

Shear stress modulates the interaction of platelet-secreted matrix proteins with tumor cells through the integrin $\alpha_v\beta_3$

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Lawler, Karen, Gerardene Meade, Gerald O'Sullivan, and Dermot Kenny. Shear stress modulates the interaction of platelet-secreted matrix proteins with tumor cells through the integrin $\alpha_v\beta_3$. *Am J Physiol Cell Physiol* 287: C1320–C1327, 2004. First published July 7, 2004; doi:10.1152/ajpcell.00159.2004.—Interaction of tumor cells with the vascular wall is required for metastasis from the bloodstream. The precise interaction among metastatic cells, circulating platelets, the vessel wall, and physiological flow conditions remains to be determined. In this study, we investigated the interaction of shear on metastatic cell lines adherent to lipopolysaccharide (LPS)-treated endothelium. Tumor cells were perfused over LPS-treated human umbilical vein endothelial cells (HUVECs) at incremental venous shear rates from 50 to 800 s^{-1} . At a venous shear rate of 400 s^{-1} , 3% of adherent tumor cells formed pseudopodia under shear, a process we termed shear-induced activation. Because platelets promote tumor dissemination, we then investigated the effect of pretreating tumor cells with platelet releasate collected from activated platelet concentrate. We found that in the presence of platelet releasate, the number of tumor cells adhering to HUVECs increased and tumor “activation” occurred at a significantly lower shear rate of 50 s^{-1} . This was inhibited with acetylsalicylic acid. Depletion of fibronectin or vitronectin from the platelet releasate resulted in significantly less adhesion at higher venous shear rates of 600 and 800 s^{-1} . The integrin $\alpha_v\beta_3$ has been shown to mediate cell adhesion primarily through vitronectin and fibronectin proteins. Inhibition of $\alpha_v\beta_3$, followed by the addition of platelet releasate to the tumor cells, resulted in significantly less adhesion at higher venous shear rates of 600 and 800 s^{-1} . Collectively, our data suggest that $\alpha_v\beta_3$ promotes the metastatic phenotype of tumor cells through interactions with the secreted platelet proteins vitronectin and fibronectin under venous shear conditions.

platelet releasate; vitronectin; fibronectin

ESOPHAGEAL CANCER IS ONE of the least studied and deadliest cancers worldwide. At the time of diagnosis, >50% of patients have radiographically visible metastases (12). Metastases arise through a complex series of sequential steps involving a variety of tumor and host properties. A key step is emigration from the circulation. Metastasis occurs primarily in the venous circulation (20). This relatively low-shear venous environment facilitates the arrest of tumor cells to the vessel wall, eventually leading to penetration of the vascular wall (21). Previous experiments suggested that cancer cell binding to endothelium has a key role in metastases; however, these experiments were performed under static conditions (32). The interaction of metastatic cells with the endothelium under fluid flow conditions is not well

understood. Recent evidence suggests that tumor adherence occurring in the presence of shear stress is analogous to the manner in which platelets (6) and leukocytes tether, adhere, and roll (4).

In the present investigation, we studied a shear-specific effect on metastatic cells and further characterized the interaction of tumor cells, platelets, and inflamed endothelium. Successful cell arrest in the circulation is dependent on the balance between adhesive and antiadhesive forces and on the rate at which adhesive interactions are broken (33). The initial interaction between adherent platelets and inflamed endothelium was recently described (10). Inflamed endothelium in cancer patients has been shown to enhance metastasis formation (1). Thus we hypothesized a dynamic interaction between inflamed endothelium and flowing metastatic cells.

Platelet tumor interactions are important in metastasis dissemination. Experimental evidence published in the 1960s demonstrated that a decrease in platelet count reduced the spread of cancer (11). When platelets are activated, they secrete mitogenic and angiogenic factors that have been implicated in tumor spread (25). The effect of this secreted component, i.e., the platelet releasate on enhancing bone cell migration and recruitment *in vitro*, has only recently been appreciated (26). We therefore examined the effect of preincubating tumor cells with platelet releasate under venous shear conditions. We further examined the involvement of the platelet-cyclooxygenase pathway in the platelet releasate by using the antiplatelet agent aspirin [acetylsalicylic acid (ASA)]. Platelet releasate potentiated the response of tumor cells to shear, while pretreating the tumor cells with platelet releasate from aspirinated blood abrogated this enhanced response.

Integrins support tumor cell arrest during blood flow (28). To identify the role of specific integrins in this process, we further characterized the effect of shear on tumor cell adhesion to matrices used by integrins. Our results show that the platelet releasate mediates its effects partially through the integrin $\alpha_v\beta_3$. Furthermore, depletion of vitronectin or fibronectin from the platelet releasate decreases the enhanced tumor response observed in the presence of platelet releasate.

In summary, we demonstrate a novel protein interaction between tumor cells expressing $\alpha_v\beta_3$ and platelet-secreted vitronectin and fibronectin under venous shear conditions. Our findings suggest that therapies targeted at specific integrins in a shear environment may prevent tumor cell spread from the circulation, thereby controlling metastatic spread.

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EXPERIMENTAL PROCEDURES

Chemicals and materials. The following materials were purchased from Sigma-Aldrich (Poole, UK): nitrocellulose membranes, ASA, phosphate-buffered saline (PBS), lipopolysaccharide (LPS), U-46619, thrombin, vitronectin (Vn), collagen I, fibrinogen, fibronectin (Fn), collagen IV, and bovine serum albumin (BSA). Filter concentrators (1 kDa) were obtained from Pall Life Sciences (Portsmouth, UK). The SuperSignal enhanced chemiluminescence (ECL) kit was purchased from Pierce (Rockford, IL). Culture dishes (35 mm) were obtained from Corning (Corning, NY). SQ-29548 was purchased from Cayman Chemicals (Ann Arbor, MI). Protein G Sepharose beads were purchased from Amersham Biosciences (Piscataway, NJ). Phalloidin 488 was obtained from Molecular Probes Europe (Leiden, the Netherlands). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Paisley, UK).

Antibodies and cells. Monoclonal antibody against the integrin receptor $\alpha_v\beta_3$ (LM609) was obtained from Chemicon International (Temecula, CA). Monoclonal antibodies directed against Vn (clone VIT-2) and Fn (clone IST-4) were purchased from Sigma-Aldrich. Rib bone marrow micrometastases (RBM/E-3) were isolated from the rib bone marrow of a patient who underwent resection for primary esophageal cancer. Diagnostic and clinical investigations for staging of all patients included standards of care in accordance with guidelines of the human ethics committee for clinical research, National University of Ireland. Informed consent for surgery and bone aspiration was obtained in all cases. The cells were cultured from rib bone marrow cells (26). Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described (14). MCF-7, Hs578T, and HuT cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD) and cultured as previously described (7, 12, 15).

Flow assay. The behavior of RBM/E-3 cells under physiologically relevant shear conditions was assayed using a modified parallel plate flow chamber assembly described by Lawrence et al. (22). The flow chamber was purchased from GlycoTech (Rockville, MD) and consisted of a flow deck and silicon rubber gasket, with the flow path defined precisely by gasket thickness and gasket flow path width. HUVECs were grown on 35-mm culture dishes (Corning, Corning, NY), which fit precisely within the flow chambers. Fifteen hours before performing the flow assay, HUVECs were pretreated with LPS (10 $\mu\text{g}/\text{ml}$) to upregulate the expression of adhesive proteins (30).

RBM/E-3 ($1 \times 10^6/\text{ml}$) were suspended in PBS, prewarmed to 37°C, and kept at this temperature throughout the experiments. RBM/E-3 cells were injected via a side port into the flow path and allowed to settle on LPS-stimulated HUVECs in the parallel plate flow chamber for 5 min before being exposed to flow. The chamber was then perfused with PBS at a venous shear rate of 50 s^{-1} for 1 min using a syringe pump (Harvard Apparatus, Holliston, MA). The cells were visualized in bright field with phase-contrast microscopy ($\times 40$ and $\times 63$ LD-Acroplan objectives) using a Zeiss Axiovert-200 epifluorescence microscope (Carl Zeiss, Gottingen, Germany).

As soon as flow was initiated, images were captured every 200 ms up to 1 min with a liquid-chilled Quantix-57 charge-coupled device camera (Photometrics, Tucson, AZ), resulting in 300 frames. Flow was then stopped for 5 min to analyze tumor adhesion. After the 5-min interval, the shear rate was increased incrementally to 100, 200, 400, 600, and 800 s^{-1} , with 5-min intervals of no flow. This resulted in six 1-min movies, each corresponding to an individual shear rate. Please refer to the Supplementary Material for this article to view renderings (Movies 1–3).¹ The interaction of the RBM/E-3 cells and HUVECs was analyzed offline using the commercial software package MetaMorph (version 4.6.8; Universal Imaging, Downingtown, PA). Flow

rates were calculated using the equation for laminar flow to give a venous shear rate between 50 and 800 s^{-1} . MCF-7, Hs578T, and HuT cells were also investigated as described above. In additional experiments, RBM/E-3 cells were incubated for 30 min with 70 μl of either platelet releasate or platelet releasate immunodepleted of Vn or Fn and then perfused over HUVECs as described above.

Isolation of platelet releasate. Gel-filtered platelets were prepared after being obtained from healthy volunteers who had not taken ASA for at least 10 days. Collection of blood was approved by The Royal College of Surgeons, Ireland, ethics committee, and informed consent was obtained from the volunteers. Blood was collected in acid-citrate-dextrose (ACD; in mM: 38 citric acid, 75 sodium citrate, 124 dextrose) as anticoagulant (0.15 vol/vol). The blood was centrifuged for 10 min at 150 g at room temperature. Platelet-rich plasma was then acidified to pH 6.5 with ACD. PGE₁ (1 μM) was added to prevent platelet activation. Platelets were pelleted by centrifugation at 720 g for 10 min, and then the supernatant was removed and the pellet was resuspended in *buffer A* (in mM: 130 NaCl, 10 trisodium citrate, 9 NaHCO₃, 6 dextrose, 0.9 MgCl₂, 0.81 KH₂PO₄, and 10 Tris, pH 7.4). The platelets were gel filtered using Sepharose B resin and supplemented with 1.8 mM CaCl₂. Gel-filtered platelets were stimulated with thrombin (0.1 U/ml) and collagen (0.19 mg/ml) and stirred for 15 min. The platelet aggregate was centrifuged at 720 g for 10 min, and the supernatant was aspirated and filtered through 0.22- μm -pore disks to remove platelet microparticles. The filtrate was then centrifuged at 6,000 g in 1-kDa cutoff filters for 12 h to concentrate the platelet releasate. For ASA-treated platelet releasate, whole blood was collected into ACD with ASA, and the releasate was collected.

Drug experiments. To assess the effects of ASA on the effects induced by platelet releasate, we added ASA (200 μM) directly to platelet releasate and preincubated this with RBM/E-3 cells or treated the tumor cells directly with ASA alone (200 μM). In separate experiments, we investigated the effects of adding a thromboxane antagonist (SQ-29548) to the platelet releasate with RBM/E-3 cells for 30 min or directly added a thromboxane mimetic (U-46619) to the tumor cells. The cells were then perfused over the HUVECs matrix as described above.

Tumor adhesion assays on matrix proteins. Glass coverslips were coated overnight with 10 $\mu\text{g}/\text{ml}$ of Vn, Fn, fibrinogen, and collagen type I or IV. RBM/E-3 cells were grown to $\sim 80\%$ confluence and then suspended in prewarmed 37°C DMEM at a concentration of 1×10^6 cells/ml. The flow chamber containing the matrix-coated glass coverslip was assembled. RBM/E-3 cells were injected into the flow chamber, allowed to settle on the matrix for 5 min, and then exposed to a continuous shear rate of 100, 200, or 400 s^{-1} for 1 h.

Blockade of $\alpha_v\beta_3$ in tumor cells. To investigate the role of $\alpha_v\beta_3$ in tumor adhesion under flow in the presence of platelet releasate, we pretreated RBM/E-3 cells with 10 $\mu\text{g}/\text{ml}$ of the antibody LM609 for 30 min before addition of 70 μl of platelet releasate. LM609 inhibits the binding of $\alpha_v\beta_3$ to matrix protein. The RBM/E-3 cells were then perfused over anti- $\alpha_v\beta_3$ -treated HUVECs at shear rates from 50 to 800 s^{-1} . The rates of adhesion and pseudopod formation were analyzed as described above.

Immunodepletion of vitronectin and fibronectin from platelet releasate. Immunoprecipitation was performed with monoclonal anti-Vn, anti-Fn, or anti-IgG isotype antibodies. Immunoprecipitates were recovered with protein G-Sepharose beads. Immunodepleted platelet releasates and immunoprecipitates were separated by performing 7.5% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with the above antibodies. The protein-antibody complexes were then visualized with a horseradish peroxidase-linked α -mouse secondary antibody and SuperSignal ECL reagents.

Effect of shear on $\alpha_v\beta_3$ clustering in tumor cells. Glass slides were coated with Vn (100 $\mu\text{g}/\text{ml}$) for 2 h at room temperature, blocked in 1% BSA in PBS for 1 h, and then washed three times with PBS before use. RBM/E-3 cells were suspended in serum-free DMEM at a concentration of 1×10^6 cells/ml. The tumor cell suspension (400 μl)

¹ Supplemental data for this article may be found at <http://ajpcell.physiology.org/cgi/content/full/00159.2004/DC1/>.

was added to each slide and incubated for 1 h at room temperature. In separate experiments, the tumor cells were allowed to adhere to Vn for 30 min and then exposed to a continuous shear rate of 400 s^{-1} for 30 min at 37°C . Both sheared and nonsheared adherent tumor cells were washed three times and fixed in 3.7% formaldehyde for 15 min at room temperature. Slides were incubated with $50 \mu\text{l}$ of LM609 ($5 \mu\text{g/ml}$) for 1 h at room temperature and then washed and incubated with a secondary goat anti-mouse IgG antibody for 10 min. The cells were then permeabilized with ice-cold acetone for 2 min. Phalloidin 488 was added at a concentration of $5 \mu\text{g/ml}$, incubated at room temperature for 20 min to stain actin, washed, and incubated with a secondary antibody for 10 min. The cells were then washed and mounted using Dako fluorescent mounting medium. Slides were analyzed using a LSM510 Axioplan 2 upright confocal microscope (Carl Zeiss). Rhodamine fluorescence was detected at 546 nm, and FITC was detected at 488 nm.

Statistical analysis. Numerical values are means \pm SD. Data were analyzed by unpaired *t*-tests or by one-way ANOVA.

RESULTS

Venous shear activated tumor cells. To simulate the fluid mechanical environment of the venous circulation, RBM/E-3 cells were perfused over confluent monolayers of HUVECs stimulated with LPS at venous shear rates of $50\text{--}800 \text{ s}^{-1}$. The tumor cells were allowed to settle on the inflamed endothelium for 5 min, and then perfusion was initiated. We and others have documented that a brief period of minutes facilitates interactions between receptors and their ligands under various shear conditions (29). We examined the flow chamber area where tumor cells were adhering to HUVECs, and a representative $170 \times 180\text{-}\mu\text{m}$ section was visualized. The resting tumor cells were then exposed to a shear rate of 50 s^{-1} for 1 min, which was digitally recorded at an interval of 200 ms/frame, corresponding to a 1-min movie sequence. Flow was then discontinued for 5 min. Adherent cells were further subjected to incremental shear rates for 1-min intervals and digitally recorded. The 1-min movie sequences were then reviewed for cell adherence, cell rolling, and structural morphology differences at varying shear rates.

The patterns of adherence for shear rates of $50, 100,$ and 200 s^{-1} were similar to those on the total chamber area, where ~ 490 cells remained adherent. The number of adherent cells decreased as the shear rates were increased from 400 to 600 s^{-1} . A significant decrease in tumor cell adhesion occurred at 600 s^{-1} (Fig. 1A), with only 12% of the initial adherent cell population remaining adherent ($P = 0.001$). Increasing the shear rate to 800 s^{-1} did not affect this cell population. We observed some tumor cells clustering together at shear rates $< 400 \text{ s}^{-1}$, but at higher shear rates, only individual cells remained adherent to the endothelium.

Under venous flow conditions, adherent metastatic cells remained quiescent until a shear rate of 400 s^{-1} was reached, corresponding to 20 min of adherence to the HUVECs. At 400 s^{-1} , a rapid morphological response occurred within 200 ms of the onset of shear (Movies 1 and 2). A significant number (3%) of the adherent cells rapidly formed pseudopodia ($P = 0.003$), which extended and retracted over the cell surface (Fig. 1B). We termed this response shear-induced activation, dependent on shear and time variables. A representative cell is shown in Fig. 2. Pseudopod formation was independent of the shear rate in that increased shear rates did not result in any greater degree of

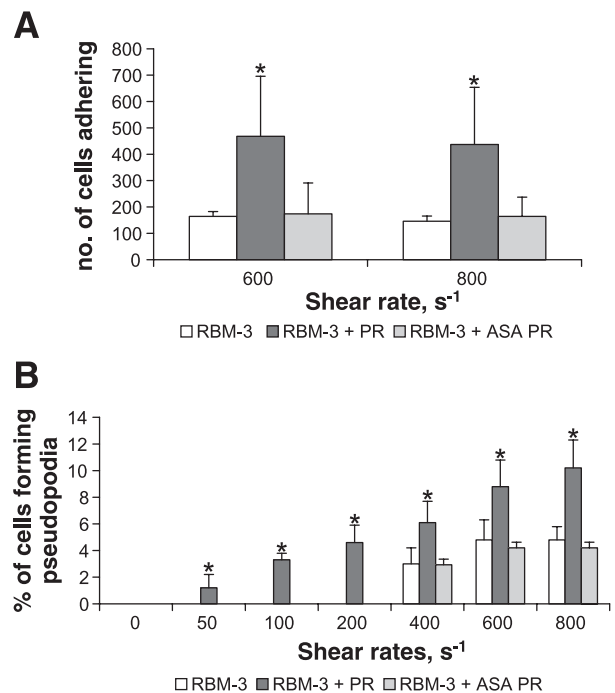


Fig. 1. Adhesion and shear-induced activation are enhanced by platelet releasate. Aspirin [acetylsalicylic acid (ASA)] abrogates the enhanced effects of platelet releasate (PR). RBM/E-3 cells were perfused across LPS-stimulated human umbilical vein endothelial cells (HUVECs) at incremental shear rates from 50 to 800 s^{-1} . The results shown in *A* represent the average number of untreated and PR-treated cells adhering at shear rates of 600 and 800 s^{-1} . There was significant increase in the number of PR-treated cells (RBM-3 + PR) adhering to HUVECs compared with untreated cells (RBM-3) or aspirin-PR-treated cells (RBM-3 + ASA PR) ($*P < 0.001$). *B*: percentages of cells actively forming pseudopodia at incremental shear rates ($n = 10$). RBM/E-3 cells activated at significantly lower shear rates in the presence of PR ($*P = 0.003, n = 6$), and this effect was abolished by aspirin treatment.

activation. Increasing shear rates $> 800 \text{ s}^{-1}$ did not result in greater retraction and formation of pseudopods (data not shown).

Pretreatment of RBM/E-3 cells with platelet releasate enhanced cell adhesion and shear-induced pseudopod formation. Metastatic cells were preincubated with the platelet releasate and assayed under the physiologically relevant shear conditions described above. A significant increase ($P = 0.0005$) in adhesion was observed at all shear rates compared with non-treated platelet releasate tumor cells (Fig. 1A). The number of adherent cells gradually decreased with increasing fluid shear up to 400 s^{-1} . At this point, exposure to the incremental shear rates resulted in ~ 490 cells remaining after 30 min, with 6% of these cells exhibiting activated pseudopodia. In contrast, the nonplatelet releasate-treated cells had ~ 160 adherent cells at the end of the assay, with only 3% of these cells forming pseudopodia. Therefore, addition of platelet releasate to tumor cells greatly enhanced cell adhesion to vascular endothelium.

The tumor cells in the presence of the platelet releasate exhibited pseudopodia at a significantly lower shear rate of 50 s^{-1} ($n = 6$) compared with the shear rate of 400 s^{-1} , which caused activation in the absence of the releasate. These results are shown in Fig. 1B. We also noted the formation of filopodia, i.e., cylindrical projections from the surface of the tumor cell. The percentage of adherent cells forming pseudopods is 1.2%

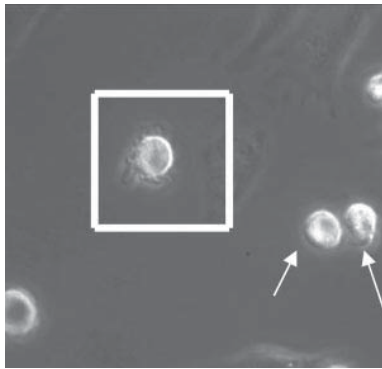


Fig. 2. RBM/E-3 cells activate in response to a shear rate of 400 s^{-1} . At a shear rate of 400 s^{-1} , a rapid morphological response occurred wherein 3% of adhering RBM/E-3 cells rapidly extended and retracted pseudopodia within 200 ms over the surface of the cell. A representative activated cell is shown within box at center. Quiescent cells are present at right and are indicated by arrows. Please refer to the Supplementary Material for this article (published online at the *American Journal of Physiology-Cell Physiology* web site) to view Movies 1–3.

at 0 s^{-1} , and this percentage progressively increased as shear rates strengthened, with a maximum response at 800 s^{-1} .

Platelet releasate from ASA-treated platelets abrogated the effects mediated by the platelet releasate in RBM/E-3 cells and was not due to thromboxane A_2 . High concentrations of thromboxane A_2 (TxA_2) and low concentrations of prostacyclin are observed in patients with cancer (16). ASA impairs TxA_2 synthesis through acetylation of platelet cyclooxygenase. Because we had found that the platelet releasate potentiated the activation of RBM/E-3 cells, we investigated the effect of platelet releasate from platelets pretreated with ASA ($200 \mu\text{M}$).

The potentiation of shear-induced RBM/E-3 cell activation by the platelet releasate was completely abolished by ASA treatment. The interaction of tumor cells with HUVECs decreased and displayed a pattern similar to that observed in RBM/E-3 cells in the absence of the releasate (Fig. 1A).

To further characterize the effect of ASA in the platelet releasate, we pretreated RBM/E-3 cells directly with ASA alone or with ASA added to platelet releasate. The addition of ASA alone or ASA added directly to platelet releasate did not result in significant differences in tumor activation compared with platelet releasate derived from aspirinated blood (data not shown).

We investigated whether thromboxane caused increased tumor activation upon addition of the platelet releasate. We used a thromboxane receptor antagonist, SQ-29548, and a thromboxane mimetic, U-46619, as described in EXPERIMENTAL PROCEDURES. Both rates of adhesion and pseudopod formation did not significantly change when the tumor cells were exposed to shear rates from 50 to 800 s^{-1} , confirming that TxA_2 was not involved in the process (data not shown).

Perfusion of MCF-7 and Hs578T cells over LPS-treated HUVECs resulted in shear-dependent pseudopod formation. We further characterized the behavior of other cell lines that frequently metastasize to bone. We tested the behavior of a metastatic breast cancer cell line (Hs578T) and a nonmetastatic breast cancer cell line (MCF-7) under venous shear conditions. The cells were injected at the same concentrations used for RBM/E-3 cells, allowed to settle on LPS-treated HUVECs for 5 min, and analyzed similarly for adhesion and pseudopod

formation. Both MCF-7 and Hs578T cells were more adhesive than RBM/E-3 tumor cells at all shear rates; however, no significant differences were detected (data not shown).

Shear sensitivity in HuT cells. To determine whether our shear response was a nonspecific response of metastatic cells, we examined the behavior of a cell line derived from circulating T cells (HuT cells). In these cells, we observed an entirely different response to shear. These cells formed a long-tailed pseudopodal structure at low venous shear rates of $50\text{--}100 \text{ s}^{-1}$. RBM/E-3 cells demonstrated a different morphology at shear rates of $\geq 400 \text{ s}^{-1}$. Increasing the shear rate to $>200 \text{ s}^{-1}$ resulted in rapid retraction of the tail in HuT cells (Fig. 3), demonstrating a shear-sensitive mechanism (Movie 3). As shear rates were increased to $>400 \text{ s}^{-1}$, fewer HuT cells adhered and no tail formation was observed.

Maximal RBM/E-3 adhesion and pseudopod formation occurred on Vn and Fn. To analyze the mechanism of tumor cell ligand binding during flow, we perfused RBM/E-3 cells over

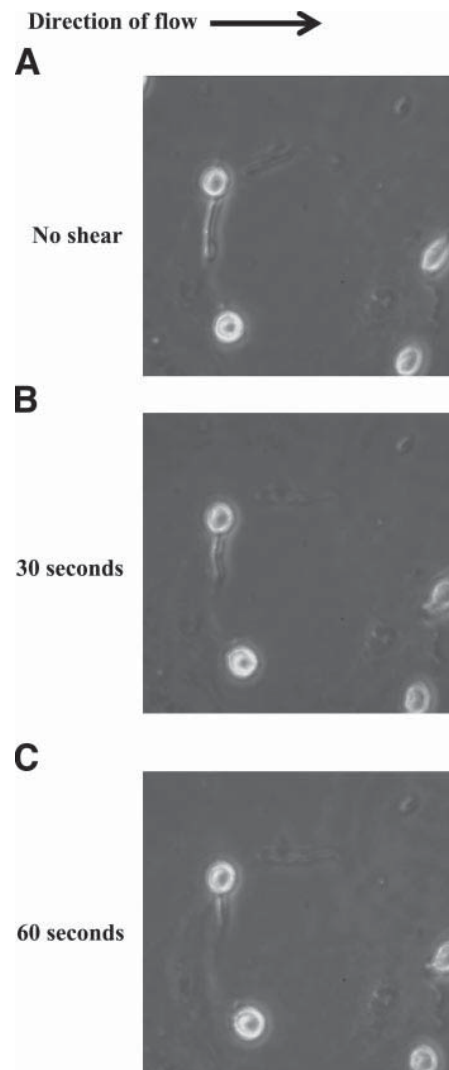


Fig. 3. The morphological response of HuT cells under shear stress is different in malignant cells. HuT cells were perfused across LPS-stimulated HUVECs. Between shear rates of 0 and 100 s^{-1} , a long, single pseudopodal structure formed (A, no shear). At a shear rate of 200 s^{-1} , rapid retraction of the tail occurred over a 1-min period (B, 30 s, C, 60 s).

the most abundant matrix proteins found in basement vascular membranes (collagen IV), human plasma (collagen I, fibrinogen, Fn, and Vn) and also those secreted by platelets (fibrinogen, Fn, and Vn). Glass slides were coated with matrix protein. RBM/E-3 cells were allowed to adhere to these matrices for 5 min and then were exposed to a continuous shear rate of 100, 200, or 400 s^{-1} for 1 h. The rates of adherence and morphological changes were analyzed. We observed a dramatic response in RBM/E-3 cells adherent to the matrices of Vn and Fn. (Fig. 4A). Collagen I, collagen IV, and fibrinogen resulted in low levels of tumor cell adherence with little or no spreading (Fig. 4B). However, on Fn and Vn, a significant increase ($P < 0.0001$) in tumor cell adherence and rapid tumor cell spreading was observed within minutes of adherence (Fig. 4B). The results indicate a rapid morphological change upon tumor cell ligation to Vn and Fn under venous flow conditions. In the absence of shear, adherent tumor cells were activated on Vn and Fn after a period of 40–50 min (results not shown), suggesting that shear forces influence the ability of tumor cells to adhere and activate rapidly upon ligation to different matrix proteins.

Platelet releasate mediated its effects through $\alpha_v\beta_3$ at high venous shear rates. The rapid activation response of RBM/E-3 tumor cells on Vn and Fn suggested a potential role for the integrin $\alpha_v\beta_3$. Because the platelet releasate contains both Vn and Fn, we examined whether $\alpha_v\beta_3$ inhibition would negate the effects of the platelet releasate. Blocking $\alpha_v\beta_3$ alone in RBM/

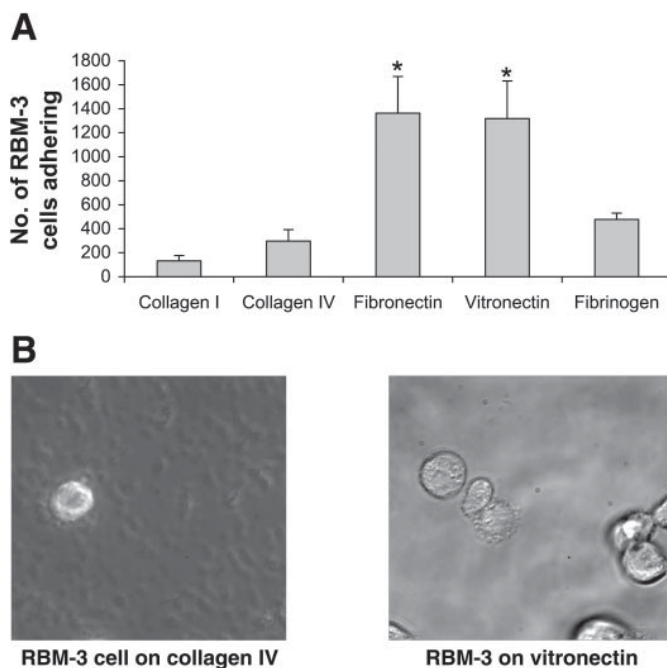


Fig. 4. RBM/E-3 cells adhere and activate more on vitronectin (Vn) and fibronectin (Fn) than on other extracellular matrices. The cells were exposed to a continuous shear rate of 100, 200, or 400 s^{-1} for 1 h. Results shown in *A* represent the number of adherent RBM/E-3 cells after a continuous shear rate of 100 s^{-1} . Maximal adhesion occurred on Vn and Fn, with nearly 3 times the number of RBM/E-3 cells adhering, compared with collagen (types I and IV) and fibrinogen ($P < 0.0001$). *B*: different morphological responses of different matrix proteins are demonstrated by the RBM/E-3 cells on collagen IV (*left*) and Vn (*right*). The formation of pseudopodia and spreading were not evident on collagen IV; however, on Vn, rapid formation of pseudopodia preceded spreading within minutes.

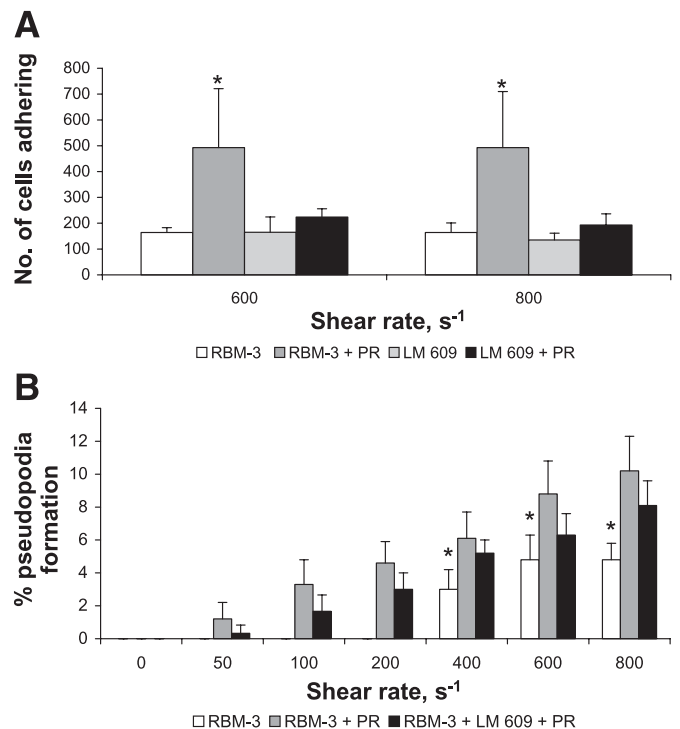


Fig. 5. Monoclonal antibody anti- $\alpha_v\beta_3$ inhibits PR-induced cell adhesion and activation to HUVECs under shear conditions. Blocking $\alpha_v\beta_3$ alone in RBM/E-3 cells results in less adhesion at shear rates of 50–800 s^{-1} . *A*: pretreatment of RBM/E-3 cells with anti- $\alpha_v\beta_3$ antibody LM609 significantly reduced the potentiating effect of PR on RBM/E-3 cell adhesion to HUVECs under shear conditions. The number of adherent cells decreased 2-fold compared with PR-treated cells ($*P < 0.001$) to levels similar to those for untreated cells or cells treated with LM609 alone. *B*: a decrease in pseudopod formation was observed when PR-treated cells were preincubated with $\alpha_v\beta_3$ antibody. However, this was not significant ($P = 0.4711$). At all shear rates, pseudopod formation was significantly lower in untreated RBM/E-3 cells compared with PR- and LM609 + PR-treated cells ($*P < 0.001$).

E-3 cells resulted in less adhesion at all shear rates (Fig. 5A). Blockade of $\alpha_v\beta_3$ followed by addition of the platelet releasate resulted in increased numbers of adherent RBM/E-3 cells, but these numbers were significantly less than the RBM/E-3 cells treated with platelet releasate alone ($P < 0.001$). At higher venous shear rates of 600 and 800 s^{-1} , average tumor cell adherence in the presence of $\alpha_v\beta_3$ antibody and platelet releasate was half that of RBM/E-3 cells treated with platelet releasate. There was some decrease in pseudopod formation in anti- $\alpha_v\beta_3$ -treated tumor cells with platelet releasate; however, the difference was not statistically significant (Fig. 5B).

Secreted platelet Fn and Vn contributed to tumor cell arrest at high venous shear rates. To further characterize the effect of Vn and Fn in platelet releasate and its physiological importance in tumor activation, we pretreated RBM/E-3 cells with immunodepleted platelet releasate. Western blot analysis confirmed the presence of Vn and Fn in the platelet releasate and their corresponding depletion from platelet releasate, as shown in Fig. 6, *A* and *B*. These immunodepleted releasates were incubated with RBM/E-3 tumor cells, perfused over HUVECs, and subsequently examined for adhesion and pseudopod formation.

Shear rates of 50–400 s^{-1} resulted in similar rates of tumor adhesion for platelet releasate and immunodepleted platelet releasate-treated cells. However, at higher venous shear rates

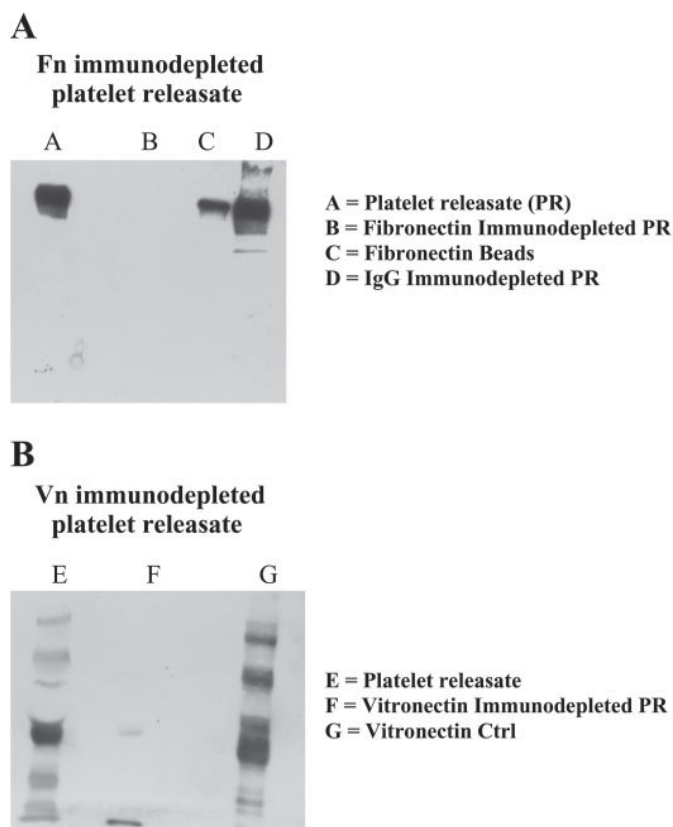


Fig. 6. Immunodepletion of Fn and Vn from platelet releasate. The physiological importance of platelet-secreted Vn and Fn in tumor activation was further examined. Western blot analysis confirmed the presence of Vn and Fn in platelet releasate and their corresponding depletion from platelet releasates (A and B).

of 600 and 800 s^{-1} , significant differences in tumor adhesion were observed. Average tumor cell adhesion in RBM/E-3 cells treated with platelet releasate immunodepleted of Vn at 600 and 800 s^{-1} was 203 and 162, respectively (Fig. 7A). Platelet releasate immunodepleted of Fn at 600 and 800 s^{-1} resulted in 181 and 156 adhering tumor cells, respectively. In contrast to platelet releasate-treated tumor cells, with 493 cells adherent at both of these shear rates, a significant decrease in tumor cell adhesion was observed in the absence of platelet Vn and Fn. This corresponded to the results obtained in the $\alpha_v\beta_3$ inhibition experiments, which showed significant differences at venous shear rates of 600 and 800 s^{-1} (224 and 193 adherent cells, respectively), suggesting that both Vn and Fn in the platelet releasate enhanced RBM/E-3 activation at higher venous shear rates ($P = 0.0001$).

Pseudopod formation was analyzed similarly at all shear rates. Both immunodepleted Vn and immunodepleted Fn resulted in less pseudopod formation at all shear rates. At 100 s^{-1} , 3% of adherent cells formed pseudopodia in the presence of platelet releasate, while 0.3% of tumor cells formed pseudopodia in the presence of platelet releasate with no Fn and 1.7% of adherent tumor cells exhibited pseudopodia in the presence of platelet releasate with no Vn (Fig. 7B). Increasing shear rates resulted in increased pseudopodia formation, with a significant difference detected with immunodepleted Fn platelet releasate at 100 and 600 s^{-1} ($P = 0.0033$).

Taken together, these data suggest that both platelet-derived Vn and Fn contribute to tumor cell adhesion in a high venous shear environment.

Localization of $\alpha_v\beta_3$ in tumor cells exposed to shear. Clustering of integrins is associated with actin rearrangement leading to cell motility (17). Because our results demonstrate that $\alpha_v\beta_3$ played an important role in the adhesion to endothelium, we examined the clustering of this integrin in the absence and presence of shear. RBM/E-3 cells were stained with an anti- $\alpha_v\beta_3$ antibody, shown in red in Fig. 8A. To assess the effect of shear on these cells, we perfused RBM/E-3 cells over Vn as described in EXPERIMENTAL PROCEDURES and stained the remaining adherent cells. Notable differences in the intensity of $\alpha_v\beta_3$ staining were observed between sheared and nonsheared tumor cells, as shown in Fig. 8B. This suggests a possible role for shear in maximizing integrin clustering as well as tumor cell surface area, thus enhancing potential tumor cell arrest.

DISCUSSION

The results of the present investigation demonstrate that metastatic cells adherent to HUVECs pretreated with LPS responded to specific shear rates by extending and retracting pseudopodia. This response was enhanced in the presence of the platelet releasate, which mediated its effects partially through the integrin $\alpha_v\beta_3$. In addition, the secreted component from ASA-treated platelets abrogated the effects mediated by the platelet releasate.

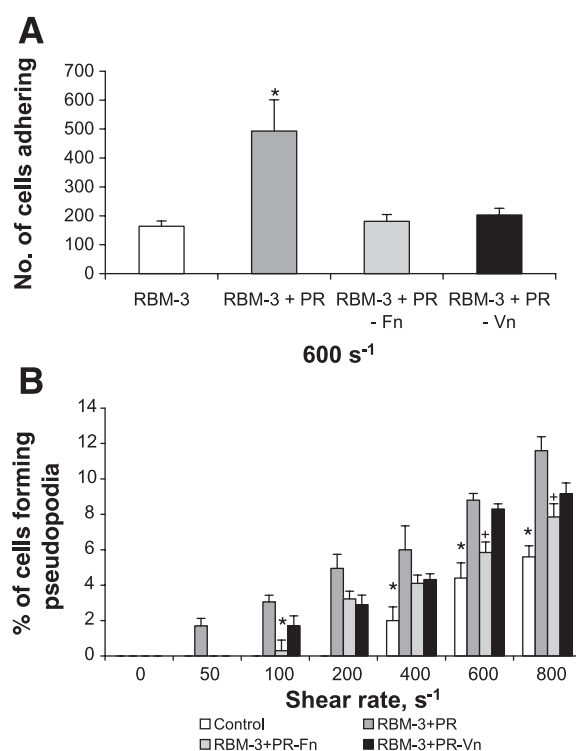


Fig. 7. Secreted platelet Vn and Fn contribute to tumor cell arrest at high venous shear rates. The immunodepleted releasates were preincubated with RBM/E-3 cells before flow. A: at venous shear rates of 600 and 800 s^{-1} , a significant decrease in tumor cell adhesion was detected ($*P < 0.0001$ compared with RBM-3 + PR). Results in B represent pseudopod formation at all shear rates. Less pseudopod formation was observed with immunodepleted Fn releasates than with immunodepleted Vn releasates ($+P < 0.0033$).

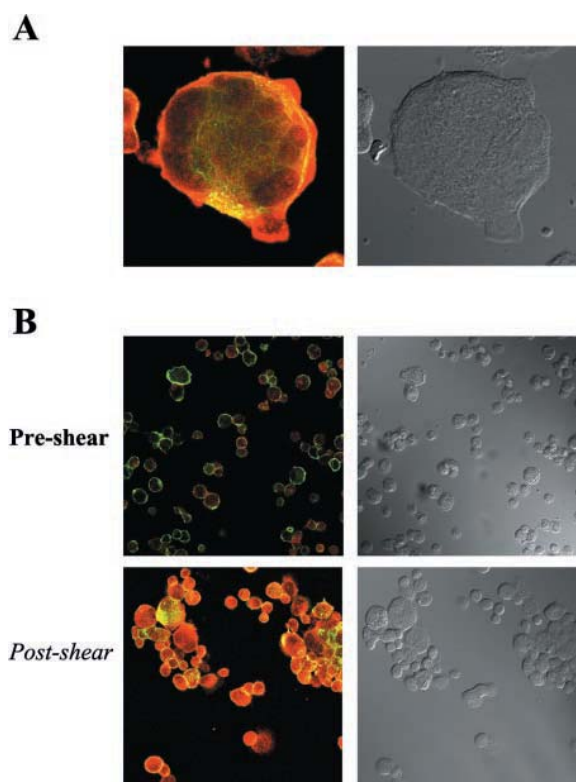


Fig. 8. Shear augmented the expression of $\alpha_v\beta_3$ in tumor cells. We investigated the clustering effect of $\alpha_v\beta_3$ in tumor cells pre- and postshear. *A*: RBM/E-3 cells were exposed to a continuous shear rate of 400 s^{-1} and then stained with anti- $\alpha_v\beta_3$. *B*: distinct differences between sheared and non-sheared cells were observed, with sheared cells showing a significant increase in staining intensity.

Esophageal cancer is a relatively uncommon malignant condition with a low likelihood of cure. Once the cancer develops, it spreads rapidly via metastases through the bloodstream. A prerequisite for the progression of hematogenous metastasis is that circulating cancer cells lodge in the vascular bed of the target organ, a process that is opposed by flowing blood (3). Because leukocytes and platelets tether and roll in a shear-dependent manner, we hypothesized that cancer cells would respond similarly. In the present investigation, we used RBM/E-3 cells isolated from the rib bone marrow of a patient who underwent resection for primary esophageal cancer. Nonmetastatic MCF-7 and metastatic Hs578T breast cancer cells also were examined for their behavior under shear conditions. We have demonstrated that both metastatic and nonmetastatic cells activated at shear rates between 50 and 400 s^{-1} . In contrast, a cell line derived from circulating T cells behaved in an entirely different manner. RBM/E-3 adherence occurred in the continuous presence of shear stress, similar to leukocyte and platelet tethering and rolling on inflamed endothelium. Several researchers have reported the ability of cytokine-stimulated endothelium to cause E selectin-dependent rolling of cancer cells on the endothelial surface under conditions of fluid flow (13). Similarly, stimulating endothelial cells with endotoxin promotes the formation of mediators, which enhance tumor cell-endothelium interaction. To simulate the environment of circulating metastatic cells interacting with activated endothelium, we perfused RBM/E-3 cells over LPS-stimulated endothelium.

Investigators in numerous studies have examined the adhesion of cancer cells to endothelium, but they have used a static adhesion assay to quantify adhesive strength (18). More recently, the use of parallel plate flow chambers has enabled researchers to quantify the adhesion of cancer cells to endothelial monolayers under controlled flow conditions, which exposes both tumor and endothelial cells to shear stress. Shear stress affects both endothelial cell function and tumor cells (8). Exposing endothelial cells to shear stress is known to alter the surface expression of adhesion molecules (31), an effect that could explain why RBM/E-3 tumor cells adherent to inflamed HUVECs form pseudopodia at specific shear rates. We were unable to demonstrate a shear-induced activation response when RBM/E-3 cells were perfused over matrices such as fibrinogen, collagen I, and collagen IV. This suggests a specific response of metastatic cells that adhere to the endothelium. Previous studies have subjected tumor cells to shear conditions for periods of hours (24), thus maximizing the shear response in the tumor cells. In contrast, we investigated the initial response of tumor cells under hydrodynamic conditions.

Tumor cell activation is characterized by the protrusion of pseudopods (5), which ultimately leads to extravasation of the vascular wall. Under shear conditions, we did not observe pseudopod protrusion in the RBM/E-3 cells until a shear rate of 400 s^{-1} was reached, which corresponded to 20-min adherence on endothelium. At shear rates $>400\text{ s}^{-1}$, there was no increase in the formation and retraction of pseudopods, suggesting that once activated, the formation of pseudopods becomes independent of shear. In the absence of shear, pseudopod formation did not occur until ≥ 40 min had elapsed.

We also investigated the shear-induced response in MCF-7 and Hs578T tumor cells. These cells also activated at a precise shear rate. The results of the present investigation suggest that a critical shear stress is necessary for tumor cells to activate and remain adherent. This may also reflect the point in the vasculature where these cells metastasize. Our results demonstrate that different tumor cells exhibit different activation states under the influence of venous flow. It is well known that malignant tumors preferentially metastasize to particular distant organ sites (19). Our results suggest that metastatic spread may reflect the shear forces encountered in that region.

Although it is well accepted that platelets play an important role during hematogenous metastasis, the direct involvement of the platelet releasate in tumor cell behavior under shear conditions has not yet been investigated. We examined the effects of fluid shear stresses on RBM/E-3 cells adherent to HUVECs in the presence of platelet releasate and releasate from ASA-treated platelets. We observed a dramatic response in tumor cells preincubated with the platelet releasate. First, the number of cells adherent to the HUVECs surface increased. Second, the minimum shear rate required to induce pseudopod formation was reduced to 50 s^{-1} , and in some cases, the cells became activated before shear was initiated. Thus one or more components present in the platelet releasate had an effect on the behavior of RBM/E-3 tumor cells under flow conditions. ASA-treated platelet releasate did not potentiate the activation response of the tumor cells. The effects of platelet releasate from ASA-treated platelets on tumor activation warrants further study, but in an initial evaluation, we have found that the effects of ASA platelet releasate are not due to ASA alone and

that a thromboxane antagonist and mimetic do not cause significant differences in tumor activation under flow.

Characterization of the specific proteins responsible for increased tumor adhesion in the presence of platelet releasate was further investigated. Because integrins are the main mediators of tumor cell arrest in the vasculature (28), we examined the behavior of RBM/E-3 cells under flow on different matrix components that are ligands for specific integrins. Maximal tumor cell arrest and activation occurred on the matrix proteins Vn and Fn, suggesting a potential role for the integrin $\alpha_v\beta_3$. In addition, we found that blockade of $\alpha_v\beta_3$ in RBM/E-3 cells followed by addition of platelet releasate resulted in less adhesion and activation at high venous shear rates of 600 and 800 s^{-1} . Examination of $\alpha_v\beta_3$ further via confocal microscopy in pre- and postsheared tumor cells resulted in distinct differences in the intensity of $\alpha_v\beta_3$ staining, with greater staining observed in sheared cells. The differences may be due to clustering of integrins, which has been intimately linked to actin rearrangement leading to cytoskeletal movement (23), or they may be due simply to the mechanical force of shear exposing maximal cell surface area to interacting proteins.

In summary, we have shown that the mechanical stimulus of shear evokes specific changes in the morphology of tumor cells at defined shear rates. The activation state of tumor cells was enhanced in the presence of the platelet releasate through secreted Vn and Fn, while platelets pretreated with ASA abrogated the effects mediated by the platelet releasate. This may in part explain the potential benefit of ASA therapy in the treatment of cancer and suggests that therapies targeted to defined regions of shear and specific integrins may be of potential benefit.

GRANTS

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REFERENCES

- Balkwill F and Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 357: 539–545, 2001.
- Boettiger D, Lynch L, Blystone S, and Huber F. Distinct ligand-binding modes for integrin $\alpha_v\beta_3$ -mediated adhesion to fibronectin versus vitronectin. *J Biol Chem* 276: 31684–31690, 2001.
- Brooks DE. The biorheology of tumor cells. *Biorheology* 21: 85–91, 1984.
- Butcher EC and Picker LJ. Lymphocyte homing and homeostasis. *Science* 272: 60–66, 1996.
- Coates TD, Watts RG, Hartman R, and Howard TH. Relationship of F-actin distribution to development of polar shape in human polymorphonuclear neutrophils. *J Cell Biol* 117: 765–774, 1992.
- Cranmer SL, Ulsemer P, Cooke BM, Salem HH, de la Salle C, Lanza F, and Jackson SP. Glycoprotein (GP) Ib-IX-transfected cells roll on a von Willebrand factor matrix under flow: importance of the GPIb/actin-binding protein (ABP-280) interaction in maintaining adhesion under high shear. *J Biol Chem* 274: 6097–6106, 1999.
- Denton RR, Koszewski NJ, and Notides AC. Estrogen receptor phosphorylation: hormonal dependence and consequence on specific DNA binding. *J Biol Chem* 267: 7263–7268, 1992.
- Dewey CF Jr, Bussolari SR, Gimbrone MA Jr, and Davies PF. The dynamic response of vascular endothelial cells to fluid shear stress. *J Biomech Eng* 103: 177–185, 1981.
- Enzinger PC and Mayer RJ. Esophageal cancer. *N Engl J Med* 349: 2241–2252, 2003.
- Frenette PS, Moyna C, Hartwell DW, Lowe JB, Hynes RO, and Wagner DD. Platelet-endothelial interactions in inflamed mesenteric venules. *Blood* 91: 1318–1324, 1998.
- Gasic GJ, Gasic TB, and Stewart GC. Antimetastatic effects associated with platelet reduction. *Proc Natl Acad Sci USA* 61: 46–52, 1968.
- Gazdar AF, Carney DN, Bunn PA, Russell EK, Jaffe ES, Schechter GP, and Guccion JG. Mitogen requirements for the in vitro propagation of cutaneous T-cell lymphomas. *Blood* 55: 409–417, 1980.
- Giavazzi R, Foppolo M, Dossi R, and Remuzzi A. Rolling and adhesion of human tumor cells on vascular endothelium under physiological flow conditions. *J Clin Invest* 92: 3038–3044, 1993.
- Gimbrone MA Jr. Culture of vascular endothelium. *Prog Hemost Thromb* 3: 1–28, 1976.
- Hackett AJ, Smith HS, Springer EL, Owens RB, Nelson-Rees WA, Riggs JL, and Gardner MB. Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs578T) and the diploid myoepithelial (Hs578Bst) cell lines. *J Natl Cancer Inst* 58: 1795–1806, 1977.
- Hart IR. “Seed and soil” revisited: mechanisms of site-specific metastasis. *Cancer Metastasis Rev* 1: 5–16, 1982.
- Honn KV and Chen YQ. Prostacyclin, hydroxy fatty acids and cancer metastasis. In: *Prostacyclin: New Perspectives for Basic Research and Novel Therapeutic Indications: Proceedings of the Symposium on Novel Perspectives in Prostacyclin Research, Vienna, Austria, 25th September 1991*, edited by Rubanyi GM and Vane JR. Amsterdam: Elsevier, 1992.
- Honoré S, Pichard V, Penel C, Rigot V, Prévôt C, Marvaldi J, and Briand C, Rognoni JB. Outside-in regulation of integrin clustering processes by ECM components per se and their involvement in actin cytoskeleton organization in a colon adenocarcinoma cell line. *Histochem Cell Biol* 114: 323–335, 2000.
- Iwai K, Ishikura H, Kaji M, Sugiura H, Ishizu A, Takahashi C, Kato H, Tanabe T, and Yoshiaki T. Importance of E-selectin (ELAM-1) and sialyl Lewis(x) in the adhesion of pancreatic carcinoma cells to activated endothelium. *Int J Cancer* 54: 972–977, 1993.
- Koch M, Weitz J, Kienle P, Benner A, Willeke F, Lehnert T, Herfarth C, and von Knebel Doeberitz M. Comparative analysis of tumor cell dissemination in mesenteric, central, and peripheral venous blood in patients with colorectal cancer. *Arch Surg* 136: 85–89, 2001.
- Kramer RH and Nicolson GL. Interactions of tumor cells with vascular endothelial cell monolayers: a model for metastatic invasion. *Proc Natl Acad Sci USA* 76: 5704–5708, 1979.
- Lawrence MB, McIntire LV, and Eskin SG. Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. *Blood* 70: 1284–1290, 1987.
- Machesky LM and Hall A. Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization. *J Cell Biol* 138: 913–926, 1997.
- Moss MS, Siskin B, Zimmer S, and Anderson KW. Adhesion of nonmetastatic and highly metastatic breast cancer cells to endothelial cells exposed to shear stress. *Biorheology* 36: 359–371, 1999.
- Nash GF, Walsh DC, and Kakkor AK. The role of the coagulation system in tumour angiogenesis. *Lancet Oncol* 2: 608–613, 2001.
- Oprea WE, Karp JM, Hosseini MM, and Davies JE. Effect of platelet releasate on bone cell migration and recruitment in vitro. *J Craniofac Surg* 14: 292–300, 2003.
- O’Sullivan GC, Sheehan D, Clarke A, Stuart R, Kelly J, Kiely MD, Walsh T, Collins JK, and Shanahan F. Micrometastases in esophago-gastric cancer: high detection rate in resected rib segments. *Gastroenterology* 116: 543–548, 1999.
- Pilch J, Habermann R, and Felding-Habermann B. Unique ability of integrin $\alpha_v\beta_3$ to support tumor cell arrest under dynamic flow conditions. *J Biol Chem* 277: 21930–21938, 2002.
- Ruggeri ZM, Dent JA, and Saldívar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood* 94: 172–178, 1999.
- Schleimer RP and Rutledge BK. Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters. *J Immunol* 136: 649–654, 1986.
- Takahashi M, Ishida T, Traub O, Corson MA, and Berk BC. Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. *J Vasc Res* 34: 212–219, 1997.
- Varani J, Lovett EJ, Elgebaly S, Lundy J, and Ward PA. In vitro and in vivo adherence of tumor cell variants correlated with tumor formation. *Am J Pathol* 101: 345–352, 1980.
- Weiss L. Biomechanical interactions of cancer cells with the microvasculature during hematogenous metastasis. *Cancer Metastasis Rev* 11: 227–235, 1992.