SUPPLIMENTAL MATERIAL

MATERIALS AND METHODS

Isolation of human platelets

Human blood collection was performed in accordance with the Declaration of Helsinki and ethics regulations with institutional review board approval. Platelets were isolated from healthy volunteers as described.[1] Healthy volunteers did not ingest known platelet inhibitors such as aspirin or nonsteroidal anti-inflammatory drugs for at least 10 days prior to blood collection. *In vitro* tamoxifen exposure was performed by treating platelet-rich plasma (PRP) with tamoxifen (Sigma; Catalog No: T9262) at a final concentration of 10 μM or 20 μM, (Z)-4-hydroxytamoxifen (Sigma ; Catalog No H7904 at a final concentration of 25 μM or 50 μM or with DMSO (Sigma; Catalog No: D2650) vehicle control for 1 hour at 37C. Platelets were washed extensively in wash buffer (140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, pH 5 6.0) to remove the tamoxifen and then resuspended in platelet buffer (10 mM N-2-hydroxyethylpiperazine- N9-2-ethanesulfonic acid, 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl2, 5 mM NaHCO3, 10 mM glucose, pH 7.4).

Activation of platelets

Platelets were activated in vitro by exposure to 5-10 μM thrombin receptor-activating peptide (TRAP) (Sigma; Catalog No: T1573), 25 μM adenosine diphosphate (ADP) (Biodata, Horsham, PA; Catalog No: 101312) or exposure to $3x10^6$/mL MCF-7 or MDA-MB-231 human breast tumor cells (ATCC, Manassas, VA; Catalog No: ATCC® HTB-22™ and HTB-26™ respectively).
Platelets were exposed to agonist for 10 minutes at 37°C prior to collecting the releasate or processing for flow cytometry or immunofluorescence microscopy. The activation state of platelets following activation was determined by P-selectin antibody (BD Biosciences, San Jose, CA; Catalog No: 5555524) labeling on flow cytometry (BD Canto II, BD Biosciences).

Angiogenic protein quantification

VEGF concentrations were determined using the Quantikine human enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN; Catalog No: DY293B) using 100 μL of platelet releasate run in duplicate.

Antibody Array

The RayBio Human Angiogenesis Antibody C-1000 (RayBiotech, Inc; Catalog No: AAH-ANG-1000-8) membrane-based array kit was used according to the manufacturer's protocol to screen for 43 proteins in releasates generated from tamoxifen treated, 4-hydroxytamoxifen or vehicle control treated platelets.

Angiogenesis Assays

Capillary tube formation was used to assess the angiogenic potential of releasates made from resting platelets or platelets stimulated with agonists using the Millipore Capillary Tube Formation Assay kit (Billerica, MA; Catalog No: ECM625) in duplicate. Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD; Catalog No: CC-2517) and cultured in EBM media (Lonza; Catalog No: CC-3124) according to their protocol. After an incubation of HUVEC cells with platelet releasate for 6 hours, capillary tube formation was quantified. Five fields were imaged (at x4 and x20 magnification) with differential-interference-contrast microscopy using a Seizz Axiovert microscope, and the degree of tubulogenesis was quantified by counting branch points (nodes with 3 or more branches).

Endothelial migration assay

The bottom chamber of a transwell plate (Corning Inc., Corning, NY; Catalog No: 3421) was pre-coated with 0.5% gelatin. HUVECS in serum-free media were seeded in the media and 1 X 10^4/mL cells were inoculated into the upper chamber of each transwell with the releasate from 2 X 10^8/mL platelets generated under experimental conditions placed in the bottom chamber. After 24 hours of incubation, the cells were fixed and stained with Diff-quik (Siemens, Newark, DE; Catalog No: NC0674866), and the cells at the bottom were counted in 4 microscope fields. The results are shown as the number of cells that migrated to the bottom of the transwell. Independent assays were averaged, and statistical analysis was performed using the Student *t* test.

Metastasis Assays

Invasion assays was used to study the cell invasion of breast tumor cells. The bottom chamber of a transwell plate was pre-coated with BD Matrigel basement membrane matrix (BD Catalog No: 354234). Breast tumor cells in serum free media were seeded in the media and 5 X 10^4/mL cells were added to the upper chamber of each transwell. Releasate made from 2 X 10^8/mL platelets previously generated under experimental conditions were placed in the bottom chamber. After 24 hours of incubation, the cells were fixed and stained with Diff-quik (Seimens, Newark, DE; Catalog No: NC0674866), and the cells attached to the underside of the transwell membrane were counted in 4 microscope fields. Results are calculated as the percentage of cells that invaded through the matrix compared to control wells with no matrix present. Transendothelial migration assays, tranwells were endothelialized by seeding HUVECs into the top well and growing to confluence. MCFf-7 breast tumor cells were labeled with 5 nM CMFDA (Life Technologies Catalog #C7025) plated into the top chamber of endothelialized transwells at 5x10^5/ml with or without washed human platelets. Following a 24 hour incubation migrated CMFDA-labeled MCF-7 cells on the bottom of the membrane were counted in 4 distinct fields of view per well.

Patient Samples

Blood was collected from patients taking adjuvant tamoxifien therapy for no less than one month as treatment for breast cancer. Patients taking platelet inhibitors were excluded from this study. Consent was obtained from all donors and samples were collected in accordance with Dana-Farber Cancer Institute clinical trial 11-358. A total of 5 patient samples were obtained. Platelets were isolated as described above and

activated *ex vivo* by exposure to $3x10^6$/mL MCF-7 human breast tumor cells. The activation state of platelets following activation was determined by P-selectin antibody (BD Biosciences, San Jose, CA; Catalog No: 5555524) labeling on flow cytometry (BD Canto II, BD Biosciences).

Statistical Analysis

All statistical analyses were performed with GraphPad Prism. All data are representative of >3 separate experiments unless specifically noted. In all graphs, error bars represent the standard error of the mean (SEM) and were calculated using GraphPad Prism. Specific statistical tests used were ANOVA, paired and unpaired *t* tests as appropriate and p values < 0.05 were considered statistically significant.

1. Italiano JE, Jr., Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, Ryeom S, Folkman J, Klement GL. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood.* 2008;111(3):1227-1233.