Tamoxifen Directly Inhibits Platelet Angiogenic Potential and Platelet-Mediated Metastasis

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Objective—Platelets, which are mainly known for their role in hemostasis, are now known to play a crucial role in metastasis. Tamoxifen is a selective estrogen receptor modulator that is widely used for the treatment of breast cancer. Tamoxifen and its metabolites have been shown to directly impact platelet function, suggesting that this drug has additional mechanisms of action. The purpose of this study was to determine whether tamoxifen exerts antitumor effects through direct platelet inhibition.

Approach and Results—This study found that pretreatment with tamoxifen leads to a significant inhibition of platelet activation. Platelets exposed to tamoxifen released significantly lower amounts of proangiogenic regulator vascular endothelial growth factor. In vitro angiogenesis assays confirmed that tamoxifen pretreatment led to diminished capillary tube formation and decreased endothelial migration. Tamoxifen and its metabolite, 4-hydroxytamoxifen, also significantly inhibited the ability of platelets to promote metastasis in vitro. Using a membrane-based array, we identified several proteins associated with angiogenesis metastasis that were lower in activated releasate from tamoxifen-treated platelets, including angiogenin, chemokine (C-X-C motif) ligand 1, chemokine (C–C motif) ligand 5, epidermal growth factor, chemokine (C-X-C motif) ligand 5, platelet-derived growth factor dimeric isoform BB, whereas antiangiogenic angiopoietin-1 was elevated. Platelets isolated from patients on tamoxifen maintenance therapy were also found to have decreased activation responses, diminished vascular endothelial growth factor release, and lower angiogenic and metastatic potential.

Conclusions—We demonstrate that tamoxifen and its metabolite 4-hydroxytamoxifen directly alter platelet function leading to decreased angiogenic and metastatic potential. Furthermore, this study supports the idea of utilizing targeted platelet therapies to inhibit the platelet’s role in angiogenesis and malignancy.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:664-674. DOI: 10.1161/ATVBAHA.116.308791.)

Key Words: blood platelets • neoplasm metastasis • platelet activation • tamoxifen

The role of platelets in malignancy is emerging as an important and attractive area of investigation. It has now been demonstrated that platelets are essential to all stages of primary tumor growth, as well as metastatic spread. Platelets aid disseminating tumor cells by protecting them from high shear forces and immune surveillance within the circulation, forming tumor cell-platelet aggregates that facilitate embolization, promoting adhesion of tumor cells to the vascular endothelium, and releasing a variety of soluble factors that promote tumor growth, metastasis, and angiogenesis. Platelets carry a plethora of angiogenic factors within their α-granules, including the potent proangiogenic protein vascular endothelial growth factor (VEGF). In fact, platelets seem to be the main storage site for angiogenic proteins; 80% of circulating VEGF is stored in platelet α-granules. Previously, our group and others have demonstrated that platelets differentially release pro- and antiangiogenic factors, such as VEGF and endostatin in an agonist-dependent manner. Platelets are also activated on exposure to breast tumor cells, and this leads to the release of potent proangiogenic mediators. In breast cancer in particular, angiogenesis is key to tumor growth and metastasis, including ductal carcinoma in situ. Overexpression of angiogenic factors, such as VEGF, in breast tumor tissue is associated with poor clinical outcome and lower response to chemotherapeutic and hormonal-based treatment regimens. Recently, we have demonstrated that intervention with aspirin and anticoagulant drugs can significantly diminish the release of granule components from platelets. As such, the angiogenic potential of aspirin-treated platelets is inhibited in response to breast cancer tumor cells.

See accompanying editorial on page 611

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Tamoxifen is a selective estrogen receptor modulator that is used widely as antiestrogen therapy for breast cancer. Tamoxifen treatment is associated with a 50% reduction in the risk of invasive and noninvasive breast cancer in women who used the drug for at least 5 years. Interestingly, tamoxifen has demonstrated anticancer efficacy in estrogen-negative cancers, suggesting that this drug has additional mechanisms of action. Platelets express ER-α and ER-β (the receptors for estrogen), and a role for estrogen in platelet function has been investigated previously. Some studies have suggested that tamoxifen may stimulate platelet aggregation in vitro and in vivo, however, more recent data support an inhibitory role in platelet activation. Mechanistically, Chang et al have recently demonstrated that tamoxifen inhibits platelet activation through the inhibition of PLC-2-PKC-p38 signaling. Although the impact of tamoxifen on platelets has been examined in the context of cardiovascular disease, to date no studies have investigated the effect of platelet inhibition by tamoxifen on breast cancer angiogenesis or metastasis.

In this article, we aim to demonstrate that tamoxifen and its metabolites significantly diminish the release of platelet angiogenic factors and metastatic factors, leading to decreased tumor cell support. The mechanism is directly linked to the inhibitory role of tamoxifen in platelet activation, causing altered release of key angiogenic and metastatic factors during tumor cell and platelet crosstalk.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Patients on tamoxifen therapy release less VEGF and have diminished platelet angiogenic potential.

Previous work from our group has shown that drugs that inhibit platelet function, such as aspirin and anticoagulants, can disrupt proangiogenic platelet-tumor cell crosstalk. Recent in vitro studies have shown that tamoxifen can directly alter platelet function; therefore, we hypothesized that tamoxifen therapy may augment the proangiogenic response of platelets to tumor cells. Mechanically, Chang et al have recently demonstrated that tamoxifen inhibits platelet activation through the inhibition of PLC-2-PKC-p38 signaling. Although the impact of tamoxifen on platelets has been examined in the context of cardiovascular disease, to date no studies have investigated the effect of platelet inhibition by tamoxifen on breast cancer angiogenesis or metastasis.

To further examine the effect of tamoxifen on platelet activation, we pretreated platelets from healthy human donors with tamoxifen before activation by MCF-7 cells (Figure 1C). To determine whether tamoxifen treatment alone had no effect on P-selectin expression in resting platelets (Figure 2A). However, pretreatment of platelets with tamoxifen caused a dose-dependent decrease in ADP-induced activation (Figure 2B). Tamoxifen did not alter activation in response to the strong agonist TRAP at either concentration examined. Importantly, pretreatment of platelets with tamoxifen significantly reduced MCF-7–induced activation. Tamoxifen treatment alone had no effect on P-selectin expression in resting platelets (Figure 2C). Representative histograms showing P-selectin expression are depicted in Figure 2B. These results confirm that tamoxifen directly and dose-dependently inhibits tumor cell–induced platelet activation, disrupting platelet-tumor cell crosstalk.

Tamoxifen Directly Inhibits Angiogenic Potential of Tumor Cell–Activated Platelets
Next, we sought to further examine the effect of tamoxifen on the angiogenic potential of tumor cell–activated platelets that we observed in our patient cohort. We pretreated platelets with tamoxifen before activation by MCF-7 cells and measured the release of VEGF (Figure 3A). Activation with MCF-7 cells increased the release of proangiogenic VEGF, whereas tamoxifen pretreatment reduced VEGF release to baseline levels (Figure 3B).
to determine whether tamoxifen could directly alter the net angiogenic effect of platelets. We have previously shown that releasate from platelets activated by MCF-7 cells increases endothelial cell migration and capillary tube formation. Here, we pretreated platelets with tamoxifen (10 or 20 μmol/L) before activation with MCF-7 cells and tested the angiogenic potential of the resulting releasate using functional angiogenesis assays (Figure 3A, schematic). We also tested the angiogenic potential of these releasates in capillary tube formation assays. MCF-7–activated platelet releasate induced increased capillary tube formation compared with releasate from resting, unactivated (resting) platelets (Figure 3C and 3D). Releasates from platelets pretreated with tamoxifen before activation induced significantly less capillary tube formation after 6 hours compared with controls (Figure 3D). Endothelial migration, a critical step in angiogenesis, was sharply and significantly increased in response to releasate from MCF-7–activated platelets (Figure 3E and 3F). However, no significant increase in endothelial cell migration was observed when platelets were treated with tamoxifen before MCF-7 activation (Figure 3E and 3F). These results suggest that tamoxifen lowers the net angiogenic potential of platelets and that this may be one of the ways by which tamoxifen works to control tumor growth.

Tamoxifen Decreases the Metastatic Potential of Platelets

Because platelets are also known to have prometastatic effects in breast cancer, we next examined the effect of tamoxifen on platelet-mediated metastasis. To test this, we examined the effect of tamoxifen on the metastatic potential of platelets using standard in vitro metastasis assays: tumor cell invasion and transendothelial migration. For invasion assays, human platelets were pretreated with 20 μmol/L tamoxifen or dimethyl sulfoxide vehicle control, washed, and activated with MCF-7 tumor cells to generate a releasate. We measured the effect of these releasates on MCF-7 tumor cell invasion through matrix gel and found that activated platelet releasate increased MCF-7 invasion compared with resting releasate (Figure 4A and 4B). Pretreatment with tamoxifen diminished the ability of activated releasates to promote MCF-7 invasion by 76% (Figure 4A and 4B). Transendothelial migration, or the ability of tumor cells to cross an endothelial barrier, is a critical step in the metastatic process. Platelets can aid tumor cells in this process, as demonstrated by the ability of live platelets to increase the migration of MCF-7 tumor cells across an endothelialized membrane by 2.6-fold over platelet-free control (Figure 4C). In contrast, tamoxifen pretreatment completely prevented platelets from promoting the transendothelial migration of tumor cells (Figure 4C).
Tamoxifen Metabolite 4-OH Inhibits Platelet Activation and VEGF Release

Tamoxifen is a prodrug and is metabolized into more active forms in the body. Therefore, we hypothesized that a metabolite may contribute to the dramatic results seen in patient samples (Figure 1). 4-Hydroxytamoxifen (4-OH), a tamoxifen metabolite found in the blood, has been shown to inhibit platelets activation and aggregation. Therefore, we tested whether 4-OH could also inhibit platelet-mediated angiogenesis and metastasis. Platelets from healthy human donors were pretreated with 25 or 50 μmol/L of 4-OH or vehicle control, washed to remove the drug, and then activated by various agonists. Unlike tamoxifen, 4-OH significantly and dose dependently inhibited platelet activation in response to the strong agonist TRAP (Figure 5A). 4-OH also lowered activation in response to MCF-7 tumor cells; however, this reduction did not reach statistical significance (Figure 5A). More significant inhibition was, however, achieved in response to the aggressive, triple negative breast cancer cell line MDA-MB-231. VEGF release also trended toward a reduction with 4-OH (Figure 5B), prompting us to look for other angiogenic factors through angiogenic protein array studies as shown in Figure 5C through 5E. The robust response, particularly against the strong agonist TRAP and the aggressive MDA-231 cells, suggests that the 4-OH metabolite of tamoxifen has potent effects on platelets. Therefore, we further characterized the effect of 4-OH on platelet-mediated angiogenesis and metastasis.

Tamoxifen Metabolite 4-OH Dampens Release of Proangiogenic and Metastatic Factors

To further characterize how 4-OH alters the release of platelet factors, we performed a membrane-based array (C1000; RayBiotech) to simultaneously detect 43 proteins known to be involved in angiogenesis and cancer. Releasates generated from MCF-7 or TRAP-activated platelets after treatment with 50 μmol/L 4-OH or vehicle control were compared by membrane-based array. Proteins that showed a 1.5-fold or greater change in response to tamoxifen or 4-OH are reported (Figure 5C through 5E). We found that 4-OH inhibited the release of key regulators of angiogenesis and metastasis, including angiogenin, CCL5.
Figure 3. Tamoxifen decreases the angiogenic potential of platelets. Platelets were pretreated with 0, 10, or 20 μmol/L tamoxifen, washed, and activated with MCF-7 tumor cells or left unactivated (resting) to generate releasates (A). Vascular endothelial growth factor (VEGF) was quantified in releasates by ELISA (B). Capillary tube formation in human umbilical vein endothelial cells (HUVECs) was assessed after 6 hours of exposure to platelet releasates and quantified as the average number of branch points per field of view (D), with representative images shown (C). Endothelial migration in the presence of resting or MCF-7–activated releasates generated from tamoxifen (20 μmol/L) or control-treated platelets was quantified (D and E). Representative images are shown (E), and data from all replicates are quantified as the average number of migrated HUVECs per field (D). Bars indicate SEM. F, indicates **P<0.01 and n.s. is not significant. *P<0.05, **P<0.01 by ANOVA, n=3 independent replicates per treatment group. Scale bars represent 100 μm.
Tamoxifen Metabolite 4-OH Decreases Platelet Angiogenic and Metastatic Potential

The tamoxifen metabolite 4-OH caused potent inhibition of activation and protein release above and beyond what we observed with tamoxifen. Therefore, we next examined the impact of 4-OH on the net angiogenic and metastatic effects of platelets. Platelets were pretreated with 4-OH, washed, and activated with tumor cells or TRAP to generate releasates. Both TRAP and tumor cell–activated platelet releasates significantly increased capillary tube formation over releasate from resting platelets (Figure 6A and 6B). Similar to what we observed with tamoxifen, 4-OH pretreatment abolished the increase in capillary tube formation caused by activated platelet releasate (Figure 6A and 6B). Metastatic potential was also diminished by 4-OH; activated platelet releasates promoted the invasion of MCF-7 tumor cells through matrigel, whereas 4-OH pretreatment abrogated this effect, returning invasion to baseline (Figure 6C and 6D).

Overall, our work suggests that tamoxifen and its 4-OH metabolite directly alter platelet function, leading to dampened activation responses and an alteration in angiogenic and metastatic protein release. The net effect leads to platelets that have significantly reduced angiogenic and metastatic effects in response to breast tumor cells (Figure 6E, model). Translational studies using platelets isolated from patients undergoing adjuvant tamoxifen therapy confirm that therapeutic, systemic tamoxifen use leads to platelet inhibition and lower net angiogenic potential.

Discussion

Previous studies have shown that tamoxifen can directly alter platelet function and that tamoxifen use may impact the role of platelets in thrombosis and cardiovascular disease.19,21–23 Although platelets have a well-established role in cancer progression and metastasis to date, this study is the first to examine the effect of tamoxifen on platelet function in the context of breast cancer, the disease for which tamoxifen is most widely used. Overall, our studies reveal that tamoxifen directly inhibits tumor cell–induced platelet activation and substantially dampens the proangiogenic and prometastatic effects of platelets.

The existing body of literature on the effect of tamoxifen on platelets is somewhat contradictory, with a few studies showing platelet activation or aggregation in response to tamoxifen.19
However, the majority of recent studies report inhibition of platelet activation; the discrepancy between studies could be because of doses, agonists used, or aspect of platelet function analyzed. Serum levels of tamoxifen and its metabolites have been reported to be highly variable, with up to a 10-fold difference detected among patients on comparable doses. Interestingly, Kisanga et al. reported that breast tumor tissue contains significantly higher concentrations of tamoxifen.
Figure 6. Tamoxifen metabolite 4-hydroxytamoxifen inhibits platelet angiogenic and metastatic potential. Releasates from platelets treated with 50 μmol/L 4-hydroxytamoxifen (4-OH) or vehicle control were assayed for angiogenic potential using capillary tube formation assays. Capillary tube formation was quantified as the average number of branch points per field of view (B), with representative images shown (A). To determine the metastatic potential of 4-OH–treated platelets, releasates were generated as previously described and used in standard transwell invasion assays. The ability of MCF-7 tumor cells to invade through Matrigel in response to platelet releasates was quantified (C) and representative images are shown (D). Model: tamoxifen and its metabolites directly inhibit platelet activation in response to breast tumor cells, leading to a decreased release of tumor-supporting proangiogenic and prometastatic factors from platelets (E). Bars indicate SEM. **P<0.01, ***P<0.001 by ANOVA, n=3 independent replicates per treatment group.
compared with serum. It is well established that platelets interact with tumor cells in both the blood and the tumor tissue and they may, therefore, encounter different doses of tamoxifen based on location. Although these findings make it challenging to directly compare in vitro and in vivo studies, we have demonstrated decreased activation and angiogenic potential in platelets treated with tamoxifen ex vivo and more importantly in platelets isolated from patients on tamoxifen therapy.

The exact mechanism by which tamoxifen and its metabolites impact function has not been fully elucidated, but some key studies provide insight. Platelets express estrogen receptors $\alpha$ and $\beta$, but it remains unclear whether tamoxifen exerts its effects on platelets through these receptors.23 Of note, use of estrogen receptor blockers, such as ICI 182.780, do not reverse the effects of tamoxifen, suggesting that the effects of tamoxifen on platelet inhibition are not mediated directly through the estrogen receptor.21,22 Mechanistically, Chang et al21 have demonstrated that tamoxifen inhibits the PKC pathway via PLC$\gamma$ as well as the p38 MAPK pathway in platelets. Interestingly, this group and others have shown that tamoxifen also causes a rise in platelet intracellular calcium, which would suggest a proactivation effect; however, abrogation of downstream pathways, such as PKC or cAMP production, could explain inhibition in the presence of elevated calcium.21,22 Our results are in line with the majority of studies, confirming that tamoxifen and 4-OH inhibit platelet activation. Furthermore, we went on to show that tamoxifen and 4-OH also specifically inhibit platelet activation in response to breast tumor cells.

In addition to inhibiting activation, tamoxifen altered the release of angiogenic mediators from platelets. We found that tamoxifen inhibited the release of VEGF. This finding is supported by the work of Holmes et al21 who reported altered levels of VEGF in patients on tamoxifen therapy. Furthermore, our array results show that 4-OH inhibits the release of other proangiogenic factors, including angiogenin and PDGF. Our array results also detected elevated angiopoietin-1 in releasates from tamoxifen and 4-OH–treated platelets. Angiopoietin-1 is primarily involved in vessel stabilization and has a well-known antiangiogenic role in cancer.22,23 Overall, these changes could lead to a platelet releasate with enhanced antiangiogenic properties. Indeed, releasates from tamoxifen-treated platelets had a dramatically reduced net angiogenic potential in functional assays. Because angiogenesis is critical for breast cancer progression and platelets are a main source of angiogenic regulators, including VEGF, these results strongly suggest that tamoxifen may improve breast cancer outcomes by limiting the proangiogenic effects of platelets.

The importance of platelets in cancer progression and metastasis is now widely appreciated. In this study, we found that tamoxifen pretreatment potently inhibited the ability of platelets to promote metastasis in vitro. Platelets support metastasis through direct, paracrine effects on tumor cells that have been shown to enhance tumor cell migration, invasion, and epithelial to mesenchymal transition. Platelets also support metastasis by exerting effects on other cells in the tumor microenvironment, such as endothelial cells and cells of the immune system. Our array results identified decreased levels of CXCL1, CCL5, EGF, and CXCL5 in tumor cell–activated releasate from 4-OH–treated platelets. These proteins are all known to play a role in cancer progression and metastasis.27–41 Future studies are needed to examine these factors individually in the context of tamoxifen and look at how tamoxifen repurposes platelets in ways that limit cancer progression.

Recently, interest in antiplatelet agents as cancer therapeutics has grown. GPIIb/IIIa blockers and the P2Y12 antagonist clopidogrel show antitumor and antimetastasis properties in vitro and in murine models of cancer.42,43 Anticoagulants, including low-molecular-weight heparins and fondaparinux, inhibit tumor cell–induced platelet activation and attenuate the angiogenic potential of platelets in vitro.14 Aspirin is perhaps the most intriguing antiplatelet agent that has been studied to date, and several epidemiological studies have suggested that individuals who take aspirin daily are less likely to be diagnosed with cancer and show improved survival if they do develop cancer.44 The randomized phase III ABC (Aspirin for Breast Cancer) Trial will prospectively study whether adjuvant aspirin can reduce the risk of breast cancer recurrence. Tamoxifen, a selective estrogen receptor modulator, is predominantly indicated for the treatment of hormone receptor–positive cancers; however, our findings build on a growing body of evidence, demonstrating that tamoxifen also has direct antiplatelet activity. This report highlights the need to examine the efficacy of tamoxifen in estrogen receptor–negative cancers as an antiplatelet agent and supports the exploration of antiplatelet agents as cancer fighting therapeutics.

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**Disclosures**

J.E.I. has financial interest in and is a founder of Platelet BioGenesis, a company that aims to produce donor-independent human platelets from human-induced pluripotent stem cells at scale. The interests of J.E.I. were reviewed and are managed by the Brigham and Women’s Hospital and Partners HealthCare in accordance with their conflict-of-interest policies. The other authors report no conflicts.

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**Highlights**

- Breast cancer patients undergoing adjuvant tamoxifen therapy exhibit platelet inhibition and lower net angiogenic potential.
- Tamoxifen and its metabolite directly inhibit platelet activation and alter the release of angiogenic and prometastatic factors.
- Tamoxifen and its metabolite directly diminish platelet net angiogenic and metastatic potential.
Tamoxifen Directly Inhibits Platelet Angiogenic Potential and Platelet-Mediated Metastasis

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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. 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 37°C. 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) 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 10^6/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 (Siemens, 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. MCF7 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.