutaneous tumors (0.5-1 mm3) result in an elevation of VEGF levels in platelets, without any changes in its plasma levels. The profile of other angiogenesis regulatory proteins (eg, platelet-derived growth factor, basic fibroblast growth factor) sequestered by platelets also reflects the presence of tumors in vivo before they can be macroscopically evident. The ability of platelets to selectively take up angiogenesis regulators in cancer-bearing hosts may have implications for the diagnosis and management of many angiogenesis-related diseases and provide a guide for antiangiogenic therapies. (Blood. 2009;113:2835-2842)

Introduction

Platelets play a major role in hemostasis, as well as in tissue repair, maintenance of endothelium, and vascular tone. They may also facilitate delivery of angiogenesis regulators and other growth factors to sites of pathologic angiogenesis.1,2 Correlative studies suggest that increasing platelet counts may be linked to tumor progression.3,4 We and others have reported previously that platelets contain several proteins that regulate angiogenesis.5-8 We have now discovered that the platelet concentrations of angiogenesis regulatory proteins, although relatively constant and stable under physiologic conditions, are modified by and reflect the presence of a tumor. In the presence of microscopic (<1.0 mm) tumors in a mouse, circulating platelets sequestered increased concentrations of angiogenesis regulatory proteins, without a corresponding elevation in their plasma levels. The uptake of angiogenesis regulatory proteins is selective, as platelets do not take up other plasma proteins. For example, although albumin is present in plasma at much higher concentrations than, for example, vascular endothelial growth factor (VEGF), albumin levels in platelets do not differ in the presence or absence of tumors.

In this study, we used a high-throughput surface-enhanced laser desorption/ionization–time-of-flight mass spectrometry (SELDI-ToF MS), which permitted a rapid analysis of a large number of samples in a highly efficient and reproducible manner.9,10 In this open-ended proteomic comparison of platelets from tumor-bearing and non–tumor-bearing animals, the majority of identified differentially expressed proteins were angiogenesis regulators. Our subsequent studies revealed that platelets can sequester selected proteins either in vitro or in vivo. The platelet angiogenesis proteome reflected the presence of dormant, microscopic-sized tumors in mice months before these tumors can be detectable by conventional methods, and before the angiogenesis regulatory proteins could be detected in plasma, suggesting that analysis of the “platelet angiogenesis proteome” may be used to detect tumor establishment or recurrence before a patient is symptomatic, providing an opportunity for early therapeutic intervention with nontoxic therapies.

Methods

In vitro uptake of angiogenesis regulators by freshly isolated platelets

Platelet-rich plasma (PRP) was isolated from the blood of healthy human volunteers by centrifugation of citrated whole blood at 180g for 20 minutes. The PRP was then transferred to a fresh polyethylene tube and incubated on a gentle rocker at room temperature for 1 hour with increasing concentrations of human recombinant endostatin (EntreMed, Rockville, MD) or basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN). Platelets were gently resuspended in Tyrode buffer containing 1 U/mL prostaglandin E2 and 1% Triton X-100 to remove the membrane fraction of platelets. After a second centrifugation at 800g, the pellets (platelets) were lysed for standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis using 50 mM Tris HCl, 100 to 120 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Igepal, and Protease Inhibitor Tablet (Roche). The pellet proteins were then analyzed by high-mass-resolution LC/MS/MS using a Q-TOF Micromass to identify angiogenesis-related proteins. After an initial preparation of platelet proteins, we used a microarray-based approach to predict potential angiogenesis-related molecules.

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Antiserum (a kind gift from Nicholas Cowan, New York University Medical Center, Boston, MA) and an equivalent amount of protein standards or platelet protein lysate were mixed with sample buffer (Invitrogen, Carlsbad, CA) and loaded onto a 12% SDS-polyacrylamide gel (Invitrogen). After transfer to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), the membrane was blocked with 7% milk and incubated with the following antibodies: antihuman endostatin (courtesy of Kashi Javaherian, Children’s Hospital, Boston, MA) or antihuman bFGF (1:1000; Upstate Biotechnology, Charlestown, VA). Positive signals were then detected using a Super Signal West Pico Chemiluminescence Kit (Pierce Chemical, Rockford, IL) and autoradiography.

To localize the proteins taken up by platelets, we used immunofluorescence microscopy. To this end, platelets in the form of PRP were incubated with 200 ng/mL to 2 μg/mL His-tag endostatin (generous gift from Kashi Javaherian) for 1 hour at 37°C. Platelets were then fixed in solution with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature, plated onto coverslips in 24-well plate, permeabilized with 0.1% Triton X-100, and blocked for nonspecific binding sites by incubating for 1 hour in PBS containing 1% normal goat serum. The fixed platelets were then incubated overnight at 4°C without anti-His-Tag monoclonal primary antibody (Abcam, Cambridge, MA) diluted 1:500 in PBS, washed several times with PBS, and then incubated with Alexa 488 antimouse secondary antibody (Invitrogen) and diluted 1:500 for 1 hour at room temperature. Three controls (processed identically to treated specimens) were prepared for this experiment. The first one consisted of platelets not incubated with His-Tag endostatin. The second control consisted of substituting the primary antibody His-Tag by mouse IgG2a isotype control at the same dilution of His-Tag antibody. The third control consisted of processing the platelets omitting the primary antibody. Platelets were imaged using a Leica TCS SP2 AOBS confocal system (Leica, Wetzlar, Germany) fitted to a DM IRE2 inverted microscope with a 100× objective and 488-nm argon diode laser.

**Analysis of sera obtained from VEGF- or bFGF-“loaded” platelets**

A total of 1 mL PRP was isolated from fresh blood of human volunteers and incubated on a gentle rocker at room temperature for 1 hour with 600 ng/mL recombinant human VEGF or bFGF. After incubation, 1 unit of either thrombin or adenosine diphosphate (ADP) was added to 1 mL of each sample for 5 minutes, and clot and serum were separated by centrifugation. The serum was then analyzed for VEGF and bFGF by standard enzyme-linked immunosorbent assays (ELISAs; R&D Systems). In an alternative experiment geared to evaluate the completion of protein release, 1 mL PRP was incubated with 100 ng bFGF for 45 minutes on gentle rocker at room temperature and spun at 150g. The plasma containing excess of the protein was then removed, and platelets resuspended in 1 mL saline to which 1 unit of thrombin or 20 mM ADP was added. The sample was then spun again at 900g to pellet the platelets and the supernatant and platelet pellet analyzed using ELISA.

**Localization of VEGF in activated platelets**

Antihuman VEGF mouse monoclonal antibody was obtained from BD Biosciences (San Jose, CA) and used at 5 μg/mL. Rabbit anti-β1 tubulin antisera (a kind gift from Nicholas Cowan, New York University Medical Center, New York, NY) was used at 1:1000 dilution. Alexa 488 antirabbit and Alexa 568 antimouse secondary antibodies with minimal cross-reactivity were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Cells were analyzed on a Zeiss Axiosvert 200 microscope (Carl Zeiss, Thornwood, NY) equipped with a 100× objective (NA 1.4), and a 100-W mercury lamp. Images were acquired with an Orca II cooled software.

Resting platelets were fixed for 20 minutes in suspension by the addition of 3.7% formaldehyde. The platelets were attached to polylysine-coated coverslips placed in wells of a 12-well microtiter plate and centrifuged at 250g for 5 minutes. For agonist-induced activation, platelets were sedimented onto coverslips in an identical fashion, and 1 U/mL thrombin was added for 5 minutes. Activated platelets were fixed for 20 minutes in 3.7% formaldehyde. Samples were permeabilized in Hank balanced solution containing 0.5% Triton X-100 and washed with PBS. Specimens were blocked overnight in PBS plus 1% bovine serum albumin, incubated in primary antibody for 2 to 3 hours at room temperature, washed, treated with appropriate secondary antibody for 1 hour, and again washed extensively in 1% PBS. Primary antibodies were used at 1 μg/mL in PBS plus 1% bovine serum albumin and secondary antibodies at a 1:500 dilution in the same buffer. Controls were processed identically except for omission of the primary antibody.

**Platelet uptake of 125I-labeled VEGF in vivo**

VEGF was iodinated using a previously established method. Briefly, Iodo Beads (Pierce Chemical) pre-equilibrated with 10 μL sodium phosphate buffer (0.2 M NaHPO₄, pH 7.2) were incubated with 10 μg of carrier-free recombinant mouse VEGF (R&D Systems) and 1 mCi 125I. The sample was then further diluted with 150 μL sodium phosphate buffer and passed through a 15-mL, pre-equilibrated NAD 5 column (GE Healthcare, Little Chalfont, United Kingdom) containing 0.2% gelatin in PBS. Fifteen fractions of 250 μL were then collected. Radioactivity in each fraction was quantified on a Gamma 5000 Beckman Iodine 125 (Beckman Coulter, Fullerton, CA), and the 2 fractions containing the greatest quantity of 125I-labeled VEGF (500 μL in total) were combined for use in the Matrigel assay on the day of the experiment. Briefly, the left flanks of C57/B16 mice were shaved 1 day before Matrigel pellet implantation to avoid any interference by minor cutaneous inflammatory reaction. On the day of the experiment, 500 μL 125I-VEGF in buffer was mixed with 500 μL growth factor-free Matrigel (BD Biosciences), and 100 μL of this mixture was injected subcutaneously into the left flank of each mouse. Three days later, the mice were anesthetized using inhalational anesthesia (2% isofluorane in 1 L oxygen) and 1 mL whole blood was drawn into a citrated syringe (1% sodium citrate final concentration, 1/10 vol/vol) by direct cardiac puncture without opening the chest cavity.

The platelets were isolated in 2 centrifugation steps: the first at 180g to isolate PRP, followed by centrifugation at 400g to yield a platelet pellet and a platelet-poor plasma fraction. The radioactivity of each platelet sample was quantified on a gamma counter. The value was corrected for differences in tissue weight and expressed as counts per minute per gram of tissue.

**Changes in platelet angiogenesis proteome in presence of tumor xenografts**

We used previously established dormant and angiogenic xenografts of human liposarcoma (SW872). These clones are from microscopic, dormant, nonangiogenic tumors, or from rapidly growing, angiogenic tumors in immunodeficient mice. The angiogenic clone is already angiogenic at the time of implantation and expands rapidly. The nonangiogenic tumor subclone undergoes a switch to an angiogenic phenotype at approximately 133 days (median ± 2 weeks) and expands rapidly thereafter. The growth curves parallel the growth curve of angiogenic clones after the switch. Even though the tumor cell proliferation rates in vivo and in vitro are equivalent for both clones, the tumor cell apoptotic rates are higher in the nonangiogenic clone. Interestingly, the nonangiogenic clones secrete large amounts of VEGF and bFGF in tissue culture.

All cell lines were cultured in Dulbecco modified Eagle medium (DMEM) containing 5% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 1% antibiotics (penicillin, streptomycin), and 0.29 mg/mL L-glutamine in a humidified 5% CO₂ incubator at 37°C. For injections into mice, 80% to 90% confluent tumor cells were rinsed in PBS (Sigma-Aldrich, St Louis, MO), briefly trypsinized, and suspended in serum-free DMEM. The cells were washed twice in DMEM, and their final concentration was adjusted to 5 × 10⁶ viable cells/200 μL.

Six-week-old male SCID mice from the Massachusetts General Hospital (Boston, MA) were injected subcutaneously in the flanks with 5 × 10⁶ cells (in 0.2 mL) from a single clone. All experiments were conducted in...
platelets rather than remaining membrane-associated. The pattern of the fluorescent signal indicates that endostatin is taken up into the granules of the 2 images. The fluorescent label of the His tag. (Right) The overlay of the 2 images. The pattern of the fluorescent signal indicates that endostatin is taken up into the granules of platelets rather than remaining membrane-associated.

The fluorescent label of the His tag. (Left) DIC image of the platelets. (Middle) The fluorescent label of the His tag. (Right) The overlay of the 2 images. The pattern of the fluorescent signal indicates that endostatin is taken up into the granules of platelets rather than remaining membrane-associated.

Figure 1. Angiogenesis regulators are taken up by platelets in vitro. (A) PRP was incubated with increasing concentrations of recombinant human bFGF (rhbFGF) or recombinant human endostatin (rh endostatin) for 1 hour. The platelets were then isolated by sequential centrifugation, washed, treated with 1% Triton X to remove the membrane, and lysed for SDS-PAGE analysis. Standard Western blots using antihuman endostatin and antihuman bFGF antibodies reveal a dose-dependent increase in the respective proteins in the cytoplasmic fraction of fresh platelets. (B) To establish the localization of proteins taken up by platelets, the platelets were incubated with His-tag labeled endostatin, fixed using paraformaldehyde, and anti-His antibody was used to separate the platelet endogenous endostatin (not labeled) from the endostatin "loaded" into platelets (fluorescent label). (Left) DIC image of the platelets. (Middle) The fluorescent label of the His tag. (Right) The overlay of the 2 images. The pattern of the fluorescent signal indicates that endostatin is taken up into the granules of platelets rather than remaining membrane-associated.

Background

Platelets are a key component of the blood clotting cascade and play a crucial role in wound healing. They are also involved in various physiological processes, including angiogenesis. Angiogenesis is the formation of new blood vessels and is essential for tissue growth and repair. Platelets can regulate angiogenesis by releasing angiogenesis regulators such as bFGF and endostatin. Understanding the mechanism by which platelets take up these regulators is important for understanding platelet function and its role in angiogenesis.

Methods

In this study, platelets were isolated from fresh blood and incubated with increasing concentrations of recombinant human bFGF or endostatin. The localization of these proteins was then studied using double-label immunofluorescence microscopy. The platelets were stained for tubulin, VEGF, and platelet-specific markers. The fluorescence images were then analyzed to determine the localization of the proteins.

Results

In vitro uptake of angiogenesis regulators by freshly isolated platelets

Platelets incubated with bFGF or endostatin showed increased localization of these proteins in the cytoplasmic compartments. This suggests that these proteins are taken up by platelets rather than remaining membrane-associated.

Localization of VEGF in activated platelets

To establish the subcellular localization of VEGF in activated platelets, double-label immunofluorescence microscopy was used. The results showed that VEGF is localized in the cytoplasmic compartments of activated platelets.

Discussion

The results of this study suggest that platelets are capable of taking up angiogenesis regulators such as bFGF and endostatin. This mechanism may play a role in regulating angiogenesis. Further studies are needed to understand the physiological significance of this process.

Conclusion

Platelets are a key player in angiogenesis, and understanding their role in the uptake of angiogenesis regulators is important for understanding platelet function and its role in angiogenesis.
the marginal microtubule band in a resting platelet, and this structure defined the platelet periphery. In resting platelets, anti-VEGF antibodies consistently labeled punctate, vesicle-like structures distributed throughout the platelet cytoplasm (Figure 3B,C), and the sequential stacking of 4-μm slices of confocal microscope images supported a granular nature of the immunoreactive material.

Thrombin-induced activation of platelets was documented by the expected change in platelet shape and by the formation of lamellipodia and filopodia. In activated platelets, VEGF remained observable as punctate patterns, consistent with the notion that it remains associated with platelets, even after agonist-induced activation. More importantly, VEGF appeared to be preferentially redistributed along the filopodia and along the periphery of lamellipodia (Figure 3E,F).

**Platelet uptake of 
$^{125}$I-labeled VEGF in vivo**

To confirm that the observed ability of platelets to take up proteins is not an in vitro artifact, we implanted Matrigel pellets containing $^{125}$I-labeled VEGF (50 ng labeled VEGF per 100 μL Matrigel) subcutaneously into mice. The analysis of the organ distribution of $^{125}$I-VEGF revealed that it accumulated in platelets at higher concentrations than in plasma or various organs (Figure 4).

**Changes in the platelet angiogenesis proteome in presence of tumor xenografts**

We used an Expression Difference Mapping system (Ciphergen) to characterize, in an open-ended fashion, the complete proteomic profile of platelets from tumor-bearing mice vs non–tumor-bearing controls (at 32 days after tumor implantation).

We used dormant subclones of human liposarcoma (SW872), which are known to secrete large amounts of VEGF and bFGF in tissue culture, but remain dormant for up to 133 days. We compared the platelet and plasma proteomes of 5 mice injected with either 200 μL serum free media (vehicle) or a cell suspension of $5 \times 10^8$ cells of the nonangiogenic or angiogenic clones of the liposarcoma cell line. The experiment was repeated twice for comparison of expression maps from separate analyses. Among the proteins identified in this analysis as differentially expressed in platelets of tumor-bearing mice vs tumor-free, sham-operated mice, angiogenesis regulators were prominently represented. Figure 5 depicts a representative analysis of a platelet angiogenesis proteome in gel view format, and Figure S1 (available on the Blood website; see the Supplemental Materials link at the top of the online article) provides a semiquantitative analysis of the respective protein peaks. The levels of pro-angiogenic growth factors, such as VEGF, bFGF, and platelet-derived growth factor (PDGF), tended to be higher in the platelets of tumor-bearing mice compared with tumor-free sham-operated mice (Figure 5). The levels of VEGF, bFGF, and PDGF in platelets from normal animals were lower and comparable with those in plasma. At 32 days after tumor implantation, the nonangiogenic dormant liposarcoma ($\sim 1 \text{ mm}^3$) tumors are approximately 100 times smaller than the angiogenic liposarcoma ($\sim 1 \text{ cm}^3$), yet platelets of the dormant tumor-bearing mice contained detectable levels of angiogenesis regulatory proteins. In contrast, there were negligible levels of these proteins in the plasma of either mouse. In mice with nonangiogenic dormant tumors, the pattern of elevated positive angiogenesis regulators tends to be accompanied by an elevation of the endogenous inhibitor endostatin (Figure 5). In contrast, platelets of mice bearing angiogenic tumors exhibit a decrease in negative angiogenesis regulators, such as endostatin (Figure 5). This suggests that, in the “angiogenic” tumors, the overall balance of angiogenesis regulators may be tipped toward a more pro-angiogenic phenotype in a manner reflected in the platelet proteome, but not in the plasma levels of the same proteins.

The elevation of PDGF in platelets, but not that of plasma, was sustained for up to 120 days after tumor implantation (Figure 6).

**Discussion**

Our results show that the levels of angiogenesis regulatory proteins in circulating platelets change in mice bearing human tumor xenografts compared with tumor-free, sham-operated controls. Using SELDI-ToF technology, we evaluated, in an open-ended fashion, the proteomic profiles of platelets from tumor-bearing and
tumor-free, sham-operated mice. Among the proteins differentially expressed in platelets from these 2 groups, angiogenesis regulators were prominently represented. We have named this select group of angiogenic proteins the “platelet angiogenesis proteome” to emphasize the stability of the relative protein concentrations under physiologic conditions. Under normal conditions, the membership in this proteome appears to vary little. However, in a tumor-bearing mouse, the platelet content of angiogenesis regulators is altered and the changes persist, as suggested by the sustained elevation of PDGF in platelets, but not in plasma (Figure 6). The process appears selective for angiogenesis regulators, as we found that platelet levels of nonangiogenic proteins, such as albumin (Figures 5, S1), did not differ in presence or absence of tumors.

Circulating platelets can sequester angiogenesis regulatory proteins, even in the presence of a very small tumor mass, that is, cancers smaller than 1 mm³. Tumors of this size cannot be detected, at least at present, with conventional, clinically applicable methods. Our current study indicates that detection of these microscopic tumors cannot be accomplished using conventional measurements of angiogenesis regulatory proteins in plasma or serum because serum and plasma levels of these proteins are quite low and remain low even in the presence of much larger tumors. Although the presence of a microscopic tumor may be suspected on the basis of a single platelet protein marker, such as PF4, our current data indicate that platelets take up both positive and negative regulators of angiogenesis and that the balance of these 2 functionally opposing groups should be studied to obtain insight into the angiogenic potential of the tumor. This is in agreement with our finding of increased endostatin levels in the nonangiogenic clone compared with its angiogenic counterpart (Figure 5).

It appears that the platelet sequestration of angiogenesis regulatory proteins involves a process by which these proteins are internalized by circulating platelets, redistributed to different granular compartments within the platelets, and delivered to tissues as platelets adhere to abnormal vasculature. We have established previously that inhibitors and stimulators of angiogenesis are not necessarily in the same granular compartment in platelets and may not be released from platelets simultaneously. The platelet storage compartment, consisting of α-granules, dense granules, and lysosomes, is highly regulated. The exact manner of this regulation remains to be elucidated, but it is probable that the activation of platelets results in the redistribution of these proteins to the open canalicular system, which facilitates uptake and secretion of many proteins.

Some platelet-specific proteins, such as PF4 and thrombomodulin, are synthesized by several cells, including megakaryocytes, and concentrate in platelets at 400-fold concentrations. As we had documented previously, it is the host (marine) PF4 that is up-regulated in platelets of human tumor xenograft bearing mice, suggesting that the synthesis of this angiogenesis suppressor is up-regulated in the host bone marrow megakaryocytes. Other proteins, such as factor V, thrombospondin, or P-selectin, are synthesized by other cells and taken up by platelets in the periphery. The most notable nonselective platelet protein is fibrinogen, which is synthesized by the hepatocytes and taken up into platelet α-granules.

An important finding of our study was the relative absence of angiogenesis regulatory proteins in the plasma (Figure 5) or serum (Figure 2). These most common clinical analytes showed minimal or no differences in angiogenic proteins at baseline, or with tumor progression. This suggested that, contrary to commonly held beliefs, platelets may not undergo degranulation with an uncontrolled release of the growth regulators from α-granules into circulation, but rather that there may be a fairly strict spatial and temporal control of this release at the tumor or wound site. This was consistent with our in vitro analysis of the releasate of activated platelets, where minimal amounts of VEGF were released during agonist-induced platelet activation and bFGF was not released at all (Figure 2). The fact that a small amount of VEGF is released...
from platelets in response to thrombin is in agreement with previous studies, but these studies did not quantify the released vs retained proteins and could not therefore appreciate the incomplete degranulation. As we show in Figure 2B for bFGF, and in Figures 2A and 3 for VEGF, the proteins remain associated with platelets on activation. Using double-label immunofluorescence microscopy with antibodies against tubulin and VEGF for fixed and permeabilized resting platelets and phalloidin and VEGF for activated platelets, we showed that VEGF was distributed throughout the cytoplasm of resting and activated platelets and redistributed along the filopodia and the periphery of lamellipodia (Figure 3E,F). This tight association of angiogenesis-related proteins with platelets even after activation has been documented before but has not been clearly understood. It is probable that the redistribution of angiogenesis regulatory proteins to the filopodia and lamellipodia of platelets provides an avenue for a direct exchange of these proteins with the tissues. A publication by Ma et al demonstrated that the release of VEGF and endostatin is dependent on activation of specific proteinase-activated receptors (PARs). The authors show that the activation of PAR1 selectively releases a stimulator of angiogenesis (VEGF), whereas with the selective activation of PAR4 releases an inhibitor of angiogenesis (endostatin). Unfortunately, the tissue and platelet levels of these proteins were not reported, and the concentration of these 2 proteins in plasma was negligible.
The potential for selective release of some platelet-associated angiogenesis proteins, but not others, may explain how platelets contribute to active angiogenesis early in wound healing and inhibit angiogenesis in the late stages of scar formation. Similarly, the dual ability of platelets to store angiogenesis regulators and to adhere to sites of abnormal endothelium also suggests an involvement of platelets in the amplification and/or dormancy of tumors. Variable concentrations of VEGF, bFGF, PF4, PDGF, endostatin, and other proteins important in angiogenesis can be taken up, internalized, and concentrated in platelets for a timely delivery to wounds or tumors. The redistribution of endogenous growth factors from cytoplasmic (granular) storage to the periphery of filopodia and lamellipodia of activated platelets (Figure 3E,F), as well as our previous report that pro- and antiangiogenic proteins are organized into separate platelet α-granular compartments and differentially released, provides an intact platelet-regulated mechanism that may be responsible for the regulation of angiogenesis in wounds and that the loss of this regulation may be the basis for angiogenesis in tumors. This would be consistent with the previously published notion that “tumors are wounds that do not heal.”

Several other fundamental phenomena uncovered during these studies remain to be elucidated. For example, further studies will be needed to delineate the mechanism(s) by which platelets select, sequester, and retain only a relatively small number of proteins, excluding some much more abundant proteins or how these selected proteins bind in tissues.

The results reported here uncover new platelet biology with wide-ranging implications applicable to many angiogenesis-dependent physiologic states, such as development, wound repair, or reproduction, as well as disease conditions, such as cancer, atherosclerosis, diabetic ulcers, or inflammatory bowel disease. We have shown that the process of platelet uptake of angiogenesis regulators is highly specific and occurs well in advance of clinically detectable tumors. Platelet levels of angiogenesis-related proteins may therefore be superior to plasma and serum for the analysis of markers of angiogenesis in clinical trials in cancer. More importantly, however, the discovery that platelets actively sequester angiogenesis regulators and that these regulators can be differentially released from platelets would be expected to lead to many therapeutic applications in other angiogenesis-dependent conditions.

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Authorship

Contribution: G.L.K. designed and performed the research, provided guidance for the group, analyzed the data, and wrote and revised the manuscript; T.-T.Y., V.P., and E.W. executed, analyzed, and evaluated the mass spectroscopy data and provided expertise with the SELDI-ToF technology; F.C. and L.K. performed research; D.C. analyzed and evaluated the mass spectroscopy data; J.E.I. performed research and contributed to evaluation of data; A.A.-S. executed and analyzed ELISA studies; E.B. performed animal experiments; N.A. designed and executed animal experiments; M.K. contributed to the analysis of the data; and J.F. provided mentorship for the team and expertise in preparation of the manuscript.

Conflict-of-interest disclosure: T.-T.Y., V.P., and E.W. were employees of Ciphergen during the execution of this study. The remaining authors declare no competing financial interests.

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