Normal ranges of angiogenesis regulatory proteins in human platelets

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Platelets sequester angiogenesis regulatory proteins early in tumor growth, which suggests a new avenue for monitoring disease. To date, there are no clinically relevant reference ranges for markers of early angiogenesis. We introduce a new ELISA-based method for accurate and reproducible measurement of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), platelet factor 4 (PF4), thrombospondin-1 (TSP-1), fibroblast growth factor, basic (bFGF), and endostatin in platelets. To facilitate clinical applicability, the platelet levels in isolated samples were determined utilizing a new actin ELISA method. Platelets from healthy donors at single and repetitive time points were used for the assessment of normal ranges of these proteins. The physiological levels in platelets were: VEGF (0.74 ± 0.37 pg/10^6 platelets); PDGF (23 ± 6 pg/10^6); PF4 (12 ± 5 ng/10^6); TSP-1 (31 ± 12 ng/10^6); bFGF (0.44 ± 0.15 pg/10^6); and endostatin (5.6 ± 3.0 pg/10^6). There was an excellent correlation (R^2 = 0.7) between the platelet levels calculated with the actin ELISA and complete blood count. The levels of the platelets were higher than those in platelet-poor plasma by factors of: VEGF (215-fold); PDGF (914-fold); PF-4 (516-fold); TSP-1 (813-fold); and bFGF (17-fold). The endostatin levels were nearly equivalent. The biovariability of the platelet proteins in eight healthy subjects over a 5-week period was found to be minimal. We describe accurate and direct measurements of the concentrations of VEGF, bFGF, PDGF, TSP-1, endostatin, and PF4 in platelets of healthy human subjects. In contrast to the highly variable levels in plasma and serum, the platelet-derived measurements were accurate and reproducible with minimal biovariability. Am. J. Hematol. 00:000–000, 2010. © 2010 Wiley-Liss, Inc.

Introduction

The identification of biomarkers of early tumor recurrence, tumor growth, or therapeutic response has been of great interest in oncology. Similarly, the evaluation of angiogenesis induction is important for management of many diseases. Thus, the levels of angiogenesis regulatory proteins have been evaluated in biological fluids as potential diagnostic markers (biomarkers) for many years [1–4]. These efforts have been hindered by the low concentrations or short half-lives of these proteins in serum or plasma. Biomarker discovery investigations in plasma and serum, relevant to various cancers, have been conducted with varying levels of success as described in several reviews [5–9]. Support can be found for both serum and/or plasma measurements, but neither matrix allows for accurate measurements of biomarkers for angiogenesis-related diseases [10–13]. It has been proposed that in the microenvironment of the tumor, angiogenesis regulatory proteins are taken up (scavenged) by platelets early in tumor growth and released by platelets locally [14]. Klement et al. [15] demonstrated that the levels of angiogenesis regulatory proteins in circulating platelets of mice bearing human tumor xenografts change very early in tumor growth and can be used as markers of early angiogenesis. The early sequestration of angiogenesis regulators in platelets of tumor-bearing animals happens in the absence of any detectable changes in plasma or serum and is selective for angiogenesis regulators. Other plasma proteins such as albumin are not sequestered in platelets even though the concentration of albumin in plasma exceeds the concentration of angiogenesis regulators many fold.

A successful clinical application of the measurement of angiogenesis regulators in platelets requires establishment of normal physiological levels of these proteins in platelets. To do so, platelets must be isolated from whole blood without platelet activation. Similarly, the number of platelets in a given sample must be enumerated to normalize the measured level of protein to the number of platelets. Direct complete blood count (CBC) methodologies are typically performed to enumerate platelets and determine their volume in whole blood or plasma. Unfortunately, CBC measurements cannot be performed directly on platelet pellet samples and an alternative correction is necessary. Actin is a constitutively expressed structural protein found in all cellular structures including platelets and is absent from plasma [16]. We, therefore, developed a method in which actin levels within the platelet pellet are used as a surrogate for platelet enumeration.

This manuscript provides the first description of the methods that can be used for an accurate measurement of a selected set of angiogenesis regulatory proteins found in platelets. We describe the enzyme-linked immunosorbent assay (ELISA) based measurements of vascular endothelial growth factor (VEGF), fibroblast growth factor, basic (bFGF), endostatin, thrombospondin-1 (TSP-1), platelet-derived growth factor (PDGF), and platelet factor 4 (PF4); the method of actin enumeration of platelets; and for the first time, report the normal ranges of these proteins in platelets in a presumably healthy population. To establish the biovariability of angiogenesis-related protein levels in platelets, we measured the level of the protein in a group of presumably healthy individuals on a weekly basis over a 5-week period of time. Unlike other methods of analysis, the reported methods

Additional Supporting Information may be found in the online version of this article.

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Contract grant sponsor: Ortho Clinical Diagnostics.

Conflict of interest: Nothing to report.

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Received for publication 23 January 2010; Accepted 6 April 2010

Am. J. Hematol. 00:000–000, 2010.

Published online 13 April 2010 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ajh.21732

can be easily translated into clinical practice. We provide reference change values (RCVs) [17] of the analytes, and define normal ranges and limits, which can be used in the future as a baseline for the evaluation of levels of angiogenesis regulatory protein concentrations in disease states.

As a central feature of this article, we describe the application of a quality system using methods and controls designed to enable the detection of analytical or processing errors, which could lead to inaccurate or invalid results.

Materials and Methods

Human subjects, preparation of platelet pellet, and platelet-poor plasma

Standard citrated coagulation blood samples were collected from presumably healthy individuals. Individuals with history of cancer, inflammatory disease, diabetes, or nonsteroidal anti-inflammatory drug use were excluded from the study. Informed consent was obtained in accordance to institutional practice and guidelines at Ortho Clinical Diagnostics and Children's Hospital Boston. A total of 50 subjects of unknown ethnicity were enrolled. The samples represented both genders equally (29 females and 21 males) and the age ranged from 26 to 89 years (median 55 ± 13 years). Five weekly samples from eight other subjects; six females and two males between the ages of 40–62 years (median age 47 ± 8 years) were collected in a blinded fashion for evaluation of biological and sample variability. The menstrual status of the females was not known. The samples were processed according to standard methods for platelet collection. Briefly, whole blood was drawn by venipuncture into BD Vacutainer® Citrate tubes containing 105-mM citrate (pH 5) anticoagulant at a ratio of 1:9 (vol/vol) buffer to blood. The tubes were gently inverted to mix the blood and anticoagulant and kept at ambient temperature (20–28°C) to avoid activation of the platelets. The blood samples were centrifuged for 20 min at 150*g* using a Sorval RT swinging-bucket rotor (1000B). One milliliter of platelet-rich plasma (PRP) was conservatively recovered from the upper phase and transferred into a secondary polypropylene Eppendorf tube. The 1 mL aliquots were centrifuged for 10 min at 900*g* to sediment platelets. The supernatant [i.e., platelet-poor plasma (PPP)], was transferred to another tube and the residual plasma blotted away from the inside walls of the tube containing the platelet pellet to minimize plasma contamination of the platelet pellet. The tubes containing either the platelet pellet or plasma were then stored at −80°C until further analysis.

Platelet lysis

Platelet samples were thawed and 100 μL of lysis buffer, containing 0.5% Triton X-100 (Fluka) and protease inhibitor cocktail (Sigma P8340, diluted 1:100) in phosphate buffered saline (PBS) buffer pH 7.2 (Pierce), was added to each platelet pellet. The platelet pellets were fully solubilized by vigorous pipetting and vortexing until translucent in appearance. A volume of 1.5-mL PBS buffer was added to each lysed platelet sample, yielding a platelet lysate solution, which was diluted for analysis as described below.

Platelet lysate dilution factors

The dilution factors of the platelet lysate solution for testing in the various assays were developed, so that one platelet pellet sample isolated from 1-mL PRP could be tested in all of the assays in replicates of two or four at up to two different dilution factors. The limited volume of platelet lysate dilution factors were balanced for assay sensitivity and analyte levels. As a result, VEGF and bFGF were tested undiluted and twofold; endostatin at 5- and 10-fold; PDGF at 20- and 40-fold; actin at 32-fold; TSP-1 at 200- and 400-fold; and PF4 at 4,000- and 8,000-fold. Dilutions were made in low increments to avoid error introduced by large one-step dilutions. The diluents for dilution were the same as described for the calibrators.

Platelet-poor plasma

The PPP corresponding to a given platelet sample was tested simultaneously to provide correlative information about the relative concentrations of angiogenesis regulatory proteins in the plasma. Two replicates were tested at one dilution for each of the assays at the following dilutions: undiluted for VEGF; twofold dilution for bFGF; fourfold for PDGF; 20-fold for TSP-1; 200-fold for endostatin; and 2,000-fold for PF4. The diluents for dilution were the same as described for the calibrators. With these dilution factors, 1 mL of PPP was sufficient to perform all of the ELISA testing at two dilutions with multiple replicates.

ELISA materials and development

Some of the ELISA assays were developed from R&D Systems DuoSet reagents, which are matched sets of reagents containing coating antibody, detection antibody, standard protein (for calibration), and streptavidin horseradish peroxidase (HRP). The DuoSet reagents (with product codes) we used were: VEGF (DY293B); bFGF (DY233); endostatin (DY1098); PDGF-BB (DY220E); and PF4 (CXCL4/PF4 DY795E), which were used according to the manufacturer's recommendations. For the TSP-1 assay, the monoclonal antibody (MAB3074) and recombinant protein for calibration (3074-TH, NSO derived) were purchased from R&D Systems. The actin ELISA was developed de novo using a commercially available monoclonal antibody (Millipore MAB1501R) and actin purified from human platelets (Cytoskeleton, INC. APHL-95). The antibodies were either coated onto the solid phase for analyte capture or biotinylated for detection of captured analyte with streptavidin-HRP.

Coating of microwell plates

The coating antibodies were diluted in either BuPH buffer pH 7.2 (Pierce 28372) or BuPH buffer pH 9.4 (Pierce 28382), depending on the analyte: VEGF (1.2 μg/mL, pH 9.4); bFGF (2 μg/mL, pH 7.2); endostatin (4 μg/mL, pH 7.2); PDGF (0.5 μg/mL, pH 9.4); TSP-1 (1 μg/mL, pH 7.2); PF4 (0.75 μg/mL with 1 μg/mL bovine serum albumin (BSA) co-coat, pH 7.2); and actin (2 μg/mL, pH 7.2). High-binding microwell plates (CoStar cat# 2592) were coated with 100-μL coating antibody solutions, incubated overnight in high humidity, and washed with wash buffer (described below) three times with 400 μL per well. The plates were then postcoated with 150 μL/well of Starting Block (Pierce 37542), incubated at room temperature in a humid box for a minimum of 2 hr, aspirated (not washed) 1 × 2 sec, allowed to dry in a humidity-controlled incubator, pouched in a sealed bag with a desiccant and stored at 2–8°C until use.

Detection antibody

Antibodies not commercially available as conjugates (TSP-1 and actin) were biotinylated by conventional methods. All of the biotin conjugates were diluted to working strength in reagent diluent (RD) (R&D Systems part DY995) prediluted 1:10 in water as instructed by the manufacturer. The biotinylated antibody working concentrations were: VEGF (100 ng/mL); bFGF (250 ng/mL); endostatin (100 ng/mL); PDGF (400 ng/mL); TSP-1 (10 ng/mL); PF-4 (13 ng/mL); and actin (800 ng/mL).

Standards used for calibration

Calibrations of VEGF, bFGF, endostatin, PDGF, TSP-1, and PF4 were carried out using recombinant proteins supplied with R&D Systems DuoSet kits at the assigned value. Calibrations of actin utilized the mass values assigned by the manufacturer (Cytoskeleton). Certificates of analysis indicated 95% purity or better. The high calibrator levels and diluents used were: VEGF (1,000 pg/mL, PBS); bFGF (1,000 pg/mL, PBS); endostatin (1,000 pg/mL, RD); PDGF (1,000 pg/mL, RD); TSP-1 (100 ng/mL, RD); and PF4 (2.5 ng/mL, RD). Actin, purified from human platelets, was diluted to 2.5 ng/mL in polymerization buffer (10 mM Tris, pH 7.5, 2 mM $MgCl_2$, and 50 mM KCl) followed by serial dilutions.

Additional reagents for ELISA testing and development

Streptavidin-HRP (R&D Systems DuoSet generic reagent part 890803), diluted 1:200 in RD; 20X Wash Buffer Concentrate (Ortho Clinical Diagnostics part 933730) diluted in deionized water; tetramethylbenzidine (TMB) peroxide substrate for ELISA (Moss, part # TMBE-1000) used undiluted; 4N sulfuric acid (Ortho Clinical Diagnostics part 933040); Actin Polymerization Biochem Kit (Cytoskeleton, Cat # BK003)

Actin ELISA development

Actin exists in equilibrium between monomer and polymer. For accurate quantitation, actin was first fully polymerized using a commercially available actin polymerization assay to assess the level of polymerization under defined conditions (Cytoskeleton, BK003). In addition, since our ELISA assay detected the polymer form, samples were processed under high ionic strength to further promote polymerization. Ultracentrifugation studies confirmed that our selected lysis and diluent buffers resulted in 92% actin polymerization (data not shown). The correlation of actin (in μg) and platelet counts from standard clinical assessments (CBC) was established using additional samples from 27 presumably healthy subjects with an average age of 53 ± 7 [1 standard deviation (SD)] and female/male ratio of 17/10.

Principle of the assays

The assays were designed as quantitative sandwich enzyme immunoassays utilizing monoclonal or polyclonal antibodies specific for the analytes. In brief, 100 μL aliquots of samples, controls, and calibrators were pipetted into precoated wells, incubated for 1 hr, washed 3 × 400

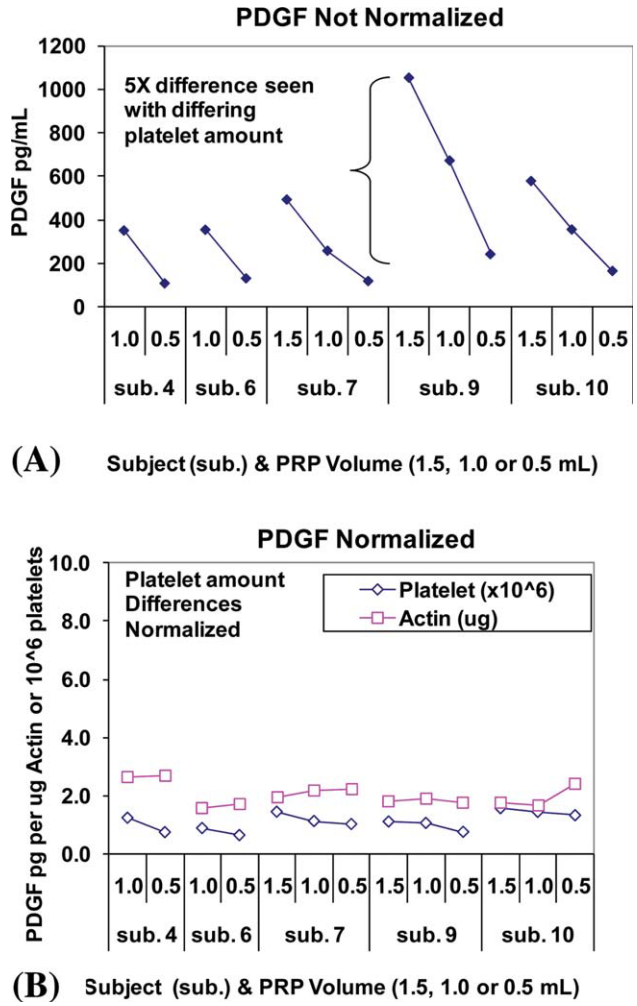

Figure 1. Platelets were isolated and tested for PDGF and actin by centrifugation of differing volumes of PRP. The non-normalized PDGF values (A) reflect the amount of platelets, whereas the normalized PDGF values (B) reflect the specific platelet content. Platelet-derived growth factor (PDGF), platelet-rich plasma (PRP), Vol (volume, mL), Est. Plt Cnt (platelet count, estimated based on the volume of PRP, with a known platelet value, centrifuged to pellet the platelet content). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

μL/well followed by addition of 100-μL biotinylated polyclonal or monoclonal antibody, incubated for 1 hr, washed 3 × 400 μL/well followed by addition of 100-μL streptavidin conjugated to HRP. After a 30-min incubation, the wells were washed 3 × 400 μL/well and 100-μL TMB substrate solution (Moss Labs) was added. The colorimetric reaction was stopped after 30 min with 50 μL 2N H_2SO_4. Color intensity was then measured with a microplate spectrophotometer at 450 nm with a 620 reference. The first and second incubations were performed on a shaking incubator (1-mm rotation at 600 rpm) at 37°C, except VEGF and bFGF, which were incubated with shaking, but at an ambient temperature (22–27°C).

Process and quality control

We utilized quality and process controls to ensure accuracy and precision. Briefly, the platelet levels were enumerated by normalization to actin. Platelet lysis, dilution, and testing were controlled by the use of "platelet controls." Plate-to-plate and day-to-day variance was controlled by the use of "run controls". Calibration was performed on each plate in order to control for run-to-run variance. All ELISA reagents were pretested for accuracy and precision prior to clinical testing. The quality of the sample results were assessed for acceptable replicate mean and SDs.

Description of controls used in the assays

Control reagents were developed to identify microwell plate results, which may be inaccurate due to an operational or reagent error. Platelet controls were prepared by centrifugation of multiple 1 mL aliquots of pooled PRP followed by removal of the supernatant PPP and storage of the platelet pellets at −80°C until use. Plasma controls were prepared from PPP, obtained as a side product of the platelet control preparations. Run controls were prepared from platelet control, which was lysed and diluted to a working dilution for each assay as described above. The diluents for dilution were the same as described for the calibrators. Control ranges were developed from multiple day and run precision testing with calculation of 95% Confidence Intervals (CIs), upper and lower bound. CBC was performed on a Beckman Coulter LH755 Analyzer by ACM Medical Laboratories, Rochester, New York according to established laboratory practices. Using this method, the normal platelet range is 130,000–400,000 platelets per μL EDTA whole blood, and the normal mean platelet volume range is 7.5–12.0 fL.

Results

Protein measurement and normalization

The number of platelets in normal, presumably healthy individuals varies widely. Because it can be difficult to determine platelet levels in a pellet sample corresponding to levels calculated by CBC in plasma, we established an alternate correction using a protein absent from plasma but present in stable values in platelets. Figure 1 summarizes the principle of the normalization process. A twofold to threefold variation in platelet volume ($n = 5$) results in a corresponding change in actin levels (Fig. 1A). Data normalized to the number of platelet (Fig. 1B rhomboid) or

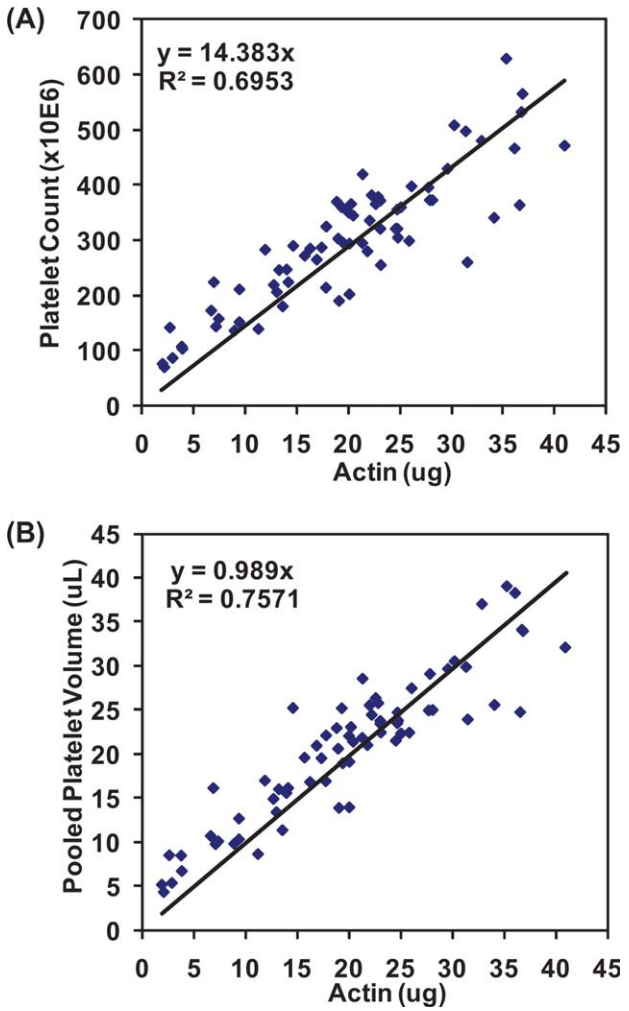

Figure 2. Platelet counts and platelet volumes were determined by CBC of PRP-containing platelet samples which were then pelleted by centrifugation for lysis and actin ELISA. The actin levels were used to predict platelet count (A) and platelet volume (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Control Results

Assay	Control type	Units	Average	Std dev	95% CI range		N	% Outside
					Lower bound	Upper bound		
VEGF	Platelet	pg/mL	145	14	117	173	24	2
	Run		126	12	103	149	48	2
	Plasma		53	20	14	91	16	0
PF4	Platelet	ng/mL	3,130	506	2,138	4,122	48	6
	Run		0.23	0.03	0.17	0.30	144	3
	Plasma		316	47	224	408	16	6
PDGF	Platelet	pg/mL	3,909	567	2,797	5,020	48	6
	Run		114	16	83	146	144	3
	Plasma		378	80	222	534	16	0
TSP1	Platelet	ng/mL	6,555	936	4,720	8,390	48	4
	Run		26	4	18	34	144	5
	Plasma		405	52	303	508	16	0
bFGF	Platelet	pg/mL	97	10	78	116	24	2
	Run		94	10	75	113	48	0
	Plasma		329	34	263	396	16	0
Endostatin	Platelet	pg/mL	1,142	115	916	1,368	24	0
	Run		121	10	102	140	48	4
	Plasma	ng/mL	97	11	76	119	16	6
Actin	Platelet	ng/mL	14,649	3,829	7,144	22,154	48	4
	Run		280	66	151	409	144	5

95% Confidence Interval (CI) ranges for platelet, run, and plasma controls, where LB is the lower bound and UB is the upper bound, *N* is the number of replicates, and % outside is the number of replicate that fell outside of the 95% CI range.

VEGF, vascular endothelial growth factor; bFGF, fibroblast growth factor, basic; PDGF, platelet derived growth factor; PF4, platelet factor 4; TSP-1, thrombospondin-1; Std Dev, standard deviation.

actin (Fig. 1B square) showed an equivalent and consistent platelet protein content. The actin measurement had the advantage of providing an estimate of platelet concentration even within a PRP obtained from varied amounts of blood. A linear regression relationship to describe platelet count relative to actin is expressed with eq. 1 and shown in Fig. 2A. The *Y* intercept was set to zero and the correlation (R^2) was 0.695. The actin level, in μg, is that found in a platelet lysate prepared from platelet material isolated from 1-mL PRP by centrifugation, as described in Materials and Methods.

$$Y\ \text{platelet number}(\times 10^6/\text{mL PRP}) = 14.383 \times (Actin,\ \mu g/mL\ PRP)$$

A linear relationship between platelet volume and actin content is described with eq. 2 and in Fig. 2B. The correlation (R^2) was 0.757. The *Y* intercept was set to zero.

$$Y\ \text{platelet volume}(\mu\text{L platelet/mL PRP}) = 0.989(Actin,\ \mu g/mL\ PRP)$$

Assessment of assay variability

The variability of the assays was estimated using analysis of variance (ANOVA), and is shown in Supporting Information Supplement 1. Utilizing the means and SDs obtained from the variance testing, we were able to define 95% CIs (2.5–97.5 percentile) for all of the controls by adding or subtracting the product of 1 SD × 1.96. The resultant ranges allowed us to predict, based on known variances, the acceptable results for 95% of the replicates of in-control process shown in Table I. By running controls at the same time as our subject samples, we were able to monitor the performance of the processes to ensure that the results had a 95% level of certainty to be accurate as long as the control results were within the defined ranges. All of the results from the analysis of the platelet samples, using 24 or 48 replicates depending on the assay fell within the expected 95% CI ranges with the exception of one replicate each for the PDGF and PF4 assay for a platelet control and one replicate for an endostatin plasma control.

TABLE II. Normal Ranges

	Matrix	Unit	Avg	Median	SD	95% CI range[a]	
						Min	Max
VEGF	Platelet	pg/10^6	0.74	0.68	0.37	0.02	1.47
		pg/μL	11	10	5.4	0	22
	Plasma	pg/μL	0.05	0.05	0.02	0.01	0.08
	X-Fold	pg/μL		215			
PF-4	Platelet	ng/10^6	12	10	5	2.4	22
		ng/μL	178	150	74	34	323
	Plasma	ng/μL	0.36	0.29	0.25	0	0.86
	X-Fold	ng/μL		516			
PDGF	Platelet	pg/10^6	23	21	6	12	33
		pg/μL	330	312	83	167	494
	Plasma	pg/μL	0.38	0.34	0.24	0	0.84
	X-Fold	pg/μL		914			
TSP-1	Platelet	ng/10^6	31	27	12	7	54
		ng/μL	449	403	178	101	798
	Plasma	ng/μL	0.56	0.5	0.27	0.03	1.09
	X-Fold	ng/μL		813			
bFGF	Platelet	pg/10^6	0.44	0.42	0.15	0.15	0.74
		pg/μL	6.4	6.17	2	2.47	10.3
	Plasma	pg/μL	0.365	0.371	0.143	0.086	0.645
	X-Fold	pg/μL		17			
ES	Platelet	pg/10^6	5.6	5.1	3	0	11.5
		pg/μL	81	74	42	0	163
	Plasma	pg/μL	119	111	34	52	187
	X-Fold	pg/μL		0.7			

Platelet concentrations relative to the platelet count and platelet volume (per μL). Plasma concentrations are shown per μL for comparison to platelet concentration. Min and Max are defined by the 95% empirical confidence interval (2.5–97.5 percentile). All biomarkers are normalized to actin and expressed per μL platelet volume or 10^6 platelets.

VEGF, vascular endothelial growth factor; bFGF, fibroblast growth factor, basic; PDGF, platelet derived growth factor; PF4, platelet factor 4; TSP-1, thrombospondin-1; ES, endostatin; SD, standard deviation.

X-Fold = Platelet concentration/plasma concentration, in same units. [a]Defined by the 95% empirical confidence interval (2.5–97.5 percentile).

Platelet and plasma levels of respective proteins in normal, presumably healthy subjects

The platelet concentration is shown relative to mass however the molar concentrations may be calculated for each

TABLE III. ANOVA of Results Obtained from Platelet Samples Collected from Eight Subjects Over 5 Weeks (Longitudinal)

			Standard deviation				% CV			
Analyte	Units	Mean	Intersubject	Intrasubject	Intraplate	Total	Intersubject (%)	Intrasubject (%)	Intraplate (%)	Total (%)
PDGF	pg/10^6	24.6	3.9	3.1	1.5	5.2	16	13	6	21
PF4	ng/10^6	16.5	3.8	3.2	0.9	5.1	23	19	6	31
TSP1	ng/10^6	28.2	3.8	2.2	3.1	5.4	14	8	11	19
ES	pg/10^6	6.9	1.4	1.7	0.2	2.2	21	24	3	32
VEGF	pg/10^6	0.88	0.38	0.15	0.02	0.41	44	17	3	47
bFGF	pg/10^6	0.47	0.13	0.07	0.02	0.15	28	14	4	32

VEGF, vascular endothelial growth factor; bFGF, fibroblast growth factor, basic; PDGF, platelet derived growth factor; PF4, platelet factor 4; TSP-1, thrombospondin-1; Std Dev, standard deviation; CV, coefficient of variability; Intersubject, subject-to-subject; Intrasubject, week-to-week; Intraplate, well-to-well.

TABLE IV. ANOVA of Results Obtained from Platelet-Poor Plasma Samples Collected from Eight Subjects Over 5 Weeks (Longitudinal)

			Standard deviation				% CV			
Analyte	Units	Mean	Intersubject	Intrasubject	Intraplate	Total	Intersubject (%)	Intrasubject (%)	Intraplate (%)	Total (%)
PDGF	pg/mL	314	130	158	43	209	41	50	14	66
PF4	ng/mL	319	95	143	14	172	30	45	5	54
TSP-1	ng/mL	573	213	138	77	265	37	24	13	46
ES	ng/mL	104	11	6	2	12	10	5	2	12
VEGF	pg/mL	351	519	65	25	524	148	19	7	149
bFGF	pg/mL	478	305	75	27	315	64	16	6	66

VEGF, vascular endothelial growth factor; bFGF, fibroblast growth factor, basic; PDGF, platelet derived growth factor; PF4, platelet factor 4; TSP-1, thrombospondin-1; Std Dev, standard deviation; CV, coefficient of variability; Intersubject, subject-to-subject; Intrasubject, week-to-week); Intraplate, well-to-well.

analyte using the molecular weights of 45 kDa for VEGF [18], 18.7 kDa for bFGF [19], 20 kDa for endostatin [20], 450 kDa for TSP-1 [21], 28 kDa for PDGF [22], 31.2 kDa for PF4 (tetramer) [23], and 41.8 KDa for actin [24] (calculations not shown). Comparisons to levels published in the literature are in the Discussion section. The levels of the above-listed proteins in platelet and plasma of presumably healthy donors are shown in Table II. Platelet concentrations are expressed relative to 10^6 platelets as determined by the actin method. The plasma results are shown relative to μL, to compare concentrations with platelets. The minimum and maximum concentrations are defined by the 95% empirical CI (2.5–97.5 percentile) [25]. The differences in concentrations between platelet and plasma (based on median values) are: VEGF (215-Fold), PF-4 (516-fold), PDGF (914-fold), TSP-1 (813-fold), and bFGF (17-fold). The smallest difference between platelets and plasma was detected in the case of endostatin (0.7-fold).

Week-to-week (longitudinal) variance

We studied the degrees of variance for the six analytes over 5 weeks, with longitudinal samples taken weekly from eight individuals. The group, comprised of two males and six females, had an average age of 49 ± 8 (1 SD) years. We obtained blood samples once a week over a 5-week period of time, $N = 39$ (Subject #6, one time point was not collected). The concentrations of each of the analytes were normalized to platelet number (with the actin ELISA) and analyzed by nested ANOVA (MiniTab 15). The results of the ANOVA, depicting the sources of variance are shown in Table III for platelets and Table IV for PPP. Tables and plots of each analyte in the platelets of each subject over the 5 weeks are shown in Supporting Information Supplements 2–9. The categories of variance are defined as: intersubject, the difference from subject to subject; intrasubject, the difference from week to week within the same subject; intraplate, also called replicate error, is the difference between results within the same test. The samples for the individual time points for a given individual were tested on the same microwell plate. The intersubject results, therefore, are confounded by plate-to-plate variance. The total CV is the pooled cumulative error (variance) from all of the factors. The intersubject variance of platelet was lowest for TSP-1 (14%) and highest for VEGF (44%) in platelets. The intrasubject variance was highest for endostatin (24%) and lowest for TSP-1 (8%) in platelets. The plasma levels of the angiogenesis regulatory proteins were more variable than the levels found in platelets for all analytes except endostatin. VEGF had the highest degree of intersubject variability in plasma at 148%.

TABLE V. Reference Change Value Calculated as Described in eq. 3

Analyte	CV_A	CV_A^2	CV_I	CV_I^2	RCV (%)
PDGF	27	727	13	158	130
PF4	25	614	19	373	138
TSP1	24	597	8	63	113
Endo	26	672	24	599	156
VEGF	23	535	17	298	126
bFGF	25	643	14	195	127

VEGF, vascular endothelial growth factor; bFGF, fibroblast growth factor, basic; PDGF, platelet derived growth factor; PF4, platelet factor 4; TSP-1, thrombospondin-1; Endo, endostatin; CV_A, change value, analytical; CV_I, Change value, individual.

A statistical relationship was developed by Fraser [17] wherein the analytical variability combined with an individual's biovariability enabled a prediction of a normal observable analyte range. Results falling outside of this range in clinical testing would be considered outside of normal ranges. The relationship called the RCV is defined in eq. 3.

$$\mathrm{RCV} = n^{1/2*} Z^* (\mathrm{CV}_\mathrm{A}^2 + \mathrm{CV}_\mathrm{I}^2)^{1/2}$$

where n (number of serial samples) = 5; Z-score = 1.96 for a bidirectional probability of 95%; CV_A = total analytical variation, platelet control results for each analyte; CV_I = total individual variation, five serial samples from eight subjects drawn 1 week apart.

The CV_A was calculated with 52 replicates each for PDGF, PF4, and TSP-1 and 26 replicates each for endostatin, VEGF, and bFGF over the course of 13 runs. The CV_I (for the individuals) was calculated by ANOVA as the intrasubject (interweek) component from 156 replicates each for PDGF, PF4, and TSP-1 and 78 replicates each for VEGF,

bFGF, and endostatin, from eight donors over 5 weeks. The CV_A, CV_I, and the calculated RCV are shown in Table V.

Discussion

Angiogenesis regulatory proteins are sequestered in platelets. While the relative concentrations of these proteins in platelets remain stable under physiologic conditions, their levels change significantly in the presence of a tumor. Our manuscript is the first to describe endogenous levels of selective angiogenesis regulators (VEGF, bFGF, PDGF, TSP-1, endostatin, and PF4) in platelets. The sample sizes are small and caution is required in interpreting the results. For accurate and consistent measurements of platelets, we developed process controls and a method to measure the number of platelets in a pellet sample. While actin levels have been routinely used for normalizing gene or protein expression, a rapid ELISA method has not been previously reported and platelet levels in pellet samples could not be accurately described. The combination of reliable and reproducible assays and baseline values of the individual proteins provides future investigators with the basic tools to use platelet-associated angiogenesis regulators as markers of tumor growth or therapeutic response.

Our data is in agreement with earlier studies of angiogenesis-related proteins in platelets. For example, Hart et al. [22] measured PDGF-BB in 100 mL whole blood and arrived to a value of 266–570 ng of PDGF-BB, depending on the buffer. On the basis of the average platelet levels and volumes of 130–400 K of platelets per μL EDTA whole blood and mean platelet volume of 7.5–12.0 fL, we extrapolated their results to range from 188 to 411 pg/μL platelet volume depending on the buffer. Our finding of 330 pg of PDGF/μL (Table II) fell well within the range described by Hart et al. Similarly, plasma PF4 levels were previously described as 102 ± 23.5 IU/mL [12], in studies where the PF4 levels were used to assess platelet activation. A PF4 international unit (IU) is 1 ng (WHO). This value falls within a 1 SD range of our finding of 363 ± 255 ng/mL. We found no direct measurement data on platelet levels of PF4 in the literature for comparison. A review by Kut et al. [26] reports plasma VEGF to fall between 19 ± 8 and 47 ± 8 pg/mL. These values, generated from a variety of methods, were similar to our average value of 46 ± 18 pg/mL. We also report VEGF levels in platelets of normal individuals to be 0.74 ± 0.37 pg/10^6 platelets (range 0.15–1.86), values very similar to those reported by Salven et al. [27] [0.5 pg/10^6 platelets (range 0.1–1.6)]. The levels of TSP-1 in plasma have been described as 180 ng/mL [28], which was lower than those determined in our studies (559 ± 272 ng/mL). We found no direct measurements of TSP-1 in platelets in the literature. Martyre et al. [29] described bFGF levels in platelets in normal donors to be 0.13 ± 0.058 pg/10^6 platelets based on the difference in levels found in PRP and PPP. Endostatin levels in plasma were described by Feldman et al. [30] as 43.2 ± 15.1 ng/mL. This was within our range of 52–187 ng/mL. We found no direct measurements of endostatin in platelets in the literature.

The concentrations of the selected angiogenesis regulators investigated in this study were higher in platelets than in plasma. The presence of these biologically active proteins in platelets is thought to play regulatory roles in tissue repair and blood vessel development [31]. Some proteins are scavenged by the platelet and organized in the α-granules of platelets [32,33]. We document, for the first time, that the concentrations of VEGF, PF-4, PDGF, TSP-1, and bFGF in platelets of normal human subjects differs significantly from plasma. While this has always been suspected [15,34−36], the determination of these differences would not have been possible without accurate knowledge of the volume (mass) of platelets being measured.

Although serum measurements have been considered to represent the platelet levels of angiogenesis regulatory proteins [37], we have documented previously that platelets do not release angiogenesis regulators en masse [33] and the serum measurement can be misleading. Furthermore, this approach would not allow for an analytical description of the differences of corresponding concentrations between platelet and plasma. With knowledge of analytical variability (platelet control results) and the biovariability in individuals (longitudinal studies), it was possible to determine normal ranges. While some variation in angiogenesis regulatory proteins may be expected over the course of an individual's life, the differences are not manifested in the longitudinal study, which spanned 5 weeks. Within the limited population we evaluated, there was a minimal degree of variance in the levels of angiogenesis regulatory proteins in platelets in presumably healthy individuals over time.

Our study evaluated angiogenesis regulatory proteins in platelets of normal individuals, and established the methodologies necessary for clinical translation of the assays (i.e., normalization to actin). The baseline values for VEGF, bFGF, PDGF, TSP-1, endostatin, and PF4 in platelets are established by the publication of this manuscript and can serve as guidelines for the determination of differences in platelet content of angiogenesis regulators in disease states. Studies comparing platelets from individuals with cancers are presently in progress and should determine if the analysis of platelet content of angiogenesis regulators may have diagnostic or prognostic applications. If confirmed, we will be able to advance the numerous clinical trials using biologic modifiers and targeted therapies, where toxicity cannot be used as marker of efficacy. As such, the ability to quantify angiogenesis in cancer, endometriosis, inflammatory bowel disease, retinal disorders, and other diseases will lead to a paradigm shift in medicine.

Acknowledgments

This article is dedicated to the memory of Dr. Judah Folkman, without whom this work would not have begun. The authors thank Dennis Robords of Ortho Clinical Diagnostics and Susan Connors, Abdo Abou-Slaybi, and Marleike de Bruin of Children's Hospital Boston for their technical assistance. The authors also thank Marsha Oenick and John Backus of Ortho Clinical Diagnostics and Sean Downing of Children's Hospital Boston for helpful discussions.

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