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14. ABSTRACT Platelets are circulating reservoirs of proteins that both promote and inhibit angiogenesis and metastasis. The release of proteins in the tumor microenvironment can be accomplished by multiple different pathways. We determined the influence of key platelet activation pathways on angiogenesis using an endothelial cell migration assay. We found independent pathways of platelet activation resulted in differential effects on angiogenesis. Activation through the platelet collagen receptor and proteinase activated receptor-4 (PAR4) receptor resulted in significantly increased endothelial cell migration while proteinase activated receptor-1 (PAR1) receptor stimulation did not increase endothelial cell migration. The endothelial cell migration effects did not correlate directly with the amount of platelet VEGF release suggesting additional platelet proteins are important in the release reaction and subsequent angiogenesis effects. Our results suggest targeted inhibition of collagen and PAR4 receptor mediated platelet activation may decrease angiogenesis in the tumor microenvironment. Our work identifies potential new targets for angiogenesis and cancer control in cells not previously targeted in breast cancer therapy. The ability to manipulate and control a large, circulating pool of angiogenesis and metastasis modulating proteins may represent a significant step forward in breast cancer control.					
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Introduction

Platelets are circulating reservoirs of proteins that both promote and inhibit angiogenesis and metastasis. The release of proteins in the tumor microenvironment can be accomplished by multiple different pathways related to tumor procoagulant effects. We determined the influence of key platelet activation pathways on angiogenesis as measured by a HUVEC (human umbilical vein endothelial cell) migration assay. Consistent with our original hypothesis, we found independent pathways of platelet activation resulted in differential effects on angiogenesis. Under conditions of maximal stimulation, activation through the platelet collagen receptor using convulxin resulted in significantly increased endothelial cell migration as compared to control. Proteinase activated receptor-4 (PAR4) receptor stimulation similarly increased migration but to a lesser extent than seen with convulxin. Proteinase activated receptor-1 (PAR1) receptor stimulation did not increase endothelial cell migration and thrombin decreased migration due to non-platelet receptor based effects of the enzyme. The endothelial cell migration effects seen did not correlate directly with the amount of VEGF released from the platelet suggesting additional platelet proteins are important in the release reaction and subsequent angiogenesis effects. Work continues in the laboratory to determine if, similar to angiogenesis, collagen and PAR4 receptor stimulation increase breast cancer metastasis in cellular based assays. Our results suggest targeted inhibition of collagen and PAR4 receptor mediated platelet activation may effectively decrease angiogenesis in the tumor microenvironment. Our work has identified potential new targets for angiogenesis and cancer control in cells not previously targeted in breast cancer therapy. The ability to manipulate and control a large, circulating pool of angiogenesis and metastasis modulating proteins may represent a significant step forward in breast cancer control.

Body

Angiogenesis is critical for the growth and metastasis of tumors and the inhibition of angiogenesis is now validated as a viable anti-cancer strategy. Angiogenesis is controlled in part by intratumoral mechanisms; however, complex interactions between tumor cells, surrounding stroma, blood vessel wall and circulating blood cells are also required. Platelets may contribute to angiogenesis in part, through the processes of platelet activation and protein release.

In circulation, platelets uptake and store proteins that regulate angiogenesis. For example, platelets are the major serum source of the potent pro-angiogenic protein, vascular endothelial growth factor (VEGF) (1), a secreted protein that induces angiogenesis (2, 3). Importantly, the platelet pool comprises greater than 80% of total circulating VEGF in patients with cancer as well as healthy individuals (4). Platelets also contain proteins that inhibit angiogenesis, including endostatin, a C-terminal fragment of collagen XVIII (5-7). Release of angiogenic proteins from platelets has been studied following thrombin mediated platelet activation (8-12). The differential release of VEGF and endostatin from platelets based on the selective engagement of the thrombin receptors PAR-1 and PAR-4 was recently demonstrated (7). This observation suggested that stimulation of specific platelet receptors could lead to selective protein release. The release of distinct alpha-granules that selectively sequester proteins has been recently supported in the literature (6).

Multiple potential mechanisms of platelet activation are available in the tumor microenvironment. The relative importance of each pathway has not been fully ascertained and early studies suggest the mechanism of platelet activation by tumor cells may be tumor cell specific and, in some cases, mutually exclusive(13). Therefore, we undertook an investigation of the impact of agonist treated platelet releasate on angiogenesis.

Our statement of work outlined the following major objective: **To determine the influence of key platelet activation pathways on angiogenesis using a cellular model.** This was accomplished first by the establishment of a working endothelial cell migration assay in the laboratory using HUVECs (human umbilical vein endothelial cells). Our original proposal identified the source of platelets as isolated from baboon whole blood. Following an extensive investigation with commercial suppliers and US laboratory researchers, we determined this approach to be non-feasible in the allotted time period of the award. We therefore applied for and received approval from the U.S. Army Medical Research and Materiel Command's (USAMRMC) Office of Research Protections (ORP), Human Research Protection Office (HRPO) to use previously collected human blood platelet releasate samples.

Establishment of Endothelial Cell Migration Assay

Our laboratory first established a functional endothelial cell migration assay using human umbilical vein endothelial cells(HUVEC) HUVECs were obtained from Lonza (Walkersville, MD) and maintained in EGM-2 media supplemented with SingleQuot supplements and growth factors (Lonza) according to the manufacturers instructions. Cells were used at low passage numbers (< 5), corresponding to ≤ 15 doublings, as per the manufacturers instructions.

An assay to assess migration was based on a literature review of reported assay methods with slight modifications. In general, sub-confluent (60%-80%) monolayers were kept in EGM-2 basal

media (without growth factors and FBS) containing 0.1% delipidated BSA for 4-5 hours. Cells were then trypsinized and resuspended in basal medium, and cell viability was determined by trypan blue exclusion. 5×10^4 HUVECS in 0.25 mLs of medium were seeded in duplicate in the upper wells of fibronectin coated 24 –multiwell insert plates (3 micron pore size) (BD Biosciences). 0.75 mLs of platelet-derived supernatant was added to the bottom wells of the migration chamber, and the plates were kept in a humidified incubator at 37°C in 5% CO₂ for 22-24 hours. Following the incubation, non-migrating cells were removed from the upper surface of the membrane by scrubbing with a cleaning stick (Puritan), and fixed and stained using a Diff-Quik stain set (Siemens). To assess migration of HUVECs, ten fields/membrane were counted and the results expressed as # cells/ high-powered field. Control experiments to establish concentration dependent migrations using fetal bovine serum were completed. In addition, controls using agonists alone in the cell migration assay were performed to ensure no direct effect of the agonist on cell migration at the concentrations used for platelet activation.

An example of developmental experiments to assess the dependence of cell migration on releasate concentration is shown in figure 1 for the PAR4 receptor agonist, AYPGKF. An average increase in endothelial cell migration of 50-100 percent is ascribed to the platelet releasate as compared to no agonist controls.

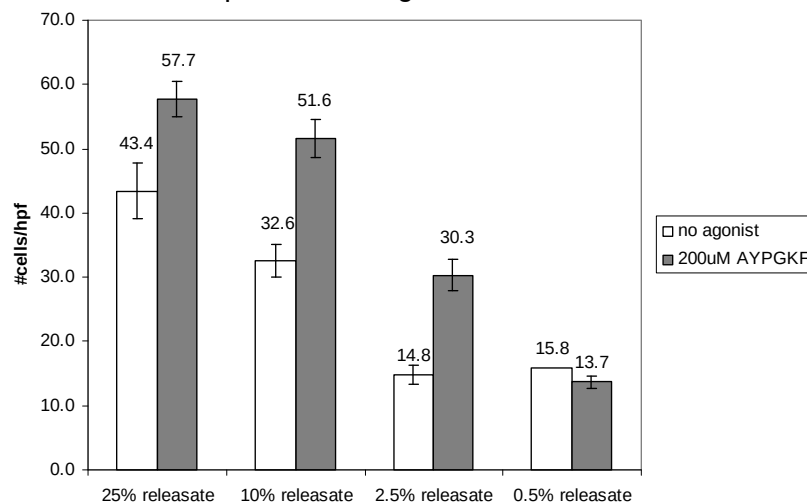


Figure 1. PAR4 receptor activated platelet releasate increases endothelial cell migration.

Endothelial cells were incubated with the releasate from washed platelets previously treated with maximal concentrations (200 μ M) of AYPGKF, a selective stimulator of the platelet PAR4 receptor. As compared to control (no activation of platelets), PAR4 stimulated platelets increased endothelial cell migration at all but the lowest concentration of releasate tested. Migration was found to increase based on the concentration of releasate used. $P < 0.05$ (as compared to no agonist) for 25%, 10%, 2.5% releasate concentrations.

Platelet Releasate Induction of HUVEC Migration is Agonist Dependent

Previously frozen human washed platelet samples were used to assess the impact of platelet activation using four agonists on endothelial cell migration. Agonist mediated activation of the washed platelet samples had been previously performed using concentrations of agonist that resulted in maximal p-selectin expression (a marker of alpha granule release). Titrations curves had established maximal and sub-maximal agonist concentrations. The selective proteinase activating receptor peptide, PAR 4AP (AYPGKF) and the selective PAR1 receptor agonist (SFLLRN) were used to selectively engage the two thrombin receptors, PAR1 and PAR4. The agonist thrombin was used to engage both the PAR1 and PAR4 receptor and is the naturally occurring *in vivo* PAR receptor activator. Convulxin selectively engages the collagen receptor to induce platelet activation. Whole platelets had been removed from the releasate using centrifugation prior to freezing and storage. The platelet agonists, lipidated tissue factor and ADP, were not pursued as originally outlined in our statement of work as no human platelet samples were available that used these agonists.

The impact of the four platelet agonists, thrombin, convulxin, PAR4AP and PAR1AP, on HUVEC migration following exposure to agonist stimulated platelet releasate was assessed. Collagen receptor stimulation resulted in maximal stimulation of endothelial cell migration at both releasate concentrations tested. As compared to endothelial cells exposed to supernatant from non-agonist treated platelets, collagen receptor stimulation resulted in a near doubling of migration under experimental conditions using 15% releasate. In comparison, the PAR 4 thrombin receptor agonist resulted in less migration than collagen receptor stimulation albeit migration remained significantly greater than the no agonist control. In contrast, maximal stimulation via the PAR1 receptor resulted in no significant increase or decrease in migration. Notably, the removal of platelet microparticles from the releasate did not impact the observed migration effects.

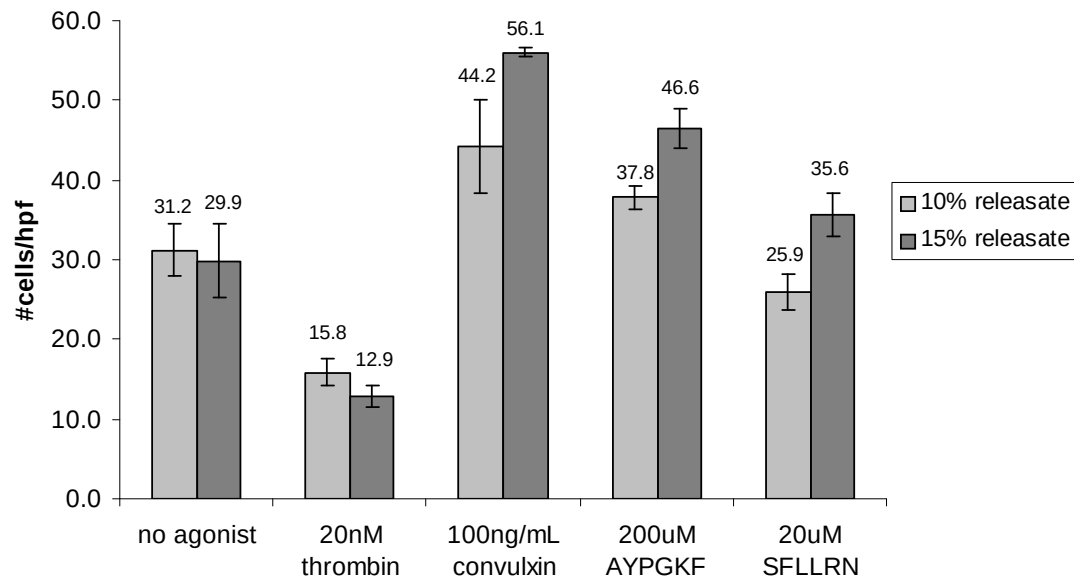


Figure 2. Agonist induced platelet releasate increases endothelial cell migration.

Endothelial cell migration was ascertained under 10% and 15% releasate conditions. Activation via the PAR1 platelet receptor (SFLLRN), the PAR4 platelet receptor (AYPGKF), the collagen platelet receptor (convulxin) and dual PAR1 and PAR4 receptor activation (thrombin) was assessed. The results represent N=3 healthy individuals. $P < 0.05$ as compared to no agonist control for thrombin, convulxin and AYPGFK (10% and 15% releasate conditions).

Platelet releasate following simultaneous activation of the PAR1 and PAR4 receptor using thrombin as an agonist resulted in a significant decrease in endothelial cell migration relative to no agonist controls. This result is in marked contrast to stimulation of either receptor alone. To further understand our observations, we treated releasate samples from PAR4AP stimulated platelets with 20 nM thrombin and assessed migration. In this series of experiments, PAR4AP mediated platelet releasate resulted in an average of 46 cells/high powered field while treatment of the PAR4AP sample resulted in 15 cells/hpf. In no agonist treated samples, 29 cells/hpf was noted. Thrombin addition resulted in a decrease in migration back to below baseline values suggesting the enzymatic properties of thrombin are degrading or modifying key enhancer(s) of endothelial cell migration that are released from the platelet. Notably, thrombin alone in the

assay (in the absence of platelet releasate) had no effect on endothelial cell migration. The pro-angiogenic protein target for the serine protease, thrombin, is currently not known but will be the focus of subsequent research proposals.

Our original hypothesis that different agonist stimulated platelet releasate would result in differing effects on endothelial cell migration was based on initial studies demonstrating VEGF release differed depending on the agonist used. Table 1 demonstrates the number of VEGF molecules released per 1000 agonist stimulated platelets for each agonist. Both thrombin and convulxin activation of platelets resulted in the largest numbers of VEGF molecules released. Only with the convulxin treated platelet releasate did this VEGF release translate into a significant increase in endothelial cell migration (as above). In contrast, while no increase in endothelial cell migration was seen for the selective PAR1 agonist, PAR1AP, the number of VEGF molecules released was essentially no different from the PAR4 selective releasate. These results suggest that while VEGF may be an important mediator of angiogenesis, the differential release of this molecule alone does not appear to explain the significant differences seen with these agonists in the endothelial cell migration assay.

	VEGF molecules/ 1000 platelets (mean (+/- SEM))	Table 1. Releasate concentrations of VEGF following agonist specific stimulation of platelet activation. Results are reported as number of VEGF molecules detected per 1000 platelets following platelet stimulation with the listed agonists at maximum concentration (as indicated). P<0.05 for all agonists tested as compared to no agonist control (N=3).
No agonist	622 (43)	
20 nM thrombin	16,673 (6029)	
100 ng/mL convulxin	15,536 (5412)	
200 µM AYPGKF	12,963 (4335)	
20 µM SFLLRN	13,168 (4457)	

Our statement of work also outlined the following major objective: **To determine the influence of key platelet activation pathways on breast cancer metastasis using a cellular invasion model.** Our work toward this aim was significantly limited due to time constraints. To date, we have established the assay in the laboratory; however, no results using platelet releasates are available to report. We will continue to pursue these studies despite completion of the formal grant period in order to compare the metastasis results with those we obtained using our assay of angiogenesis.

Key Research Accomplishments

- Established that the mechanism of platelet activation influences endothelial cell migration
- Validated our original hypothesis that each independent pathway of platelet activation uniquely impacts angiogenesis.
- Identified collagen receptor activation as the most significant inducer of endothelial cell migration as compared to thrombin receptor activation
- Determined that VEGF release alone does not account for observed differences in endothelial cell migration seen with different agonists.
- Established an endothelial cell migration assay in the laboratory.

- Established a breast cancer invasion assay in the laboratory (early stages of assay development only).

Reportable Outcomes

- Research results will be reported in a manuscript submission (not yet completed).
- Results will be used to apply for future Department of Defense and National Institutes of Health (R01) grants by the Principal Investigator

Conclusions

We have shown for the first time that independent pathways of platelet activation have differential effects on angiogenesis. This work supports the role of platelets in cancer progression and extends the field to suggest specific platelet pathways that might be targeted to decrease angiogenesis in the tumor microenvironment. This work focuses attention on a potential new target in breast cancer prevention and treatment – control of the ~3 trillion platelets found in every patient with breast cancer.

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