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Shear stress and shear rate differentially affect the multi-step process of leukocyte-facilitated melanoma adhesion

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Abstract

Previous studies have shown that neutrophils (PMNs) facilitate melanoma cell extravasation [M.J. Slattery, C. Dong, Neutrophils influence melanoma adhesion and migration under flow conditions, Intl. J. Cancer 106 (2003) 713–722] Little is known, however, about the specific interactions between PMNs, melanoma and the endothelium (EC) or the molecular mechanism involved under flow conditions. The aim of this study is to investigate a “two-step adhesion” hypothesis that involves initial PMN tethering on the EC and subsequent melanoma cells being captured by tethered PMNs. Different effects of hydrodynamic shear stress and shear rate were analyzed using a parallel-plate flow chamber. Results indicate a novel finding that PMN-facilitated melanoma cell arrest on the EC is modulated by shear rate, which is inversely-proportional to cell–cell contact time, rather than by the shear stress, which is proportional to the force exerted on formed bonds. β_2 integrins/ICAM-1 adhesion mechanisms were examined and the results indicate LFA-1 and Mac-1 cooperate to mediate the PMN–EC–melanoma interactions under shear conditions. In addition, endogenously produced IL-8 contributes to PMN-facilitated melanoma arrest on the EC through the CXC chemokine receptors 1 and 2 (CXCR1 and CXCR2) on PMN. These results provide new evidence for the complex role of hemodynamic forces, secreted chemokines and PMN–melanoma adhesion in the recruitment of metastatic cancer cells to the EC.

Keywords

β_2 integrins; ICAM-1; CXCR1/2; Interleukin-8; Extravasation

Introduction

Inflammation is a vital component of the body’s defense against infections. Human leukocytes, including polymorphonuclear neutrophils (PMNs), actively participate in the inflammatory response through adhesion to the endothelium (EC). Accumulating evidence suggests that tumor progression is governed not only by genetic changes intrinsic to the tumor cell, but also by epigenetic and environmental factors. Although the adaptive immune system reduces tumor incidence through immune-surveillance mechanisms [2], the innate immune system can promote tumor progression through inflammation-dependent mechanisms [3]. Immune and inflammatory cells and their secreted chemokines and cytokines have dramatic effects both on the host’s physiology and on cancer cell behavior, modulating the growth, invasion, migration

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and metastasis of cells within the tumor microenvironment [3,4]. These insights are fostering new approaches to cancer treatment through anti-inflammatory therapeutics.

Tumor metastasis consists of a complex cascade of adhesive interactions between tumor cells and host tissues. During their passage through the circulatory system, tumor cells undergo extensive interactions with various host cells including PMNs [5]. Previous studies have shown that colon carcinoma cell adhesion to EC involves sialylated molecules and integrins [6]. However, melanoma cells do not express β_2 integrins, Sialyl Lewis X (sLe^x) or other sialylated molecules at levels effective to adhere to inflamed EC by themselves [1]. Although several independent studies have shown that PMNs, which participate in inflammatory responses, may enhance tumor metastasis [7,8], there is little understanding of the mechanisms potentially involved. Both endothelial and melanoma cells express intercellular adhesion molecule-1 (ICAM-1), the potential ligand for β_2 integrins on PMNs; therefore, PMN–EC and PMN–melanoma cell adhesions may play roles in bringing tumor cells into close proximity to the EC, thus facilitating their subsequent migration through the EC.

Significant progress has been made in the past decades towards understanding the process of PMN adhesion to the vascular endothelium and subsequent migration to sites of inflammation. Studies indicate that endothelial selectins, including E-selectin, mediate PMN capture from the blood stream and rolling on the vascular wall [9,10], whereas the firm adhesion to the EC is mediated by β_2 integrins (e.g., LFA-1 and Mac-1) on PMNs and ICAM-1 on endothelial cells [11]. Recent studies have quantified the strength and kinetics of LFA-1 and Mac-1 in PMN heterotypic aggregation to transfected cells expressing ICAM-1 in a shear flow [12,13]. These studies showed that PMN adhesion to ICAM-1 is a cooperative and sequential process of LFA-1-dependent initial endothelial capture of PMNs followed by Mac-1-mediated stabilization. A similar adhesion mechanism has been observed for the interactions between PMNs and ICAM-1-expressing colon carcinomas [14,15].

Interleukin-8 (IL-8), which belongs to the superfamily of CXC chemokines, has a wide range of proinflammatory effects and mediates the migration of PMNs from the circulation to the site of infection by activation of CXC chemokine receptors 1 and 2 (CXCR1 and CXCR2) [16–18]. Studies have also demonstrated that melanoma cells, such as C8161 [1], SK-MEL 13 [19], A375SM [20] and WM9 (unpublished data) can produce IL-8. IL-8 could trigger the functional up-regulation of the ligand binding activity of the β_2 integrins on PMN [21]. IL-8 secretion is a marker for increasing metastatic potential and functions as an important promoter for melanoma growth [22,23]. Therefore, IL-8 secreted by melanoma cells and PMNs may play a role in the communication between PMNs and melanoma cells.

Hemodynamic flow regulates blood cell and EC interaction through the modulation of cell collisions, alterations of forces on cell–cell adhesive bonds and regulation of adhesion molecule expression, affinity and avidity. Shear rate is dependent on the flow rate and the geometry of the flow field, but is independent of fluid viscosity. It also governs the transport of cells to other cells, and thus modules cell–cell collisions and cell–cell contact time. Shear stress is the frictional force of flow and is proportional to the fluid viscosity and the shear rate. Shear stress dictates the forces applied to cells individually and the intermolecular bonds between cells.

In the present study, we utilized a parallel-plate flow chamber to study PMN-facilitated melanoma cell adhesion under shear conditions. This system has been used to characterize the role of platelets in cancer metastasis in a shear flow [24]. Studies carried out in flow chambers have quantified the interactions between cells and substrate in terms of the number of cell–cell collisions and the number of rolling and adherent cells [25,26]. The metastatic WM9 human melanoma cell line, which expresses high levels of ICAM-1, was used in this study due to its extensive characterization and use in both in vitro and in vivo assays [27–29]. Specifically, the

different roles of hydrodynamic shear stress, shear rate and β_2 integrins/ICAM-1 adhesion mechanisms were examined. Our results indicate that the number of melanoma cells in close proximity to the EC via tethered PMNs is modulated by only the shear rate (inversely proportional to the PMN–melanoma contact time) rather than by the shear stress (proportional to the force exerted on formed bonds). LFA-1 and Mac-1 cooperate to mediate the PMN–EC–melanoma interactions under shear conditions. LFA-1 binding to ICAM-1 is necessary and sufficient for the initial PMN–melanoma adhesion, while Mac-1 is important in maintaining this binding. A "two-step adhesion" mechanism is proposed that involves PMNs first tethering (rolling and arrest) to the EC and secondly capturing melanoma cells. The endothelial E-selectin and ICAM-1 affect the *first* step, modulated by shear stress and shear rate; whereas melanoma-expressed ICAM-1 affects the *second* step, mediated only by the shear rate. In addition, endogenously produced IL-8 contributes to PMN-facilitated melanoma arrest on the EC through the CXCR1 and CXCR2 receptors on PMNs.

Materials and methods

Reagents

Formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma (St. Louis, MO). Monoclonal antibodies (mAbs), mouse anti-human LFA-1, mouse anti-human Mac-1 and mouse anti-human ICAM-1 were purchased from CalTag (Burlingame, CA). Mouse anti-human E-selectin and mouse anti-human CXCR1/CXCR2 mAbs were purchased from R&D systems (Minneapolis, MN).

Cell culture

WM9 melanoma cells (Gift from Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA) were grown in RPMI-1640 (BioSource, Rockville, MD) supplemented with 10% FBS (BioSource). Prior to each experiment, WM9 cells were detached when confluent using trypsin/versene (BioSource) and washed twice with fresh medium. Then, WM9 cells were suspended in fresh medium and allowed to recover for 1 h while being rocked at a rate of 8 rpm at 37°C. In the parallel-plate flow assays where anti-ICAM-1 mAb was used, WM9 cells were incubated with mAb (5 μg/10^6 cells) for 30 min at 37°C prior to the start of the assay.

Fibroblast L-cells that had been transfected to express human E-selectin and ICAM-1 (EI cells; provided by Dr. Scott Simon, UC Davis, CA) were maintained in culture as described elsewhere [30]. EI cells express ICAM-1 comparable with IL-1β stimulated human umbilical vein endothelial cells (HUVECs) [31] and were used as an endothelial substrate for cell adhesion study. For some adhesion receptor blocking assays, the confluent EI monolayer was treated with anti-ICAM-1 mAb (5 μg/10^6 cells) or anti-E-selectin mAb (5 μg/10^6 cells).

Neutrophil isolation

Following The Pennsylvania State University Institutional Review Board (IRB) approved protocols (#990758 and #19311), fresh human blood was collected from healthy adults by venipuncture. PMNs were isolated using a Histopaque® (Sigma) density gradient as described by the manufacturer and kept at 4°C in Dulbecco's PBS (D-PBS) containing 0.1% human serum albumin for up to 4 h before an experiment. For inhibition studies, stimulated-PMNs were pretreated for 30 min at 4°C with function-blocking anti-human LFA-1 or Mac-1 mAbs (5 μg/10^6 cells). To block IL-8 receptors, PMNs were treated with anti-human CXCR1 mAb (6 μg/ml) and CXCR2 mAb (10 μg/ml) for 30 min at 4°C. All blocking mAbs were kept present during flow assays.

Viscosity measurements

Media viscosities were determined using a cone-plate viscometer (RotoVisco 1, Haake, Newington, NH). Solutions of varying viscosity were prepared with 2×10^6 M_r dextran (Sigma) in RPMI-1640 containing 0.1% BSA (Sigma). Measurements were taken over a range of shear rates at 37°C. High-molecular-weight dextran was used to minimize the change in osmolarity of the medium.

Quantification of receptor expression

To examine the surface receptor expression, selected cells were incubated with saturating concentrations of primary mAbs directed against ICAM-1, E-selectin, sLe^x (an E-selectin ligand), LFA-1 or Mac-1 for 30 min at 4°C and then washed twice with D-PBS containing 2% goat serum and 4% calf serum (BioSource). After an additional 30 min incubation with FITC-labeled IgM or TRITC-labeled IgG (1 μg/10^6 cells; Jackson ImmunoResearch, West Grove, PA), the specimens were washed twice, fixed with 2% formaldehyde and analyzed by Coulter EPICS XL (Coulter Corp.) flow cytometer (where the FITC-labeled IgM was used) or GUAVA personal cytometer (GUAVA technologies Inc., Burlingame, CA) (where the TRITC-labeled IgG was used). As a control, background fluorescence was assessed using cells treated with secondary antibodies only.

Parallel-plate flow assays

Cell collision and adhesion experiments were performed in a parallel-plate flow chamber (Glycotech, Rockville, MD) mounted on the stage of a phase-contrast optical microscope (Diaphot 330, Nikon, Japan). A syringe pump (Harvard Apparatus, South Natick, MA) was used to generate a steady flow field in the flow chamber. A petri dish (35 mm) with a confluent EI cell monolayer (acting as a ligand-binding substrate) was attached to the flow chamber. All experiments were performed at 37°C. The field of view was 800 μm long (direction of the flow) by 600 μm. The focal plane was set on the EI monolayer. The flow chamber was perfused with appropriate media over the EI monolayer for 2–3 min at a shear rate of 40 s^{-1} for equilibration before the introduction of a predetermined concentration (1×10^6 cells/ml) of PMNs and WM9. PMNs were stimulated with 1 μM fMLP for 1 min or 1 ng/ml IL-8 for 1 h before the perfusion into parallel-plate flow chamber. After allowing PMNs and WM9 cells to contact the EI monolayer at a shear stress of 0.1–0.3 dyn/cm^2 for 2 min, we adjusted the shear stresses to the experimental range of 0.6–2 dyn/cm^2 and kept constant for 6–7 min. Experiments were performed in triplicate and analyzed off-line.

Cells in contact with monolayer are easily discriminated from faster elements

PMNs and melanoma cells that were in contact with EI monolayer were easily discriminated by combining several criteria (Fig. 1). First, cells moving close to the EI monolayer had rather sharp boundaries, whereas other cells appeared as blurred moving shapes. Second, cell-substratum interaction resulted in marked irregularities of the motion, including both curvature of trajectories and temporal variations of the cell velocity, with transient or durable arrests. On the contrary, cells that were at distance from the monolayer followed straight lines. Third, the cell size differences in PMN and WM9 cell (WM9 ~16 μm and PMN ~8 μm in cell diameters) could be used to tell if melanoma cells were in contact with the EI monolayer through PMNs.

PMN tethering frequencies

The tethering frequency was determined experimentally as the number of PMNs that adhered to the EI monolayer per unit time and area in the parallel-plate flow chamber assay, 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. This normalization followed the procedure of Rinker et al. [32] based on equations derived by Munn et al. [33].

WM9–PMN aggregation

The extent of aggregation between entering WM9 cells and tethered PMNs at each time point was characterized by "normalized WM9 aggregation", which is the number of WM9–PMN aggregates normalized to the total number of tethered PMNs at that time point. Aggregation variables quantified included the total number of PMNs which were tethered (rolling or arrested) on the EI monolayer; collisions between WM9 (from the free stream near the EI) and tethered PMNs; aggregation/disaggregation of WM9 with tethered PMN as a result of the collision; and final attachment/detachment of WM9–PMN aggregates on the EI monolayer. Some PMNs which collided with and were arrested by a WM9–PMN aggregate close to the EI surface were counted as tethered PMNs. For the rare cases in which more than one tumor cell adhered to a PMN, we count such a case as two aggregates if two WM9s adhered to a PMN.

WM9 adhesion efficiency

"WM9 adhesion efficiency" can be expressed by the following ratio:

$$\text{WM9 Adhesion Efficiency} = \frac{\text{Number of WM9 cells arrested on the EI monolayer}}{\text{Number of WM9 and PMN collisions}}$$

The numerator is the number of WM9 cells arrested on the EI monolayer at the end of the entire flow assay as a result of collision between entering WM9 cells and tethered PMNs. The denominator is the total number of WM9–PMN collisions near the EI monolayer surface and is counted as a transient accumulative parameter throughout the entire flow assay.

Statistical analysis

All results are reported as the mean ± standard error of the mean (SEM) unless otherwise stated. One-way ANOVA analysis was used for multiple comparisons and *t* tests were used for comparisons between 2 groups. $P < 0.05$ was considered significant.

Results

Adhesion molecule expressions on WM9 and EI cells

Flow cytometry was used to detect expression of E-selectin and ICAM-1 on EI cells and ICAM-1, sLe^x, LFA-1 and Mac-1 expression on WM9 cells. Mac-1, LFA-1 and sLe^x were not found, whereas significant ICAM-1 expression was detected on WM9 cells (Table 1) and the expression level remained stable for 2 h experimental time (data not shown). Significant expressions of E-selectin and ICAM-1 were also detected on EI cells (Table 1).

Effects of dextran-supplemented media

The different effects of shear stress and shear rate on melanoma cell contact to the EC via tethered PMNs were analyzed by using dextran to vary the media viscosity. In order to keep shear stress ($\tau = \mu\dot{\gamma}$) constant while varying shear rate ($\dot{\gamma}$), cell suspension media viscosity (μ) was varied by adjustments in medium dextran (2×10^6 M_r) concentration. To minimize any possible shielding effects of dextran, ultra-high molecular weight dextran was used. Shielding of endothelial functions was reported previously with 4×10^4 M_r dextran [34]; however, Rinker et al. [32] reported that the 2×10^6 M_r dextran did not shield monocyte rolling on the EC. The changes in osmolarity due to the adding of 2×10^6 M_r dextran were found to be negligible

[32] and dextran was not found to affect relevant adhesion molecule expression on any of the cell types used in this study, including melanoma cells, PMNs or EI cells (data not shown).

Tethering frequencies of PMNs to the endothelium

The PMN tethering frequency to the EI substrate was quantified at different shear rates (62.5, 100 and 200 s^{-1}) and shear stresses (0.6–6.4 dyn/cm^2). As seen in Fig. 2A, the PMN tethering frequency was significantly affected by shear rate and shear stress. The normalized PMN tethering frequency decreased with increasing shear rate and shear stress. For a fixed shear rate, elevation of shear stress led to an increase in tethering frequency. At a constant shear stress of 2 dyn/cm^2, the tethering frequency was greatest when shear rate was the lowest (62.5 s^{-1}) and decreased with increasing shear rate. In contrast, WM9 adhesion efficiency was affected only by shear rate, not by the shear stress (Fig. 2B). Since WM9 does not express ligands for either E-selectin or ICAM-1, the difference between Figs. 2A and B suggests a "two-step" mechanism governing the interaction of melanoma cells with the EC (Fig. 3). The *first step* is PMNs tethering to the EC, which is mediated by endothelial E-selectin and ICAM-1 binding to sLe^x and LFA-1/Mac-1 on PMNs. This is followed by the *second step* of melanoma cells being captured by tethered PMNs, which is shear rate-dependent. The PMN–melanoma cell interaction is facilitated by β_2 integrins on PMNs and ICAM-1 expressed on tumor cells. Capture of the melanoma cells by the PMNs thus facilitates melanoma extravasation by providing a mechanism to bring the melanoma cell into close proximity to the EC.

Shear rate affects melanoma cell aggregation to tethered PMNs

The aggregation of melanoma cells to tethered PMNs on the endothelial surface was characterized over a range of flow conditions using the parallel-plate flow chamber. Fig. 4 shows that the number of WM9 cells arrested on the EI monolayer via aggregation to tethered PMNs decreased when shear rate was increased from 62.5 to 100 s^{-1}. An increase of shear rate from 100 to 200 s^{-1} did not significantly change melanoma aggregation to PMNs. At the lowest shear rate of 62.5 s^{-1}, WM9 aggregation to PMNs on the EI monolayer increased over a longer period of flow than at the higher shear rates and reached a peak at 4 min. After 4 min, WM9 cells detached from WM9–PMN aggregates faster than such aggregates formed on the EI surface. At the higher shear rates, WM9 contact with the EI monolayer via tethered PMNs increased over a period of about 2 min, followed by a steady balance between the detachment and formation of WM9–PMN aggregates. These results indicate that hydrodynamic shear forces regulate the PMN–melanoma cell binding kinetics when PMNs are tethered to the EC.

When shear stress was held constant and shear rate varied from 62.5 to 200 s^{-1}, the efficiency of melanoma adhesion to PMNs decreased dramatically, suggesting an inversely proportional relation between shear rate and melanoma adhesion efficiency (Fig. 5A). In contrast, when shear rate was held constant and shear stress ranged from 0.6 to 2 dyn/cm^2, adhesion efficiency levels were not significantly different (Fig. 5B). These results illustrate that melanoma cell adhesion efficiency is regulated by the local hydrodynamic shear rate, or the contact time ($\propto 1/\dot{\gamma}$), not by the shear stress.

Relative influence of LFA-1 and Mac-1 in PMN-facilitated melanoma cell adhesion

Function-blocking mAbs against LFA-1 and Mac-1 were used to elucidate how WM9–PMN aggregates were arrested on the EI monolayer under shear conditions. Blocking LFA-1, Mac-1 or both partially inhibited melanoma adhesion efficiency under all shear conditions tested (Fig. 6A). Blocking Mac-1 did not significantly alter the rate of aggregation between entering WM9 cells and tethered PMNs, and LFA-1 alone supported WM9 aggregation with tethered PMN on the EC up to a peak level observed at 3 min (Fig. 6B). However, after a period of 3 min, disaggregation of WM9 cells from WM9–PMN aggregates on the EI surface proceeded more rapidly in the presence of anti-Mac-1 mAb than in the control. Aggregation of entering WM9

cells with tethered PMNs was significantly reduced at the end of 5 min. In comparison, Mac-1-dependent contact with the EC (in the presence of anti-LFA-1) proceeded more slowly and reached a maximum which was approximately 25% of the control case. These aggregates remained stably adhered to the EI surface over 5 min (Fig. 6B). These results suggest that LFA-1 alone is important for the initial formation of WM9–PMN aggregates, and plays a primary role in the recruitment of WM9 cells to the EI monolayer. Mac-1 maintains the stability of formed WM9–PMN aggregates on the EI surface after the initial WM9 cell capture by PMNs.

Relative role of E-selectin and ICAM-1 in PMN-facilitated melanoma cell arrest

To assess how E-selectin is involved in WM9 into close proximity to the EI monolayer via tethered PMNs, EI cells were treated with mouse anti-human E-selectin blocking mAbs. Blocking E-selectin on the EI monolayer significantly reduced PMN tethering (Fig. 7A) and subsequent WM9 adhesion efficiency compared with the control (Fig. 7B).

To examine the involvement of ICAM-1 in PMN–EC and PMN–melanoma adhesions, WM9 and EI cells were treated separately with functional blocking mAbs against ICAM-1. Blocking ICAM-1 on the EI cells significantly reduced PMN tethering (Fig. 7A) and WM9 adhesion efficiency compared with the control (Fig. 7B). E-selectin and ICAM-1 blocking on EI cells reduced PMN tethering on EI monolayer and thus affect PMN-facilitated melanoma adhesion. Results suggest that PMN tethering to the EC, the *first* step in the “two-step adhesion” model (Fig. 3), is necessary for melanoma cell forming close contact with the EC.

Blocking ICAM-1 on WM9 significantly reduced melanoma adhesion efficiency compared with both the control and the anti-ICAM-1 on EI cases (Fig. 7B), which indicates that β_2 integrins/ICAM-1 adhesion between PMNs and melanoma cells, the *second* step in the “two-step adhesion” model (Fig. 3), is more important than that between PMN and EC for successful melanoma cell in close contact with the EC. Although the first step of PMN tethering in the “two-step adhesion” is necessary for PMN-facilitated melanoma adhesion, it is not sufficient because the PMN tethering could not guarantee the melanoma adhesion. Fig. 7B shows that melanoma adhesion decreased more significantly by blocking ICAM-1 on melanoma, however, leaving the PMN tethering unchanged.

CXCR1/2 receptors influence PMN recruitment of melanoma cell to the endothelium

To examine the possibility that chemokines could stimulate communication between PMNs and melanoma cells, unstimulated PMNs, fMLP-stimulated PMNs, IL-8-stimulated PMNs or PMNs whose IL-8 receptors (CXCR1 and CXCR2) were functionally blocked were perfused, respectively, together with WM9 cells through the parallel-plate flow chamber. WM9 adhesion efficiencies were calculated for the different cases at a shear rate of 62.5 s^{-1} and a shear stress of 2 dyn/cm^2 (Fig. 8). There was no significant difference in WM9 adhesion efficiency between the cases when PMNs were stimulated with IL-8 or fMLP, but significant differences occurred between the stimulated and nonstimulated PMN groups (Fig. 8). WM9 adhesion efficiency decreased dramatically in the presence of CXCR1/CXCR2-blocked PMNs compared with unstimulated PMNs (Fig. 8), indicating that PMN plays a role in recruiting melanoma cells to the EC. IL-8 increases PMN-facilitated melanoma cell arrest on the EC.

Discussion

Melanoma cells produce various cytokines and chemokines that attract leukocytes. The inflammatory component of a developing neoplasm may include a diverse leukocyte population–for example, neutrophils (PMNs), dendritic cells, macrophages, eosinophils and mast cells, as well as lymphocytes–all of which are capable of producing an assorted array of

cytokines. A thorough understanding of the mechanisms through which the immune/inflammatory system promotes cancer metastasis could help researchers develop new therapeutic interventions.

In this study, a parallel-plate flow chamber was used to examine how the dynamic shear environment influences the adhesive interactions between the EC, PMNs and melanoma cells. The major findings of this work are: (1) shear rate rather than shear stress modulates the PMN-facilitated melanoma cell into close proximity to the EC; (2) under shear conditions, LFA-1 and Mac-1 cooperate to achieve maximal PMN–EC adhesion and PMN–melanoma cell aggregation; and (3) E-selectin on EC and ICAM-1 on both EC and melanoma cells mediate tumor cell arrest to the EC via tethered PMNs. Together, these data provide strong evidence that the contact time for bond formation between PMNs and tumor cells, which is most affected by changes in shear rate, is the critical determinant of PMN-facilitated melanoma extravasation within the microcirculation.

The initial step in melanoma extravasation is tumor cell adhesion to the EC. PMNs typically use selectin receptors to form rolling attachments along the vessel wall and β_2 integrins to anchor firmly and support onward migration through the EC [11]. In contrast, melanoma cells do not express β_2 integrins, sLe^x or other sialylated molecules at levels to effectively adhere to the EC within the circulation [35]. Melanoma cell interaction with the EC is distinct from PMN–EC adhesion in that no known integrin or lectin molecules are involved. Published data have provided evidence that the initial microvascular arrest of metastasizing tumor cells does not occur through a “leukocyte-like” rolling adhesive interaction with vascular endothelium [36]. It becomes apparent that melanoma cell adhesion to the EC is fundamentally different from leukocytes, which requires a new paradigm. Melanoma cell adhesion to the EC may involve a cascade of cell margination, hydrodynamic rolling, melanoma–PMN aggregation as a result of collisions and final arrest via PMN-facilitated recruitment to the EC. Melanoma “margination” occurs by the same process as with PMNs. “Hydrodynamic rolling” is the traditional concept from PMN–EC interactions that describes the fast movement of cells near the EC without actual receptor–ligand based adhesion. Hydrodynamic rolling is easily detected by comparing the cell velocity with the free stream velocity at a given point in the flow stream. The “melanoma–PMN aggregation” step is a newly defined process characterizing how PMNs facilitate the recruitment of melanoma cells to close proximity with the EC without significant receptor–ligand-mediated melanoma–EC interaction.

A recent study has shown that melanoma cells by themselves cannot migrate through the EC under flow conditions; however, when PMNs are present, melanoma cell migration is observed. This process is due to ICAM-1, expressed on both melanoma and endothelial cells, binding with PMN β_2 integrins [1]. In the current study, PMNs have been directly observed facilitating melanoma cells contact with the EC. Once E-selectins initiate PMN tethering, β_2 integrins mediate stable arrest of PMNs to the EC. Since melanoma cells also express ICAM-1, a collision with PMNs in a shear flow could result in the formation of aggregates. In the case of a collision between melanoma cells in the free stream and already-tethered PMNs, the resulting aggregation would give melanoma cells access to the EC.

Results shown in Fig. 2A indicate that PMN tethering on the EI monolayer is significantly affected by both shear rate and shear stress. This agrees with previously published data on monocyte tethering to the vascular endothelium [32]. The present study provides the first report to date on how PMN tethering frequency changes in response to varying shear rate at a fixed shear stress. Results show that for a fixed shear stress, 2 dyn/cm^2, the tethering frequency decreases as shear rate increases from 62.5 s^{-1} to 200 s^{-1}. This result may be due to a longer contact time between PMNs and EC at a lower shear rate, which increases the number of bonds that will form and thus increases the tethering frequency. As shown here, PMN tethering

frequency also depends on shear stress, which is related to cell deformation. When PMNs encounter a flat surface, they deform and allow increased bond formation with the EC. Since leukocyte adhesion through selectins requires a threshold shear to support cell adhesion [37], catch bonds have been suggested to partly explain the shear threshold requirement for selectin-mediated adhesion [38]. Previous studies have shown that low forces decrease L-selectin-PSGL-1 off-rates (catch bonds) [39], therefore, an alternative hypothesis for the increase of PMN tethering frequency with increasing shear stress (especially at the low shear rate 62.5 s^{-1} and 100 s^{-1}) may be the catch bond-like behavior between E-selectin on EI and its ligand on PMN.

PMN tethering frequency reflects the number of PMNs rolling and arrested on the EC, whereas WM9 adhesion efficiency describes WM9 aggregation to tethered PMNs on the EC, thus facilitating melanoma cell arrest on the endothelial monolayer. The actual event in WM9–PMN aggregation was found by some to be reversible with WM9s attached to or detached from those tethered PMNs, but others to be apparent “irreversible”. Although PMN tethering frequency is both shear stress and shear rate-dependent, experimental results indicate that PMN-facilitated melanoma cell contact with the EC is shear rate-dependent rather than shear stress-dependent (Figs. 2B and 5). Effects of shear stress on PMN tethering may become less apparent based on the results shown in Fig. 2A (e.g., up to shear rate 200 s^{-1}) because the shear force would be too large for a successful PMN–EC adhesion to form. However, shear stress does not enhance melanoma arrest on EC via aggregation to PMNs (Fig. 2B) even under a low shear rate at 60 s^{-1}. This difference supports the “two-step adhesion” model proposed in this study: PMN tethering on the EC followed by melanoma cell-PMN aggregation bringing tumor cells into close contact with the EC (Fig. 3). A mechanism similar to that which increases PMN tethering frequency at lower shear rate is most likely responsible for the behavior of WM9 adhesion efficiency under different shear rates. Shear rate modulates the cell–cell collision frequency and contact time determined by the relative velocities of cells in the fluid. A lower shear rate increases the time that cells are in contact, thus allowing more WM9–PMN binding to the endothelial monolayer with a resulting increase in WM9 recruitment. However, since WM9 recruitment to the EC does not depend on shear stress, this suggests the mechanism of PMN-facilitated WM9 cell contact with the EC is different than that of PMN tethering. Whereas PMNs are able to adhere anywhere on the flat EC, WM9 cells only adhere to PMNs, which are not flat and only sporadically tethered to the EC monolayer. This suggests the contact between WM9 cells and PMNs is the more important step in determining PMN-facilitated melanoma margination to the EC.

PMNs form a two-way bridge by binding to ICAM-1 on both endothelial and melanoma cells through LFA-1 and Mac-1. The parallel-plate flow assay elucidates the distinct roles for LFA-1 and Mac-1 in PMN-facilitated melanoma cell interactions with the EC. Blocking LFA-1, Mac-1 or both partially decreases WM9 binding to tethered PMN under all tested shear conditions (Fig. 6A), which illustrates that both LFA-1 and Mac-1 are requisite but neither is sufficient for WM9 maintenance on the EC via WM9–PMN aggregation. Previously published work showed a similar trend for PMN binding to ICAM-1-expressing/sLe^x-low HCT-8 cells [14]. The present study shows that LFA-1 affects melanoma cell contact with the EC over the entire experimental time duration; whereas the effect of Mac-1 is only seen after 3 min (Fig. 6B). These findings are in agreement with a study by Hentzen et al. [12] of PMNs binding to ICAM-1-transfected cells, which showed that heterotypic adhesion begins with LFA-1-dependent initial endothelial capture of PMNs followed by Mac-1-mediated stabilization.

Previous studies have shown that endothelial E-selectin initiates leukocyte capture to the EC and facilitates rolling by binding sialylated ligands expressed on PMNs [40,41]. E-selectin, constitutively expressed by EI cells, has been shown to support PMN rolling in vitro using laminar flow chamber assays at near-physiologic shear stresses [42,43]. Blocking E-selectin

and ICAM-1 on EI monolayer inhibits the capture and rolling of PMNs on the EC (Fig. 7A), resulting in decreased WM9 adhesion efficiency (Fig. 7B). Thus, PMN tethering to the EC is necessary for melanoma cell contact with the EC. Blocking ICAM-1 on WM9 cells significantly reduces the WM9 recruitment efficiency, indicating that a β_2 integrins/ICAM-1 binding mechanism is involved in melanoma–PMN and PMN–EC interactions. This finding is in contrast to a published study of PMN binding to ICAM-1-expressing/sLe^x-low HCT-8 cells, which showed that the ICAM-1 blocking alone could not substantially reduce PMN–HCT-8 cell adhesion at a longer shear exposure time [15]. However, this difference may be due to the presence of another potential ligand for LFA-1 and Mac-1 with a higher affinity than ICAM-1 on the surface of HCT-8 cells [15]. In addition, blocking ICAM-1 on WM9 cells is more effective in decreasing melanoma adhesion efficiency than blocking ICAM-1 on EI cells, suggesting that the first step of PMN tethering in the "two-step adhesion" model is necessary but not sufficient for maximal melanoma adhesion to the EC. Therefore, the binding mediated by ICAM-1 between tumor cells and PMNs could be a potential therapeutic target.

There may be other ligands for melanoma cells normally found on endothelium but absent in the EI model. However, we have not found that human melanoma cells express sLe^x ligand for endothelial E-selectin (Table 1), although such ligand is abundantly expressed on human gastric or colon carcinoma cells [35]. We have tested WM9 adhesion on HUVEC in a parallel-plate flow assay, but have not seen apparent melanoma rolling or direct adhesion to the HUVECs under flow conditions (data not shown), which agrees with other in vivo finding [36] and in vitro finding [44]. Since our primary goal for this paper would be limited to E-selectin and ICAM-1 and their roles in PMN tethering and PMN-mediated melanoma arrest on EC, we used EI cell as a model. Further studies should definitely be carried out in order to fully understand melanoma–endothelium interactions.

Since melanoma cells do not express CXCR1 or CXCR2 [45], soluble IL-8 released by both melanoma cells and PMNs only acts on PMNs [1] and results in an increased Mac-1 expression on PMNs (data not shown). To examine the role that endogenous IL-8 has in PMN-facilitated melanoma cell adhesion, CXCR1 and CXCR2, the IL-8 receptors on PMNs were blocked. Blocking the IL-8 receptors on PMNs substantially inhibited melanoma cell aggregation to PMNs and subsequent contact with the EI monolayer compared to those cases using unstimulated PMNs ($P < 0.05$; Fig. 8). Furthermore, IL-8-stimulated PMNs increased WM9 adhesion efficiency significantly compared with inactivated PMNs. This confirms that the presence of certain chemokines, including IL-8, would affect PMN-facilitated melanoma adhesion and a mechanism for PMN recruitment of melanoma cell to the EC. These insights are fostering new approaches to cancer treatment through anti-inflammatory therapeutics.

In summary, this study examined molecular interactions between the EC, PMNs and melanoma cells under well-defined hydrodynamic shear conditions. Consistent with published reports [12,13], the roles of LFA-1 and Mac-1 in melanoma maintenance on the EC are to bind PMNs to both EC and melanoma cells. It is likely that the source of the distinct functions of LFA-1 and Mac-1 in regulating melanoma cell in contact with the EC may be their different responses to time and hydrodynamic shear. Two separate binding events are necessary for PMN-facilitated melanoma cell arrest on the EC. The first binding interaction is PMNs tethering to the EC and the second binding interaction is the aggregation of melanoma cells with tethered PMNs, which requires both collisions and bond formations between them. PMN tethering to an inflamed substrate mediates tumor-PMN aggregate adhesion to EC and enhances subsequent tumor cell extravasation within the microcirculation. Although PMN-endothelial cell adhesion requires the involvement of both E-selectin and ICAM-1 and is influenced by shear stress and shear rate, the adhesion between tethered PMNs and melanoma cells is regulated by β_2 integrins and ICAM-1 and is influenced by shear rate only. In addition, endogenously produced IL-8 does contribute to PMN-facilitated melanoma contact with the EC through the CXCR1 and

CXCR2 receptors on PMNs and different stimuli to PMNs or melanoma cells may alter the ability of melanoma–PMN interactions. Results from this study suggest that the significant PMN adhesion to the endothelium during the inflammation may increase the likelihood of melanoma metastasis. This study should have important implications for the development of novel therapeutics for the treatment and prevention of melanoma metastases through anti-inflammation.

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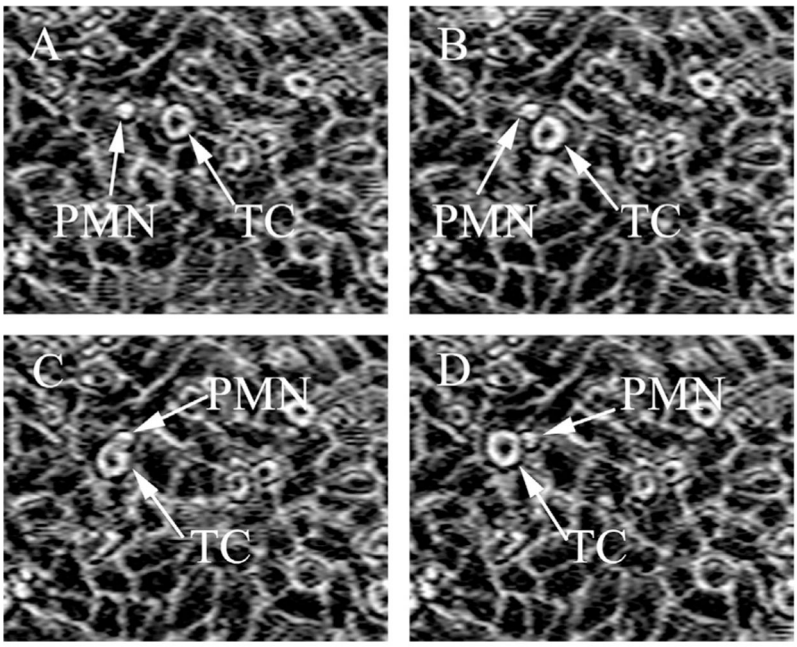

Fig. 1.

Aggregation of melanoma cell (TC) to tethered PMN on EI monolayer at a shear rate of 200 s^{-1} and shear stress of 2 dyn/cm^2. Flow direction is from right to left. (A) 0 s, melanoma cell and PMN on the EI monolayer; (B) 10 s, collision between melanoma cell and PMN; (C) 20 s, aggregation between melanoma cell and PMN due to collision; (D) 30 s, attachment of melanoma cell to EI monolayer due to the formation of aggregates.

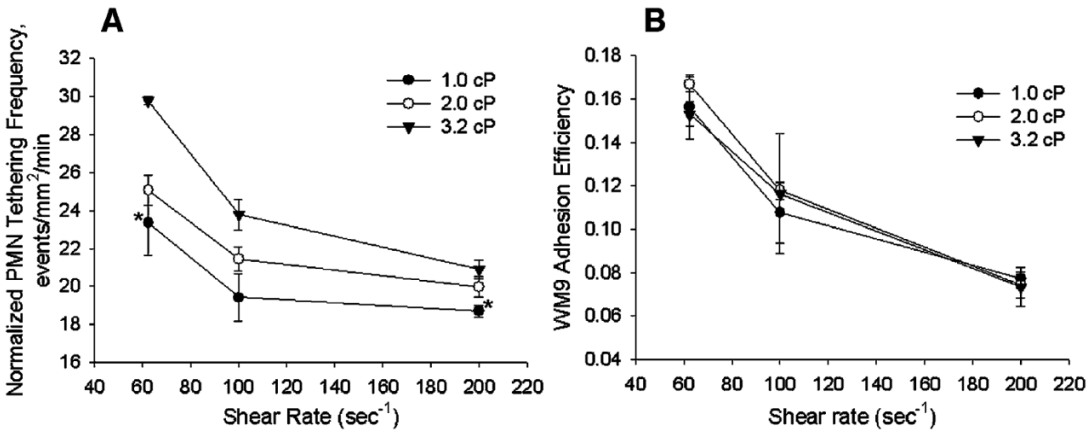

Fig. 2.

Effects of shear stress and shear rate on (A) PMN tethering frequency and (B) melanoma cell adhesion efficiency on the EC. WM9 adhesion efficiency and data correction procedure are defined in Materials and methods. **P* <0.05 compared with the 3.2 cP case at the same shear rate. Values are mean ± SEM for $N \geq 3$.

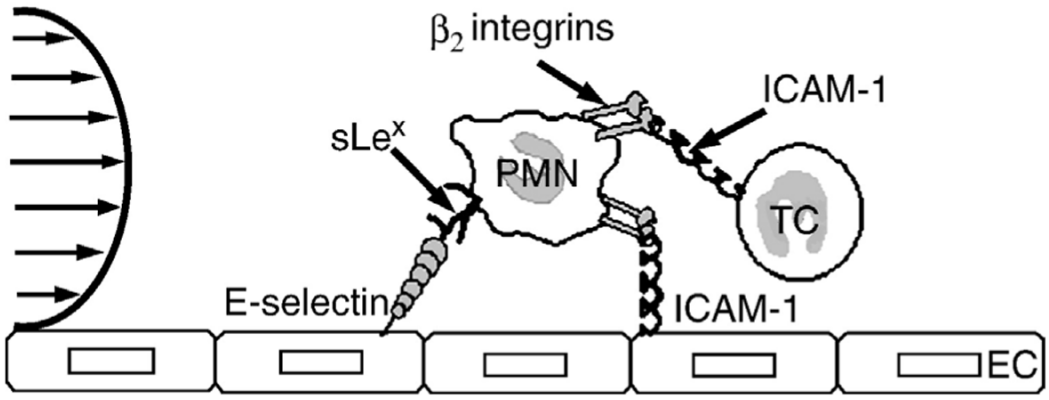

Fig. 3.

Concept of a “two-step adhesion” mechanism in bridging melanoma cell (TC) into close proximity to the EC via tethered PMN in a shear flow. The *first step* is PMN tethering on the EC followed by the *second step* in which melanoma cells are captured by tethered PMNs via β_2 integrins/ICAM-1 interactions.

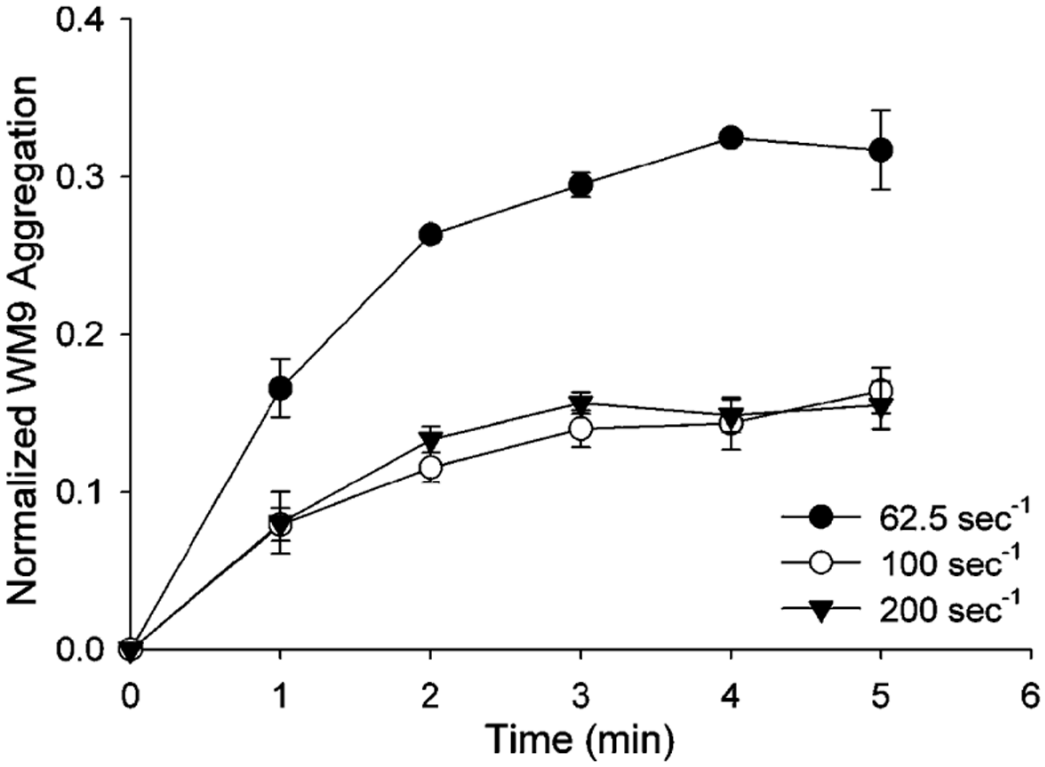

Fig. 4.

Normalized WM9 aggregation was used to quantify the extent of WM9 aggregation to tethered PMNs, which is the number of WM9–PMN aggregates normalized to the total number of tethered PMNs during each time course in the entire parallel-plate flow assay. Data show the aggregation between entering WM9 and tethered PMNs at different shear rates over the flow assay. Values are mean ± SEM for $N \geq 3$.

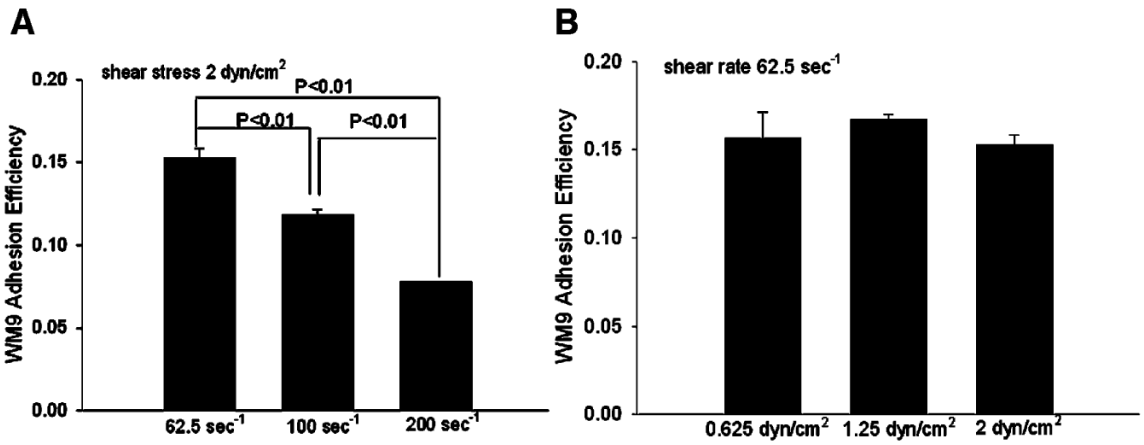

Fig. 5.

Effects of shear stress and shear rate on WM9 cell arrest on the EI monolayer via tethered PMNs. WM9 adhesion efficiency is defined in Materials and methods. Results show the melanoma cell adhesion efficiency under (A) different shear rates at the same shear stress; or (B) different shear stress at a fixed shear rate. Values are mean ± SEM for $N \geq 3$.

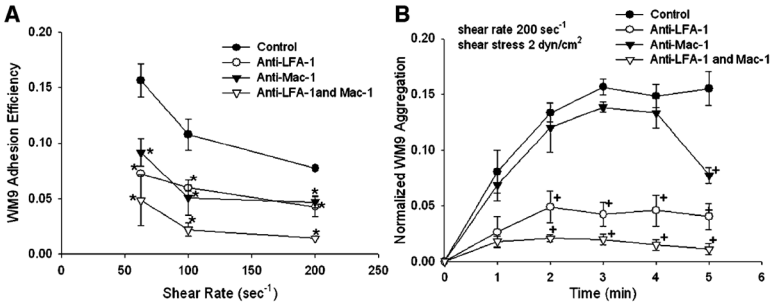

Fig. 6.

Contributions of LFA-1 and Mac-1 to WM9 arrest to the EI substrate as a result of WM9–PMN collisions. (A) WM9 adhesion efficiency at different shear rates over a period of 5 min. Shear stress was 2 dyn/cm^2 for the data shown. (B) Normalized WM9 aggregation during each time course in the entire parallel-plate flow assay. *P <0.05 compared with control at the same shear rate. ^+P <0.05 compared with control at the same time point. Values are mean ± SEM for $N \geq 3$.

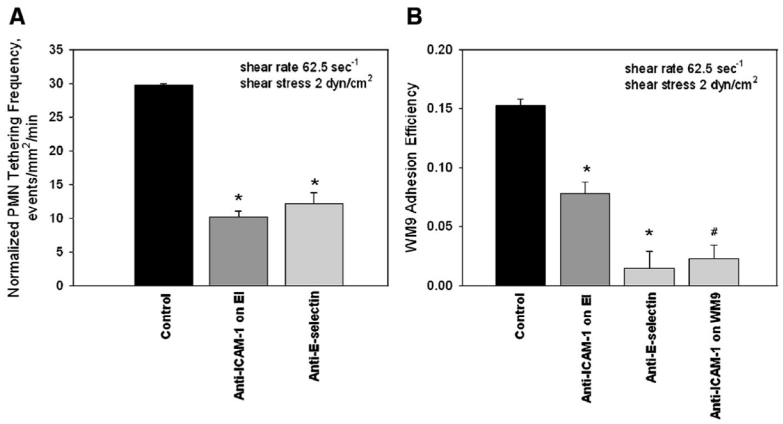

Fig. 7.

Relative role of E-selectin and ICAM-1 in (A) PMN tethering frequency and (B) PMN-facilitated melanoma cell adhesion under shear conditions. **P* <0.05 compared with control samples at the same shear condition. #*P* <0.05 with respect to concurrent ICAM-1 block of EI cells under the same shear condition. Values are mean ± SEM for $N \geq 3$.

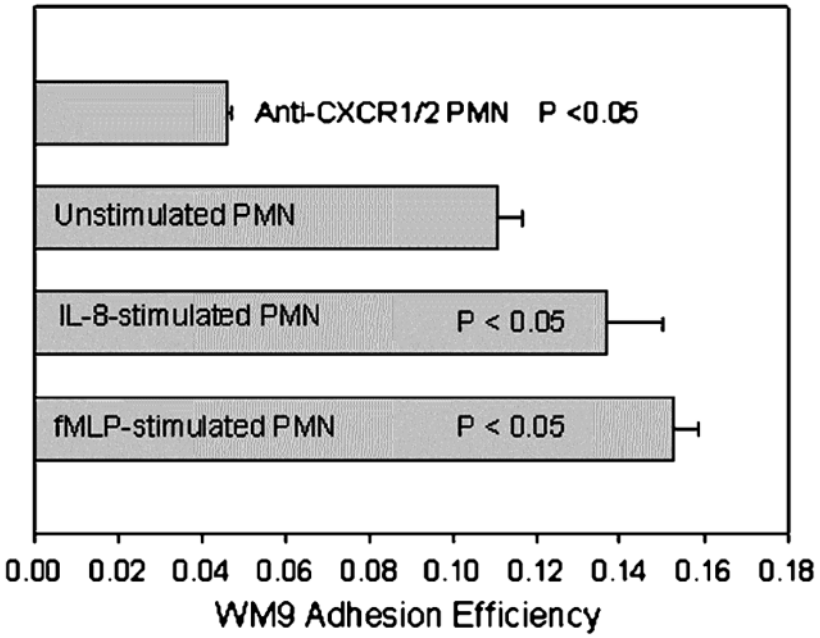

Fig. 8.

Effects of intercellular chemokine signaling and PMN activation on melanoma cell arrest on the EC under flow conditions (shear rate 62.5 s^{-1}, shear stress 2 dyn/cm^2). *P* values were with respect to unstimulated PMN case. Values are mean ± SEM for $N \geq 3$.

Table 1

Flow cytometric analysis of adhesion receptor expression on EI cells and WM9 cells[a]

Receptor	Geometric mean fluorescence	
	WM9	EI cells
Control IgG (control IgM)	0.67 ± 0.03 (6.35 ± 0.27)	0.5 ± 0
sLe^x	(5.97 ± 0.16)	–
LFA-1	0.8 ± 0	–
Mac-1	0.60 ± 0.05	–
ICAM-1	13.4 ± 0.6	4.53 ± 0.29
E-selectin	–	8.13 ± 0.58

[a]Values are geometric mean fluorescence intensities ± SEM of three experiments using different batches of cells each time.