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Distinct role of hydrodynamic shear in leukocyte-facilitated tumor cell extravasation

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Abstract

Previously, we found polymorphonuclear neutrophils (PMNs) increased melanoma cell extravasation under flow conditions (*Intl J Cancer* 106: 713–722, 2003). In this study, we characterized the effect of hydrodynamic shear on PMN-facilitated melanoma extravasation using a novel flow-migration assay. The effect of shear stress and shear rate on PMN-facilitated melanoma extravasation was studied by increasing the medium viscosity with dextran to increase shear stress independently of shear rate. Under fixed shear rate conditions, melanoma cell extravasation did not change significantly. In contrast, the extravasation level increased at a fixed shear stress but with a decreasing shear rate. PMN-melanoma aggregation and adhesion to the endothelium via β_2 -integrin/intracellular adhesion molecule-1 (ICAM-1) interactions were also studied. Lymphocyte function-associated molecule-1 (LFA-1; CD11a/CD18) influenced the capture phase of PMN binding to both melanoma cells and the endothelium, whereas Mac-1 (CD11b/CD18) affected prolonged PMN-melanoma aggregation. Blockage of E-selectin or ICAM-1 on the endothelium or ICAM-1 on the melanoma surface reduced PMN-facilitated melanoma extravasation. We have found PMN-melanoma adhesion is correlated with the inverse of shear rate, whereas the PMN-endothelial adhesion correlated with shear stress. Interleukin-8 (IL-8) also influenced PMN-melanoma cell adhesion. Functional blocking of the PMN IL-8 receptors, CXCR1 and CXCR2, decreased the level of Mac-1 upregulation on PMNs while in contact with melanoma cells and reduced melanoma extravasation. We have found PMN-facilitated melanoma adhesion to be a complex multistep process that is regulated by both microfluid mechanics and biology.

Keywords

Neutrophil; melanoma; shear stress; shear rate; β_2 -integrins; intracellular adhesion molecule-1; CXCR1/2; adhesion; migration

Tumor cell extravasation through the vascular endothelium is an important event in the metastatic cascade and currently the mechanisms regulating these phenomena are not well understood. Several previous studies (1,23) of metastasis have suggested tumor cells may exploit leukocytes to enhance their ability to adhere to an endothelium. One study (21) showed that the presence of polymorphonuclear neutrophils (PMNs) or activated macrophages increased the likelihood that rat hepatocarcinoma cells would adhere to the endothelium. In recent studies (19,25), PMNs have been reported to increase melanoma cell transendothelial migration in both static conditions and shear flow.

It is evident from in vivo studies (5,12,17,28) that the mechanisms utilized by leukocytes and metastatic tumor cells to adhere to a vessel wall before extravasation are different. An in vivo study (17) showed that under a shear flow, B16F1 melanoma cells could arrest on the walls of presinusoidal vessels in mice when pretreated with interleukin-1 α (IL-1 α). This suggests the release of cytokines into the bloodstream could result in melanoma cell arrest in portal venules by chemoattraction and adhesion rather than by size restriction only.

Welch et al. (24) showed C8161 melanoma cells are a metastatic model that is both highly invasive compared with other well-characterized melanoma cell lines and spontaneously metastatic by tail-vein injection in nude mice. Melanoma cells do not express β_2 -integrins, Sialyl Lewis X (SLe^x) or other sialylated molecules at an effective level (6,19). In contrast, the C8161 cell line expresses basal levels of intracellular adhesion molecule-1 (ICAM-1) that can be upregulated with TNF- α treatment (19). Tumor cell lines of various tissue origins also express ICAM-1 (e.g., A2058, WM9, and MDA-MB-435) (24, and personal observations). Whereas metastatic potential of melanoma cell lines does not correlate directly with ICAM-1 expression, C8161 cells express ICAM-1 in the low range of tested melanoma cells (24).

Currently little is known about specific interactions of PMN-melanoma heterotypic aggregation in a shear flow. Some recent studies (8,10,11,15) have quantified the strength and kinetic properties of lymphocyte function-associated antigens (LFA-1; CD11a/CD18; $\alpha_L\beta_2$) and Mac-1 (CD11b/CD18; $\alpha_M\beta_2$) binding in PMN homotypic (e.g., PMNs to PMNs) and heterotypic (e.g., PMNs to cells expressing ICAM-1) aggregation, especially under a hydrodynamic shear force over the time course of chemotactic stimulation. Studies by Neelamegham et al. (15) and Hentzen et al. (8) suggest that LFA-1 binding to ICAM-1 is important in initial endothelial capture of PMNs, whereas Mac-1 to ICAM-1 binding resists shear forces to stabilize the PMN-endothelium adhesion. Similar results have been published (10,11) for fluid shear effect on the interactions between PMNs and ICAM-1-expressing colon carcinomas.

Protein secretion plays an important role in tumor cell and neutrophil biology. Although several proinflammatory cytokines and chemokines have been implicated in influencing adhesive properties of transformed cells, interleukin-8 (IL-8) is of particular interest because it promotes the growth of some tumors. Tumor cells that secrete high levels of IL-8 have been characterized as having an increased metastatic potential (18,27). IL-8 is important in recruiting and activating PMNs (20) and binds to the CXCR1 and CXCR2 receptors. CXCR1 has not been detected in large quantities on melanoma cells (13), but tumor-secreted IL-8 can act on PMNs and influence their adhesion molecule expression.

The objective of this study is to understand the role of hydrodynamic forces and specifically the β_2 -integrin/ICAM-1 adhesion mechanism in PMN-mediated melanoma migration under shear conditions. We conclude that shear rate, rather than shear stress, plays a more significant role in PMN-melanoma aggregation and adhesion to the endothelium (Fig. 1). In addition, IL-8 is identified as an important chemokine, which is produced as a result of PMN-melanoma contact and facilitates melanoma-PMN-endothelium adhesion and subsequent melanoma extravasation.

MATERIALS AND METHODS

Cell culture

C8161.c9 and GFP-transfected C8161 cells were maintained and prepared as described previously (19,24). Before each experiment, C8161 cells were detached when nearly confluent and suspended at a concentration of 1×10^6 cells/ml. In migration assays where anti-ICAM-1 blocking antibodies were used (5 μ l/ml; CalTag Laboratories, Burlingame, CA), the cells were

incubated with antibody for 30 min before the start of the assay. IgG isotype control antibody was used to verify specificity of all blocking antibodies.

Fibroblast L-cells that had been transfected to express human ICAM-1 and E-selectin (EI cells; provided by Dr. Scott Simon, University of California, Davis) were maintained in culture as described elsewhere (7). E-Selectin and ICAM-1 levels were periodically checked by flow cytometry to verify expression level. ICAM-1 levels on EI cells were shown to be comparable with IL-1 β -stimulated human umbilical vein endothelial cells (7), and were used as a substrate for cell adhesion and as a model endothelium in this study. For particular assays, the EI monolayer was treated with blocking E-selectin or ICAM-1 antibodies (5 μ g Ab per monolayer; CalTag Laboratories).

PMN isolation and preparation

Fresh blood was obtained from healthy adults under informed consent following a protocol approved by the Pennsylvania State University Institutional Review Board. Histopaque gradient (Sigma, St. Louis, MO) was used to isolate and enrich the PMN population. The isolated PMN layer was first suspended in 0.1% human serum albumin (Sigma) in Dulbecco's phosphate-buffered saline (DPBS) and washed. ACK lysis buffer (0.15 M NH₄Cl, 10.0 mM KHCO₃, 0.1 mM Na₂EDTA in distilled H₂O) was used to remove erythrocytes. The cells were washed with 0.1% human serum albumin/DPBS, resuspended at a concentration of 1×10^6 cells/ml, and rocked at 4°C until they were used, no longer than 4 h. To activate LFA-1 or Mac-1 on PMNs, cells were treated with phorbol 12-myristate 13-acetate (PMA, Sigma; 100 ng/ml, 20 min) or IL-8 (R&D Systems, Minneapolis, MN; 1 ng/ml, 1 h), respectively. PMA was not found to affect Mac-1 expression on PMNs (data not shown). In blocking assays, PMNs were treated with saturating concentrations of antibodies, 5 μ g of IgG anti-human LFA-1 or Mac-1 (CalTag Laboratories) per 1×10^6 cells in blocking buffer (5% calf serum, 2% goat serum in DPBS) for 30 min at 4°C. Similarly, to block IL-8 receptors, PMNs were treated with mouse anti-human CXCR1 and CXCR2 antibodies (R&D Systems) with 6 and 10 μ g/ml, respectively, in blocking buffer for 30 min at 4°C.

Dextran-supplemented medium

RPMI 1640 medium with 25 mM HEPES (Biosource, Camarillo, CA) was supplemented with 0.1% bovine serum albumin (BSA) and 1–4% ultra-high molecular weight dextran (2×10^6 MW; Sigma). A range of dextran-supplemented media were made to achieve a range of viscosities from 0.7 cP (no dextran) to 7.0 cP (4% dextran).

Flow migration assay

The in vitro flow migration device is a recently developed, modified 48-well chemotactic Boyden chamber (Fig. 2) (6,19). In brief, the top and bottom plates of the polycarbonate chamber are separated by a 0.02-in.-thick silicon gasket (PharmElast, SF Medical, Hudson, MA). A 7 cm $\times$ 2 cm opening cut from the center of the gasket forms the flow field. The wall shear stress (τ_w) is related to the volumetric flow rate (Q) by $\tau_w = 6 \mu Q / wh^2$, where μ is the fluid viscosity, h is height, and w is width of the flow field.

A monolayer of EI cells was grown to confluence on sterilized polyvinylpyrrolidone-free polycarbonate filters (8- μ m pore size; Neuro Probe, Gaithersburg, MD) coated with fibronectin (30 μ g/ml, 3 h) (Sigma). The bottom side of the filter was scraped before use to remove any potential cell growth. Soluble type IV collagen (100 μ g/ml in RPMI 1640/0.1% BSA) (BD Discovery Labware, Bedford, MA) (2,9,26) was used as the chemoattractant in the center 12 wells and control wells were filled with medium (RPMI 1640/0.1% BSA). The chamber was assembled and the cells of interest (C8161 only; PMN only; or C8161+PMN together; 5×10^5 cells of each cell type) were pipetted into the chamber. Flow of circulating medium was

immediately perfused into the chamber, initially at a flow rate 2 ml/min, which was then increased to the desired rate (0–20 ml/min). The entire chamber was placed in a 37°C, 5% CO₂ incubator for 4 h. Experiments were run at a selected wall shear stress (0–4 dyn/cm²) or shear rate (55.5–555 s⁻¹) for a prescribed length of time (1–4 h). For assays where migration was measured, the filter was removed from the chamber and immediately stained with HEMA-3 (Fisher Scientific, Pittsburgh, PA). The cells on the bottom side of each filter, the side that had been facing the chemoattractant wells, were imaged with the use of an inverted microscope and recorded with NIH Image (version β 4.0.2) software. For assays where adhesion was characterized, the top side of the filter was immediately imaged using fluorescence microscopy. For all assays (migration and adhesion cases) three pictures were taken of each filter in different locations. The number of cells migrated or adhered was quantified and averaged for each filter. A minimum of three filters was analyzed for each data point. Background migration was subtracted from each sample as appropriate. No cells were found in the chemoattractant wells after 4 h of migration (data not shown).

Statistical significance between cases tested in the flow-migration chamber was determined by *P* values from an unpaired *t*-test, where *P* < 0.05 was considered significant (Sigma Plot; version 8). All error bars on flow migration data represent means $\pm$ SE.

Parallel plate flow assay

Adhesion and tethering experiments were performed in a parallel-plate flow chamber (Glycotech, Gaithersburg, MD) mounted on an inverted optical microscope with a $\times 10$ phase-contrast objective lens (Diaphot 330, Nikon, Toyko, Japan). The medium was perfused through the chamber using a syringe pump (Harvard Apparatus, Holliston, MA) to create a uniform laminar flow field in the chamber. A confluent EI-cell monolayer (35 mm petri dishes; Corning, Acton, MA) was used as the ligand-binding substrate on the bottom plate of the parallel plate chamber. The experiments were recorded using a video camera (model CCD72, MTI-Dage, Michigan City, IN) and video recorder (model AG-3200, Panasonic, Secaucus, NJ) equipped with a time-date generator (model VTG-33, FOR.A, Tokyo, Japan). The field of view was 800 μ m long (direction of flow) by 600 μ m wide. The EI monolayer was preinfused with warmed medium with the experimental viscosity (1–3.2 cP) for 4–5 min at a shear rate of 40 s⁻¹ before the introduction of the two cell suspensions. PMNs were either activated by IL-8 or treated with blocking antibodies (as described above). Both PMNs and C8161 cells were resuspended in appropriate dextran-supplemented medium. To allow the cell suspensions to reach the field of view on the EI monolayer, 1×10^6 /ml PMNs and 1×10^6 /ml C8161 were perfused at a low shear stress (0.1–0.3 dyn/cm²) for 120 s (accumulation phase). The flow rate was then increased to the experimental shear rate (55.5 s⁻¹), and kept constant for 6–8 min (shear application phase). Experiments were performed in triplicate. Statistical significance was tested by one-way ANOVA, where *P* > 0.05 was determined to be significant (Minitab version 14).

PMN tethering frequencies

The experimentally determined tethering frequency was the number of PMNs that adhered to the EI monolayer per unit time and area, including both rolling and firmly arrested cells. This frequency was normalized by cell flux to the surface to compensate for the different concentration of cells passing the same area of substrate at different shear rates. At higher shear rates, a higher concentration of cells would pass the endothelium and have the opportunity to adhere. This normalization followed the procedure cited in Rinker et al.'s (16) work based on equations from Munn et al. (14).

PMN-C8161 aggregation and adhesion efficiency analysis

Aggregation and adhesion of accumulated cells was counted manually from the video recording of the shear application phase of the assay. Values that were quantified include the number of collisions between entering C8161s and rolling and firmly arrested PMNs on the EI monolayer, and the number of PMN-C8161 aggregates firmly arrested on the EI monolayer as a result of a collision.

Adhesion efficiency was calculated by dividing the number of C8161 cells arrested on the EI monolayer by the number of C8161-PMN collisions. Arrested C8161 cells were quantified at the end of each assay as a result of accumulative collisions between entering C8161 cells and tethered PMNs. C8161-PMN collisions were those that occurred near the EI monolayer surface during the flow assay.

Co-culture of PMNs and C8161 cells

C8161 cells were cultured in 6-well plates (Corning). PMNs (5×10^6 per well), untreated or treated with anti-CXCR1 and CXCR2 antibodies (as described above), were added to each well either directly onto the C8161 monolayer or into a Transwell insert (0.4 μ m pore; Corning) above the monolayer. As a control, PMNs were concurrently cultured in plates without C8161 cells. The plates were then incubated for 4 h at 37°C and 5% CO₂. After 4 h, the PMNs were collected and fluorescently labeled for flow cytometry analysis (see description below).

Flow cytometry

The cells of interest were treated with murine anti-human CD marker primary antibodies (e.g., anti-LFA-1, anti-Mac-1, or anti-ICAM-1; 1 μ g Ab/ 10^6 cells) (CalTag Laboratories) for 30 min at 4°C. The cells were then treated with secondary antibody, FITC-conjugated goat anti-mouse IgG F(ab)₂ fragment (1 μ g/ 10^6 cells) (Jackson ImmunoResearch, West Grove, PA) for 25 min at 4°C. In the case of blocking CXCR1 and CXCR2 receptors on PMNs, PE-conjugated anti-Mac-1 (1 μ l/ 10^6 cells; CalTag Laboratories) was used to avoid binding secondary antibody to the existing CXCR1 and CXCR2 antibodies. The samples were fixed with 2% formaldehyde (Sigma) and analyzed with a flow cytometer (EPICS XL, Coulter, Fullerton, CA). Control cases used to determine background fluorescence were samples treated with secondary antibody only or PE-conjugated isotype control (CalTag Laboratories).

RESULTS

Shear rate rather than shear stress affects PMN-facilitated melanoma extravasation

Effects of shear rate and shear stress on PMN-mediated melanoma adhesion and migration have been compared using the novel flow-migration assay. With the use of dextran to vary the medium viscosity (μ) of the cell suspension, either shear rate ($\dot{\gamma}$) or shear stress ($\tau = \mu\dot{\gamma}$) was held constant, whereas the other was varied to determine the effects on melanoma cell adhesion and migration. Cases in which shear stress was held constant and shear rate varied from 55.5–555 s⁻¹ yielded dramatic variation in C8161 cell extravasation (183 ± 24 cells per mm² at 55.5 s⁻¹ to 290 ± 37 cells per mm² at 555 s⁻¹) that was inversely proportional to the shear rate ($P = 0.041$; Fig. 3A). In contrast, cases in which shear rate was held constant and shear stress ranged from 0.4 to 18 dyn/cm² resulted in C8161 migration levels that were not statistically different (Fig. 3, B and C). These results suggest that PMN-facilitated migration of melanoma cells is affected by local hydrodynamic shear rate, not the shear stress.

PMN tethering frequency is affected by shear rate and shear stress

Adhesion experiments were performed to examine how PMN-C8161 cell aggregates adhere to the EI cell monolayer. The PMN tethering frequency was quantified at three shear rates (60,

100, and 200 s^{-1}) and three viscosities (1, 2, and 3.2 cP). As seen in Fig. 4A, the PMN tethering frequency was significantly affected by both shear rate and shear stress. All frequencies were corrected for the decrease in cell flux with increasing shear rate and viscosity and normalized against the lowest shear stress case, as described in MATERIALS AND METHODS. These results are different from the PMN-mediated C8161 migration and adhesion efficiency results shown in Fig. 4, B and C. As clearly seen, C8161 extravasation and adhesion mediated by PMNs were affected only by shear rate, not shear stress at a fixed shear rate. The results were normalized for cell flux using the same method for Fig. 4A.

Dextran does not affect adhesion molecule expression

To elevate shear stress at a constant shear rate or change shear rate at a constant shear stress, ultra-high molecular weight dextran (2×10^6 MW) was used to vary the viscosity of the circulating medium. To assure the dextran-treated medium did not affect the cells, expression of adhesion molecules on each relevant cell type were examined. C8161 and EI cells were tested for changes in ICAM-1 expressions, and PMNs were tested for Mac-1. With the use of flow cytometry, cells from non-dextran-supplemented and dextran-supplemented suspensions showed similar fluorescent histograms (data not shown). The osmolarity of dextran-supplemented media was also tested to verify there was not a significant increase due to the addition of dextran. Osmolarity increased by <1% in a 4% solution of dextran in RPMI 1640 compared with unsupplemented media (data not shown).

Population ratio of C8161s to PMNs affects melanoma extravasation

The ratio of C8161 cells to PMNs was examined to determine whether changing the relative concentrations would change the probability of a tumor cell colliding with and binding to a PMN. Both inactivated and IL-8-stimulated PMNs were used in flow-migration experiments. Figure 5 shows that doubling the number of PMNs resulted in a greater number of C8161 migration. In the presence of unstimulated PMNs, C8161 migration increased by 92% when the concentration ratio was changed to 1:2 (C8161:PMN), whereas migration increased by 32% when the number of IL-8 activated PMNs was increased. Control results show that the use of a 1:2 ratio of C8161 cells to fixed PMNs did not alter C8161 cell extravasation level (Fig. 5), suggesting PMN-mediated melanoma cell migration is adhesion specific, not solely influenced by the total number of cells in the shear flow.

PMN-facilitated C8161 cells that adhere do migrate

The number of melanoma cells that adhered to the monolayer was examined at two time points, 1 and 2 h after the beginning of the flow migration assay (Fig. 6). It has previously been shown that after 1 h C8161 cells had not yet migrated in the flow migration chamber (6) and therefore time equal to 1 h can be considered an initial condition. Greater than 90% of the cells adhered to the monolayer after 1 h eventually migrated after 4 h under three different hydrodynamic conditions. After 2 h, the number of cells adhered to the monolayer was significantly lower than after 1 h, indicating that migration was progressing.

Mac-1 and LFA-1 differentially affect PMN-facilitated C8161 extravasation

Previously, receptor-ligand binding via β_2 -integrins (on PMNs) and ICAM-1 (on melanoma cells) has been implicated in mediating PMN-melanoma adhesion under flow conditions (19). To investigate further this mechanism, LFA-1 and Mac-1 on PMNs were targeted with functional blocking antibodies. Upregulating Mac-1 resulted in a 122% increase in melanoma cell migration compared with the C8161+PMN case. Correspondingly, blocking Mac-1 resulted in the greatest reduction in C8161 migration (66% reduction compared with C8161 +PMN; Fig. 7A). Similar results are shown in Fig. 7A for LFA-1 upregulation and blocking,

but to a lesser extent; migration increased by 63% with PMA-stimulated PMNs and decreased by 40% with LFA-1 blocked PMNs.

To understand whether changes in melanoma cell migration as shown in Fig. 7A are due to C8161-PMN adhesion or due to PMN-EI cell adhesion, ICAM-1 was functionally blocked on the melanoma cells and EI cells individually. The results, shown in Fig. 7B, indicate that each binding step between the triad of the endothelium, PMN, and tumor cell is involved in tumor cell extravasation, specifically via the interactions of β_2 -integrins and ICAM-1. Blocking E-selectin on EI cells reduced C8161 migration by 80% compared with the C8161+PMN control case (Fig. 7B). In addition, blocking E-selectin on the EI cells does not change background melanoma (C8161 only; Fig 7A, *top bar*) migration (Fig. 7B, *bottom bar*). Furthermore, because SLe^x expression was not found on C8161 (data not shown) indicates that PMNs do facilitate C8161 cell adhesion to the endothelium during the extravasation process under flow conditions.

Distinct role of LFA-1 and Mac-1 in PMN-melanoma aggregation under flow conditions

The β_2 -integrin molecules, Mac-1 and LFA-1, appear to have different roles in PMN-facilitated melanoma extravasation. To understand this phenomenon better, it is useful to look at the requisite adhesion step before extravasation. With the use of a parallel plate chamber to characterize aggregate adhesion over the course of 5 min, the distinct roles of LFA-1 and Mac-1 in melanoma extravasation can be better understood. The rate of aggregation of C8161 to accumulated PMNs on the EI monolayer at a shear rate of 55.5 s^{-1} was not significantly altered by blocking Mac-1, and LFA-1 alone supported adhesion up to the peak level observed for the untreated control at 3 min (Fig. 8). However, after 3 min, disaggregation from the EI monolayer proceeded more rapidly in the presence of anti-Mac-1 than in the untreated control. In comparison, Mac-1-dependent adhesion (in the presence of anti-LFA-1) proceeded more slowly and only reached a maximum level ~50% lower than the control. These aggregates remained stably adhered to the surface of the EI monolayer over 5 min (Fig. 8). Furthermore, upregulating Mac-1 on PMNs by IL-8 treatment did not significantly change aggregation compared with the unstimulated case. The same result was found for adhesion of C8161 to accumulated PMN on an EI monolayer at other shear rates (data not shown). These data suggest that LFA-1 alone is sufficient for the initial escalation in PMN-C8161 aggregate formation and adhesion to the EI monolayer, whereas the contribution of Mac-1 is necessary to maintain the stability of those formed aggregates in a shear flow.

CXCR1/2 receptors influence PMN recruitment in C8161 cell migration

To understand the possibility that secreted chemokines could be a stimulus responsible for melanoma-PMN communication, the IL-8 receptors on PMNs (CXCR1 and CXCR2) were antibody blocked and tested in the migration assay. C8161 cell migration dramatically decreased at a shear rate of 55.5 s^{-1} in the presence of CXCR1/CXCR2-blocked PMNs compared with unblocked PMNs (39% decrease) or IL-8-activated PMNs (73% decrease) as shown in Fig. 9A. This effect on C8161 cell extravasation will be amplified under higher shear-rate conditions.

To further understand how the IL-8 receptors affected C8161 cell adhesion and migration, Mac-1 expression on PMNs co-cultured with C8161 cells was examined using flow cytometry. Untreated PMNs and PMNs treated with blocking antibodies for CXCR1 and CXCR2 were co-cultured with C8161 cells either in contact (referred to as “Co-culture” case) or separated by a Transwell insert (referred to as “Transwell” case). Figure 9B indicates that non-CXCR1/2-blocked PMNs co-cultured with C8161 cells experienced a nearly fourfold increase in Mac-1 expression levels over those cultured alone for 4 h. The increase in Mac-1 was apparent after only 30 min of co-culture and became significant after 2 h of co-culture (data not shown).

PMNs with antibody blocked CXCR1 and CXCR2 showed no change in Mac-1 levels after co-cultured with C8161 cells compared with PMNs cultured alone (Fig. 9B).

DISCUSSION

Tumor metastasis remains a primary cause of death among cancer patients. The mechanism by which tumor cells move from the primary tumor site to a secondary tumor site and biological processes which regulate tumor cell adhesion and migration are of great importance and interest. It is well reported in the literature (5,12,17,28) that tumor cells do not use the same processes that have been described for leukocyte adhesion and migration through an endothelial wall. While not true of all tumor cells, melanoma cells characterized as highly metastatic (24) express neither ligands for endothelial selectin molecules nor β_2 -integrins at a level necessary to maintain firm adhesion to endothelial ICAM-1. One study (22) has suggested that initial microvascular arrest of metastasizing tumor cells (from cell lines of six different histological origins) does not occur through “leukocyte-like rolling” adhesive interaction with the endothelium. A recent study by Slattery and Dong (19) showed that under dynamic flow conditions, PMNs could influence melanoma cell adhesion to the endothelium by binding to ICAM-1 on both melanoma and endothelial cells with their β_2 -integrins, thereby enhancing subsequent melanoma cell migration through the endothelium.

Hydrodynamic forces play an important role in regulating melanoma cell adhesion and migration under a shear flow. Without the ability to bind to the endothelial surface alone under shear conditions, melanoma cells may recruit PMNs to aid in binding to the blood vessel wall. Determining how shear stress and shear rate effect this intercellular communication and interaction can increase the understanding of how fluid shear impacts melanoma extravasation in the microcirculation. With the use of dextran-supplemented medium to change viscosity, the shear stress was varied while holding shear rate constant or the shear rate was varied while holding shear stress constant. Melanoma cell migration was not significantly changed when shear stress was varied from 0.4 –18 dyn/cm² under a constant shear rate, but decreased significantly when the shear rate was increased 10-fold under a constant shear stress.

When the tethering, adhesion and migration stages of PMN-facilitated melanoma migration are analyzed, it becomes evident that there are two distinct stages of adhesion in PMN-facilitated melanoma adhesion and migration: PMN-endothelium adhesion and PMN-melanoma adhesion (Fig. 1). Each stage responds to the hydrodynamic environment differently. PMN tethering to the endothelial monolayer is both shear rate and shear stress dependent. In this experimental setup, this phase is mediated by SLe^x-E-Selectin adhesion and β_2 -integrin-ICAM-1 interactions. Adhesion of melanoma cells to PMN and subsequent migration are shear rate dependent and dominated by β_2 -integrin-ICAM-1 interactions. Because migration follows adhesion, this result appears to identify the adhesion stage as the determining factor for the efficiency of the melanoma migration.

The difference between the C8161 migration and the PMN tethering results under hydrodynamic forces supports the theory that PMNs facilitate C8161 migration. If C8161 cells used the traditional extravasation mechanism of binding to the endothelium itself, the response to shear would have been expected to be similar to that seen in single cell tethering (Fig. 4A). Because this is not the case, the results indicate two separate bonds are necessary for C8161 migration (Fig. 1) and the interesting shear rate dependence becomes apparent. The migration experiments examine adhesion of tumor cells to PMNs after PMNs have already adhered to the endothelium, whereas the tethering results more specifically focus on PMN to endothelium adhesion (Fig. 4A). The migration data show tumor cell-to-PMN adhesion is shear rate dependent and parallel plate flow chamber data of PMN-to-tumor cell aggregation show a similar trend (unpublished data). In contrast, the PMN-endothelium tethering is both shear rate

and shear stress dependent, which is in agreement with previously published results on monocyte tethering on human umbilical vein endothelial cells (16).

PMN tethering interactions, mediated by selectins, are a prerequisite for subsequent adherence, mediated by β_2 integrins on PMNs binding to ICAM-1 on the endothelium. Shear rate is inversely proportional to intercellular contact time (16). By decreasing shear rate, PMNs are in contact with the endothelium longer, which allows firm binding to occur. The same mechanism may be at play between PMNs and C8161 cells; a lower shear rate may increase the time the cells are in contact, therefore allowing more heterotypic binding and consequently more migration.

Rinker et al. (16) proposed when shear stress increases at a constant shear rate (and contact duration) after an initial tether is formed, the cell and its microvilli deform more, which allows more bonds to form and leads to a higher tethering frequency. This hypothesis agrees with Cao et al.'s (4) work. The PMN tethering data reported here follows the same shear stress dependence trend, signifying the importance of PMN deformation in binding between PMNs and the endothelium. However, PMN adhesion to the endothelium is necessary but not sufficient for PMN-facilitated melanoma cell adhesion or migration. Because migration data do not follow the same trend, this indicates that contact duration is more influential on C8161 migration than cell deformation.

One indication of the characteristics of the fluid flow in the migration and parallel plate flow chamber experiments is the Reynolds number. The Reynolds number local to the cell is defined as the ratio between inertia effects and viscous effects on the cell; $Re = 2\rho\dot{\gamma}R/\mu$, where ρ is the media density, $\dot{\gamma}$ is the shear rate, R is the radius of the cell, and μ is medium viscosity. When the shear rate and viscosity are changed in these experiments, this ratio is altered, which may play a role in the interactions between the cells and effect the resulting adhesion and migration. Analysis of both Rinker's and the cell tethering data reported here (Fig. 4A) reveals a correlation to the local cell Reynolds number (not shown). In contrast, a correlation between migration data and Reynolds number is not clear. Because the Reynolds number alone is not sufficient to explain the shear rate-dependent migration phenomena, additional flow characteristics and multibody dynamics are currently under investigation to determine a new correlation.

To minimize any possible shielding effects of dextran, ultra-high molecular weight dextran was used. Changes in osmolarity were found to be negligible and dextran was not found to affect adhesion molecule expression levels on any of the cell types used in this study, including melanoma cells, PMNs, or EI cells. Previously, shielding effects have been reported with 40,000 MW dextran (3); however, Rinker et al. (16) reported that the 2×10^6 molecular weight dextran did not shield monocyte rolling on the endothelium. Therefore, high molecular weight dextran was chosen to supplement the cell suspension used in this study.

Varying the PMN population with respect to the number of tumor cells lends some insight into the mechanics of PMN-facilitated tumor migration. Migration increased when the ratio of PMNs to C8161 cells was increased for both unstimulated and IL-8-treated PMN cases. By increasing the relative concentration of PMNs, the probability of PMNs and C8161 cells aggregating increased. Interestingly, doubling the concentration of PMNs increased migration in greater proportion when unstimulated PMNs were added than when IL-8-stimulated PMNs were used. This suggests both activating PMNs and increasing the number of PMNs increase C8161 migration, but the effects are not additive. Therefore, a maximum level of PMN facilitation of tumor cell migration may exist.

Comparing the number of adhered melanoma cells in the flow migration assay with cells that eventually migrate helps to characterize the transition between cell adhesion and cell migration.

The number of adherent melanoma cells at 1 h compared with the number at 2 h shows melanoma migration progression, not disassociation. Disaggregation cannot be the cause of the difference between the number of adhered cells at 1 and 2 h due to the number of migrated cells after 4 h. The ratio of the number of adhered melanoma cells after 1 h to the number of migrated cells after 4 h, which is >0.9 or 90% for each case, indicates that nearly all adhered melanoma cells eventually migrate without apparent changes due to hydrodynamic conditions. This again supports that adhesion is the determining factor in melanoma migration. The number of melanoma cells that adhere is a direct representation of the number of melanoma cells that will migrate.

LFA-1 and Mac-1, the β_2 -integrins, have sequential roles in binding leukocytes to ICAM-1; adhesion begins with LFA-1-dependent capture and is stabilized and maintained by Mac-1 (8,15). The experiments using the flow migration and parallel plate chambers allow the roles of LFA-1 and Mac-1 in PMN-melanoma cell aggregation and subsequent PMN-facilitated melanoma adhesion to the endothelium within a prescribed shear field to be elucidated. Upregulating or downregulating LFA-1 or Mac-1 showed a significant impact on the melanoma cell migration and adhesion results. Blocking LFA-1 inhibited PMN-facilitated melanoma migration through the endothelium; however, blocking Mac-1 had more of an effect. In the adhesion assays, LFA-1 affects adhesion over the entire time course, whereas the effect of Mac-1 is seen after only 3 min. This difference is apparent because of the longer time necessary for migration to occur than adhesion. This suggests that the stabilized adhesion provided by Mac-1 to ICAM-1 is more of a factor in successful C8161 migration under shear, but Mac-1 and LFA-1 are both necessary and neither is sufficient to allow PMNs to bind to C8161 cells and the endothelium.

Jadhav and Konstantopoulos (11) have shown that ICAM-1-expressing colon carcinoma cells bind to PMN under shear as a function of both contact duration and shear stress whereas sLe^x-expressing carcinoma cells (LS174T) bind to PMN as a function of only contact duration. Their experimental setup was a cone and plate viscometer, where two cell types (PMN and tumor) were allowed to collide and aggregate under a shear in a free suspension. In contrast, the data presented here is from a three-cell system (Fig. 1; PMN, tumor, and endothelial) where two separate binding events must occur near a planar surface. Jadhav and Konstantopoulos' results provided excellent insight into the kinetics of PMN-tumor cell aggregation, but might not be expected to explain PMN-tumor aggregation-mediated melanoma cell migration results presented here due to the necessary binding of PMNs on the endothelium in addition to PMN-melanoma aggregation.

PMN-facilitated melanoma adhesion to the endothelium is a multistep process, as shown in Fig. 1. Blocking either ICAM-1 or E-selectin adhesion molecules on the endothelial monolayer reduced C8161 migration, which suggests PMNs must bind to the endothelium before melanoma cells can bind to them. Blocking the ICAM-1 molecules on tumor cells also inhibited C8161 migration, which further indicates a β_2 -integrin/ICAM-1 binding mechanism is involved in PMN-facilitated melanoma cell adhesion and migration.

The level of interaction between PMNs and tumor cells has been shown to increase in the presence of inflammatory mediators such as IL-8 and TNF- α (19). Results from Slattery and Dong (19) have shown that IL-8 is secreted by PMNs after interaction with C8161 tumor cells. To examine the role that endogenously produced IL-8 has on PMN-mediated melanoma cell extravasation, the IL-8 receptors CXCR1 and CXCR2 on PMNs were blocked. The use of these blocked PMNs inhibited migration of C8161 cells compared with the use of untreated PMNs. The dramatic inhibition of Mac-1 expression on CXCR1/2 blocked PMNs clearly identifies the role of melanoma-induced IL-8 production in PMN-facilitated tumor cell migration. In addition, while physical contact between C8161 cells and PMNs produces a slightly higher

Mac-1 response, contact is clearly not requisite to stimulate a significant increase in Mac-1 expression. This suggests that a soluble factor, possibly the autocrine effect of PMN-secreted IL-8 (19), is responsible for this increase in Mac-1 expression.

PMN-facilitated melanoma transendothelial migration is a series of complex cellular and molecular interactions that are effected by the fluid dynamics of the surrounding flow. This study shows two separate bonds are necessary for PMN-melanoma-endothelium adhesion. Each step is both receptor and hydrodynamics dependent. The efficiency and strength of each receptor-ligand interaction is influenced by the surrounding hydrodynamic conditions. We have found the dominant and determining step, melanoma-PMN adhesion, to be shear rate dependent. Aggregation of melanoma cells with PMNs and subsequent migration require both a collision and bond formation between a melanoma cell and a tethered PMN. The success of this process is dominated by the time the two cell types are in contact and depends less on the cell deformation. The PMN-melanoma bond, which is influenced in a shear rate-dependent manner, is regulated by β_2 -integrin adhesion to ICAM-1, whereas the PMN-endothelial cell bond requires both E-selectin and ICAM-1 influenced by shear stress and cell deformability. Finally, endogenously produced IL-8 does contribute to PMN-facilitated melanoma migration through the CXCR1 and CXCR2 receptors.

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Fig. 1.

Schematic of endothelium-polymorphonuclear neutrophil (PMN) tumor cell adhesion. Two different cell aggregations are examined: tumor cell to PMN and PMN to endothelial cell (EC). The adhesion and migration data reveal that tumor cell to PMN adhesion varies inversely to shear rate and is dependent on contact duration while independent of shear stress. PMN tethering data indicate that PMN adhesion to endothelial cells follows a more traditional hydrodynamic relation and is proportional to shear stress and contact duration.

Fig. 2.

The top and side views of the flow migration chamber. The top and bottom plates were machined from polycarbonate. Medical grade silicone was used as a gasket (SF Medical). The melanoma cells extravasate through a cell monolayer cultured on a polycarbonate membrane with 8 μm pores (Neuroprobe).

Fig. 3.

Shear rate and shear stress were isolated by varying viscosity with dextran-supplemented medium (0 – 4%). *A*: migration varies under constant shear stress but increasing shear rate. *B* and *C*: constant shear rate data; migration is unchanged over an order of magnitude of shear stress. All error bars are means $\pm$ SE for $n \geq 3$.

Fig. 4.

Effects of shear stress on PMN tethering frequency, adhesion efficiency, and melanoma extravasation at fixed shear rates. *A* and *B*: *N*-formylmethionyl-leucyl-phenylalanine-stimulated PMNs (1×10^6 cells/ml) were perfused into a parallel plate chamber together with tumor cells (1×10^6 cells/ml). *C*: extravasation of C8161 melanoma cells with PMNs at three shear rates with media at three different viscosities. All error bars are means $\pm$ SE for $n \geq 3$ and tethering data were corrected for cell flux as described by Rinker et al. (16) and Munn et al. (14). # $P > 0.05$ compared with 1.0 cP case; + $P > 0.05$ compared with 2.0 cP case.

Fig. 5.

Migration of C8161 cells in the presence of both untreated and IL-8-treated PMNs in an equal or 1:2 ratio of C8161 to PMNs. Adding fixed PMNs demonstrated that the increase in cell number is not the cause of increased migration; instead it is adhesion specific. All error bars are means $\pm$ SE for $n \geq 3$ (P value is with respect to 1:1 case for each activation state; 55.5 s^{-1} , viscosity = 0.7 cP).

Fig. 6.

Adhesion vs. migration of melanoma cells in the flow migration assay. Under three hydrodynamic conditions, adhesion of melanoma cells to the filter after 1 and 2 h is quantified and compared with the respective migration after 4 hours. The ratio of migration after 4 h and adhesion at 1 h is >90% for all three cases.

Fig. 7.

A: relative contributions of lymphocyte function-associated antigen (LFA-1) and Mac-1 adhesion in PMN-mediated melanoma cell migration. The third and fourth bars from the top demonstrate the dramatic contribution of Mac-1 to PMN-facilitated migration. The fifth and sixth bars show that LFA-1 contributes, but to a lesser degree. As a control, the last bar shows migration with isotype-treated PMNs (P values with respect to C8161+PMN case; 55.5 s^{-1} , viscosity = 0.7 cP). **B:** relative influence of adhesion between melanoma cell and PMN vs. PMN and ICAM-1 and E-selectin (EI) monolayer in PMN-facilitated melanoma extravasation. The second bar shows the case of disrupting melanoma and PMN aggregation by blocking ICAM-1 on C8161 cells that significantly reduces migration. The third and fourth bars show the case of disrupting PMN adhesion to the EI monolayer by blocking E-selectin or ICAM-1 on EI cells, which also significantly reduces migration. Both components appear to contribute equally to PMN-facilitated C8161 migration (P values with respect to C8161+IL-8/PMN case; 55.5 s^{-1} , viscosity = 0.7 cP). All error bars are means $\pm$ SE for $n \geq 3$.

Fig. 8.

Relative contributions of LFA-1 and Mac-1 to binding of PMN-C8161 heterotypic aggregates on the surface of EI monolayer. The anti-LFA-1 case affects aggregation over the entire time course, whereas the effects of Mac-1 blocking are apparent only after 3 min. Data were normalized against the total number of rolling and arrested PMNs on the EI monolayer at each time point. Error bars are means $\pm$ SE for $n \geq 3$.

Fig. 9.

A: blocking the IL-8 receptors (CXCR1 and CXCR2) on PMNs reduces migration, implicating IL-8 as a communication factor between C8161 melanoma cells and PMNs (*P* values with respect to C8161+PMN case; 55.5 s^{-1} , viscosity = 0.7 cP). B: fold induction of Mac-1 expression on PMNs after co-culture with C8161 melanoma cells. PMNs and C8161 cells were co-cultured (in contact with each other) or Transwell cultured (not in contact but sharing the same medium) for 4 h and Mac-1 was labeled and detected by flow cytometry. Blocking the IL-8 receptors (CXCR1 and CXCR2) resulted in no increase in Mac-1 expression compared with the control case.