

Melanoma Cell Extravasation under Flow Conditions Is Modulated by Leukocytes and Endogenously Produced Interleukin 8

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Abstract: Attachment of tumor cells to the endothelium (EC) under flow conditions is critical for the migration of tumor cells out of the vascular system to establish metastases. Innate immune system processes can potentially promote tumor progression through inflammation dependant mechanisms. White blood cells, neutrophils (PMN) in particular, are being studied to better understand how the host immune system affects cancer cell adhesion and subsequent migration and metastasis. Melanoma cell interaction with the EC is distinct from PMN-EC adhesion in the circulation. We found PMN increased melanoma cell extravasation, which involved initial PMN tethering on the EC, subsequent PMN capture of melanoma cells and maintaining close proximity to the EC. LFA-1 (CD11a/CD18 integrin) influenced the capture phase of PMN binding to both melanoma cells and the endothelium, while Mac-1 (CD11b/CD18 integrin) affected prolonged PMN-melanoma aggregation. Blocking E-selectin or ICAM-1 (intercellular adhesion molecule) on the endothelium or ICAM-1 on the melanoma surface reduced PMN-facilitated melanoma extravasation. Results indicated a novel finding that PMN-facilitated melanoma cell arrest on the EC could be modulated by endogenously produced interleukin-8 (IL-8). Functional blocking of the IL-8 receptors (CXCR1 and CXCR2) on PMN, or neutralizing soluble IL-8 in cell suspensions, significantly decreased the level of Mac-1 up-regulation on PMN while communicating with melanoma cells and reduced melanoma extravasation. 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 endothelium in the microcirculation, which are significant in foster-

ing new approaches to cancer treatment through anti-inflammatory therapeutics.

keyword: Cancer, Immunoediting, Adhesion molecules, Chemokines, Signaling, Endothelium, Shear stress, Adhesion and migration.

1 Introduction

It has become evident from *in vivo* studies (Chambers *et al.*, 2000; Liotta, 1992; Scherbarth and Orr, 1997; Zetter, 1993) that the mechanisms utilized by leukocytes and metastatic tumor cells to adhere to a vessel wall prior to extravasation are very different. Human leukocytes, including neutrophils (PMN), actively participate in the inflammatory response via adhesion to vascular endothelium (Harlan and Liu, 1992; Lauffenburger and Linderman, 1993; Smith *et al.*, 1989). Detailed studies of cell-cell interactions suggest that selectins are required for the initial rolling of leukocytes on activated endothelium. L-selectin and Sialyl Lewis X (SLe X) on leukocytes and E- and P-selectin on endothelial cells (EC) participate in these interactions (Lawrence and Springer, 1991; Ley *et al.*, 1995). Stronger binding mediated by β_2 integrins expressed on the leukocyte (Mac-1 or LFA-1) and ICAM-1 (intercellular adhesion molecule) on the endothelial cells is responsible for prolong shear-resisted attachment (Springer, 1994). Recent studies have suggested that LFA-1 to ICAM-1 adhesion is important in initial endothelial capture of PMN, while Mac-1 and ICAM-1 interaction forms shear-resisted bonds to stabilize PMN-endothelium adhesion (Hentzen *et al.*, 2000; McDonough *et al.*, 2004; Neelamegham *et al.*, 1998). Several ligands for inducible endothelial adhesion molecules have been identified on various types of tumor cells (Yamada, 1993). For example, molecules containing the SLe X determinant, which is the ligand for E-selectin on endothelium, are abundantly expressed on human gastric or colon carcinoma cells (Zetter, 1993). Integrin $\alpha_4\beta_1$ molecules are expressed on RAW117 lym-

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phoma cells, which interact with VCAM-1 (vascular adhesion molecule) expressed on hepatic sinusoidal endothelial cells (Papadimitriou *et al.*, 1999). Miele *et al.* (1994) reported that a dose- and time-dependent increase in surface expression of ICAM-1 was found in human malignant melanoma cells. They also found that inhibiting ICAM-1 reduced melanoma lung metastasis *in vivo*. All these studies have supported an adhesive mechanism between tumor cells and the endothelium, rather than a simple mechanical entrapment such as vessel-size restriction.

How do tumor cells bind EC? Several studies have focused on the ability of lymphocytes (Kripke, 1994), natural killer cells (Hanna, 1985), T-cells (Willimsky and Blakenstein, 2005) and monocyte/macrophages (Key, 1983; Mantovani, 1994; van Netten *et al.*, 1993; Pollard, 2004) to mediate tumor cell binding and affect tumor progression (Dunn *et al.*, 2002). In particular, human PMN, which comprise 50-70% of circulating leukocytes and are usually cytotoxic to tumor cells, have been shown under certain circumstances to promote tumor adhesion and transendothelial migration (Wu *et al.*, 2001; Slattery and Dong, 2003). An interesting study showed that PMN and activated macrophages increased the ability of rat hepatocarcinoma cells to adhere to an endothelial monolayer (Starkey *et al.*, 1984). In addition, tumor-elicited PMN, in contrast to normal PMN, were found to enhance metastatic potential and invasiveness of rat mammary adenocarcinoma cells in an *in vivo* tumor-bearing rat model (Welch *et al.*, 1989). Using light and electron microscopy, circulating PMN were discovered in close association with metastatic tumor cells including at the time of tumor cell arrest (Crissman *et al.*, 1985). Although these studies have clearly suggested that the immune system, and PMN in particular, could enhance tumor cell metastasis, there is little understanding of the mechanisms involved (Burdick *et al.*, 2001; Coussens and Werb, 2002; Liotta and Kohn, 2001).

Although several cytokines and chemokines have been implicated in influencing adhesive properties of transformed cells, interleukin 8 (IL-8) is of particular interest. IL-8 has a wide range of pro-inflammatory effects, which mediate PMN migration from the circulation to sites of injury via activation of CXC chemokine receptors 1 and 2 (CXCR1 and CXCR2) on PMN (Walz *et al.*, 1987; Murphy and Tiffany, 1991; Holmes *et al.*, 1991). IL-8 secretion is also a marker for increasing

metastatic potentials, *e.g.*, as an important promoter for melanoma growth (Schadendorf *et al.*, 1995; Singh and Varney, 1998). IL-8 could potentially enhance PMN binding to melanoma cells and the endothelium (Singh *et al.*, 1999). Chemokines or cytokines secreted by tumor cells and PMN may play an important role in communication between tumor cells and PMN and affect the interactions between them as well (Fredrick and Clayman, 2001). However, how those chemokine expressions are regulated in a tumor microenvironment and what roles those upregulated chemokines play in mediating tumor cell extravasation within the circulation are not known.

The objectives of this paper are to apply novel *in vitro* flow and migration assays and delineate mechanisms possibly involved in PMN-mediated melanoma extravasation under the influence of hydrodynamic forces, chemokine up-regulation and melanoma-leukocyte-endothelial adhesion. We conclude that PMN-facilitated melanoma cell arrest on the endothelium and subsequent melanoma trans-endothelial migration are regulated by β_2 integrins/ICAM-1 adhesion and modulated by endogenously produced IL-8. These findings are significant in fostering new approaches to cancer treatment through anti-inflammatory therapeutics (Homey *et al.*, 2002).

2 Materials and Methods

2.1 Reagents

Type IV collagen was purchased from BD Discovery Labware (Bedford, MA). Phorbol 12-myristate 13-acetate (PMA), human serum albumin (HSA) and bovine serum albumin (BSA) were all purchased from Sigma (St. Louis, MO). Recombinant human IL-8 protein, mouse anti-human IL-8, mouse anti-human E-selectin, and mouse anti-human CXCR1/CXCR2 mAbs were purchased from R&D systems (Minneapolis, MN). Mouse anti-human CD11a, mouse anti-human CD11b, and mouse anti-human ICAM-1 were purchased from CalTag (Burlingame, CA).

2.2 Cell Culture and Choice of Melanoma Cell Lines

We utilized three different human melanoma cell lines (Table 1), to correlate tumor metastatic potentials with melanoma cell invasiveness, chemotactic migration and adhesiveness. "Metastatic" potential was qualitatively determined from the cell line origin; "Chemotactic" po-

Table 1 : Choice of Melanoma Cell Lines

Cell line	Potentials			Reference:
	Metastatic	Chemotactic (CIV)	Adhesion (ICAM-1)	
WM 9	++	+++	+++	www.wistar.upenn.edu/herlyn/melcell.htm
C8161	+++	+++	++	<i>Int J Cancer</i> 47 :227, 1991.
WM35	-	+	+	www.wistar.upenn.edu/herlyn/melcell.htm

tential was measured by cell static migration toward soluble type IV collagen (CIV; 100 μ g/ml) using Boyden chamber; and “Adhesion” potential was quantified by comparing relative mean fluorescence levels of ICAM-1 expression obtained by flow cytometry. We also used non-metastatic primary human melanocyte (BioWhittaker/Clonetics, CA) as a control.

C8161.c9, WM9 and WM35 melanoma cells were maintained and prepared as described previously (Welch *et al.* 1991; Liang *et al.*, 2005). NHEM (melanocytes) were maintained in culture according to the manufacturer’s suggested protocol (Slattery and Dong, 2003). Prior to each experiment, melanoma cells were detached when nearly confluent and suspended at a concentration of 1x10⁶cells/ml. In assays where anti-ICAM-1 blocking antibodies were used, cells were incubated with mAb (5 μ l/ml) for 30 minutes at 37°C prior to the start of an assay. IgG isotype control antibody was used to verify specificity of all blocking antibodies.

EI cells are cells that had been transfected from fibroblast L-cells to express human E-selectin and ICAM-1, which were grown in tissue culture to a monolayer as described elsewhere (Simon *et al.*, 2000). EI cells were used in this study as a substrate for cell adhesion and as a model of an “endothelial monolayer”. 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 (HUVECs) (Gopalan *et al.*, 1997). For some adhesion receptor blocking assays, the confluent EI monolayer was treated with anti-ICAM-1 mAb (5 μ g/10⁶ cells) or anti-E-selectin mAb (5 μ g/10⁶ cells).

2.3 Neutrophil Isolation and Preparation

Fresh blood was obtained from healthy adults following a Penn State Institutional Review Board approved protocol. Histopaque[®] gradient (Sigma) was used to isolate and enrich the PMN population. The iso-

lated PMN layer was first suspended in 0.1% HSA in DPBS and washed. ACK lysis buffer (0.15M NH₄Cl, 10.0mM KHCO₃, 0.1mM Na₂EDTA in distilled H₂O) was used to remove erythrocytes. The cells were washed with 0.1%HSA/DPBS, resuspended at a concentration of 1x10⁶cells/ml and rocked at 4 ° C until they were used, no longer than 4 hours. To activate LFA-1 or Mac-1 on PMN, cells were treated with PMA (100ng/ml, 20 min) or IL-8 (1ng/ml, 1 hr) respectively. PMA was not found to affect Mac-1 expression on PMN (data not shown). In blocking assays, PMN were treated with saturating concentrations of antibodies, 5 μ g of anti-human CD11a or CD11b per 1x10⁶ cells, in blocking buffer (5% calf serum, 2% goat serum in DPBS) for 30 minutes at 4 ° C. Similarly, to block IL-8 receptors, PMN were treated with mouse anti-human CXCR1 and CXCR2 antibodies with 6 μ g/ml and 10 μ g/ml, respectively, in blocking buffer for 30 minutes at 4 ° C.

2.4 Flow Migration Assay

The *in vitro* flow-migration device (Fig. 1, top right) is a recently developed modified 48-well chemotactic Boyden chamber (Dong *et al.*, 2002). In brief, the top and bottom plates of the polycarbonate chamber are separated by a 0.02 inch-thick silicon gasket (PharmElast, SF Medical, Hudson, MA). A 7cm \times 2cm 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.

An endothelial monolayer was formed by growing EI cells to confluence on sterilized PVP-free polycarbonate filters (8- μ m pore size; NeuroProbe, Gaithersburg, MD) coated with fibronectin (30 μ g/ml, 3 hr) (Sigma). The bottom side of the filter was scraped prior to use to remove any potential cell growth. Soluble type IV collagen (CIV; 100 μ g/ml in RPMI 1640/0.1%BSA) (Aznavorian *et al.* 1990; Hodgson and Dong, 2001) 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 were then introduced into the chamber. Typical experiments involved cases such as: PMN only; melanoma cells (C8161, WM9, or WM35) only; PMN + selected-type melanoma cells; 5×10^5 cells of each cell type. Flow of circulating medium was immediately perfused into the chamber, initially at a flow rate 2 ml/min, which was then increased to a desired experimental rate (0-20 ml/min). The entire chamber was placed in a 37°C, 5% CO₂ incubator for 4 hours.

To quantify migration, 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 using an inverted microscope and recorded with NIH Image (v. β 4.0.2). No cells were found in the chemoattractant wells after 4 hours of migration (Slattery and Dong, 2003; Slattery *et al.*, 2005). Three pictures were taken of each filter in different locations. The number of cells migrated was quantified and averaged for each filter. A minimum of three filters were analyzed for each data point. Background migration was subtracted from each sample as appropriate.

2.5 Parallel Plate Flow Assay

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 was attached to the flow chamber by vacuum and placed on the inverted microscope. All experiments were performed at 37°C. Flow experiments were recorded and the field of view was 800 μ m long (direction of the flow) by 600 μ m. The focal plane was set on the endothelial monolayer (Fig. 1, bottom). The flow chamber was perfused with appropriate media over the endothelial monolayer for 2-3 minutes at a shear rate of 40 sec⁻¹ for equilibration before the introduction of cells. A typical flow experiment involved perfusion of 10⁶/ml PMN and 10⁶/ml selected-type melanoma cells. After allowing cells to contact the endothelial monolayer at a shear stress of 0.1-0.3 dyn/cm² for 2 min to promote interactions with the monolayer, the shear stresses was adjusted to a desired

experimental rate and kept constant for 6-7 minutes. Experiments were performed in triplicate and analyzed off-line.

2.6 PMN Tethering Frequencies

The tethering frequency was determined experimentally as the number of PMN that adhered to the endothelial monolayer per unit time and area in the parallel-plate flow chamber assay (Fig. 1, bottom), 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.* (2001) based on equations derived by Munn *et al.* (1994).

2.7 Melanoma-PMN Aggregation and Adhesion Efficiency Analysis

Melanoma-PMN aggregation on the surface of an endothelial monolayer was analyzed by a parallel-plate flow assay. Quantification started at the onset of experimental shear rate ($t = 0$ min) and lasted for 5 min. Aggregates could be characterized by differences in cell sizes and velocities (Fig. 1, bottom). Aggregation variables to be quantified included the total number of PMN which were tethered (rolling or arrested) on the endothelial monolayer; collisions of melanoma cells (from the free stream near the endothelium) to tethered PMN; aggregation of melanoma with tethered PMN as a result of the collision; and final attachment of melanoma-PMN aggregates on the endothelial monolayer. For some cases in which more than one melanoma cell adhered to a PMN, we count such a case as two aggregates if two melanoma cells adhered to a PMN.

2.8 Melanoma Cell Adhesion Efficiency

“Melanoma adhesion efficiency” can be expressed by the following ratio:

$$\text{Melanoma Adhesion Efficiency} = \frac{\text{Number of melanoma cells arrested on the monolayer}}{\text{Number of melanoma collisions to PMN}}$$

The numerator is the number of melanoma cells arrested on the endothelial monolayer at the end of the entire flow assay as a result of collision between entering melanoma cells and tethered PMN. The denominator is the total

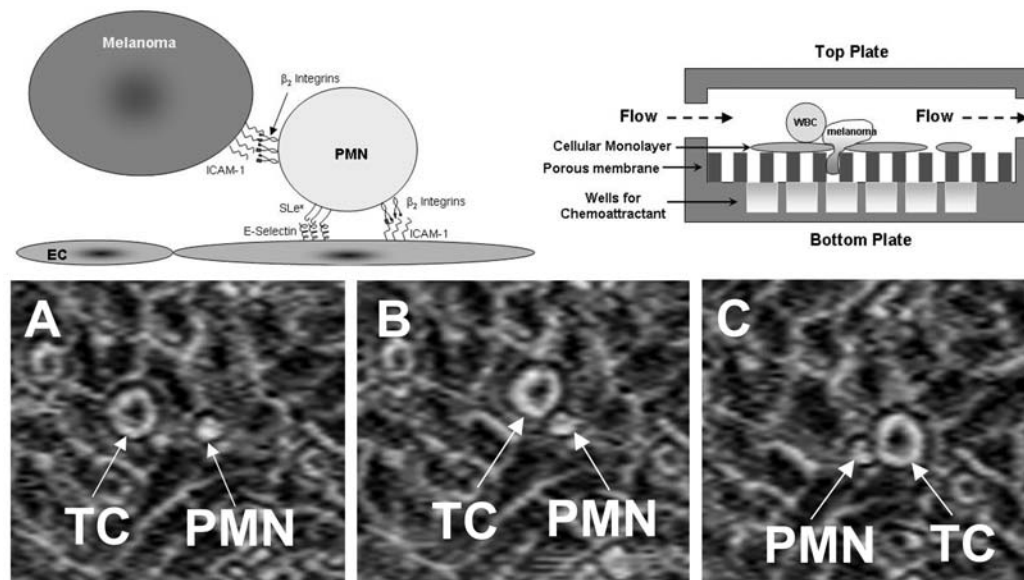


Figure 1 : Schematic of PMN-facilitated melanoma adhesion to the EC in a shear flow. Top left: a melanoma cell in close proximity to the EC via a tethered PMN. Melanoma cells are captured by tethered PMN on the EC via β_2 integrins/ICAM-1 interactions. Top right: cross-section view of the flow-migration chamber. Bottom: representative aggregation of melanoma cells (TC) to tethered PMN on an endothelial monolayer. Flow direction is from left to right: (A) a melanoma cell and a PMN on the monolayer at 0 second; (B) collision between a PMN and a melanoma cell after 20 seconds; and (C) arrest of a melanoma cell to the monolayer due to the formation of PMN-melanoma aggregates after 30 seconds.

number of melanoma-PMN collisions near the endothelial monolayer surface and is counted as a transient accumulative parameter throughout the entire flow assay.

2.9 Co-Culture of PMN and Melanoma cells

Selected-type melanoma cells were cultured in 6-well plates (Corning). PMN (5×10^6 per well), untreated or treated with anti-CXCR1 and CXCR2 antibodies (as described above), were added to each well either directly in contact with the melanoma cell monolayer or into a Transwell insert ($0.4 \mu\text{m}$ pore; Corning) above the monolayer. As a control, PMN were concurrently cultured in plates without melanoma cells. The plates were then incubated for 4 hours at 37°C and $5\% \text{CO}_2$. After 4 hours, the cell or supernatant samples were prepared for further analysis by either flow cytometry, ELISA or immunoblot (see descriptions below).

2.10 Cell Adhesion Molecule Expressions (Flow Cytometry)

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; $1 \mu\text{g}$ Ab/ 10^6 cells) for 30 minutes at 4°C . The cells were then treated with secondary antibody, FITC-conjugated goat anti-mouse IgG F(ab) $_2$ fragment ($1 \mu\text{g}/10^6$ cells) (Jackson ImmunoResearch, West Grove, PA) for 25 minutes at 4°C . In the case of blocking CXCR1 and CXCR2 receptors on PMN, PE-conjugated anti-Mac-1 ($1 \mu\text{l}/10^6$ cells; CalTag Laboratories) was used to avoid binding secondary antibody to the existing CXCR1 and CXCR2 antibodies. The specimens were washed again, fixed with 2% formaldehyde, and analyzed by GUAVA personal cytometer (GUAVA Technologies Inc., Burlingame, CA). As a control, background fluorescence was assessed using cells treated with secondary antibodies only or PE-conjugated isotype control.

2.11 Detection of IL-8 from Melanoma-PMN Co-Culture (ELISA)

Enzyme-linked immunosorbent assay (ELISA) detection of protein secretion was performed at the Penn State NIH Cytokine Core Facility. Samples were prepared from supernatant of cultured cells. Approximately 1×10^6 cells,

as counted using hemacytometer, were cultured in fresh medium for 4 hr. All supernatant samples were spun at 1500 rpm for 5 min to remove debris and stored at -80°C prior to analysis. In the cases of PMN and melanoma cell co-culture, the 2 cell types ($\sim 1 \times 10^6$ each) were cultured together in a 6 well plate (BD Biosciences) either directly in contact or with Transwell inserts on top, where melanoma cells were separated from PMN during the 4 hours co-culture.

2.12 Detection of Intracellular IL-8 Expression (Immunoblot)

Whole cell extracts were prepared from the cells co-cultured with Transwell inserts by resuspending cells in $40\ \mu\text{l}$ of lysis buffer (10mM Tris-HCl [pH 7.4], 150mM NaCl, 1mM EDTA [pH 8.0], 2mM Na_3VO_3 , 10mM NaF, 10mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 % NP-40, 1mM PMSF, 2ng/ml pepstatin A). Lysates were incubated on ice for 30 min followed by a centrifugation at 16,000 g for 1 min at 4°C . The pellet was discarded and the supernatant was mixed with 2 \times SDS running buffer (0.2% bromophenol blue, 4% SDS, 100mM Tris [pH 6.8], 200mM DTT, 20% glycerol) in 1:1 ratio. Samples were boiled for 3 min and $15\ \mu\text{l}$ was loaded onto a 15% SDS-PAGE gel and proteins were transferred to a $0.2\ \mu\text{m}$ PVDF membrane (Millipore Co., Billerica, MA) by electroblotting. Primary antibodies included rabbit anti-human IL-8 (Biosource, Inc.) and anti- β -actin IgG1 (Sigma Chemical Co.). Secondary antibodies were peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. Proteins were detected using the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech, Arlington Heights, IL).

2.13 Statistical Analysis

All results are reported as the mean \pm standard error of the mean (S.E.M.) 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.

3 Results

3.1 Adhesion Molecule Expressions on Melanoma and EI Cells

Flow cytometry was used to detect expression of cell surface adhesion molecule expressions. We did not find detectable Mac-1, LFA-1 and SLe X expressions on all

three-type melanoma cells tested, whereas significant ICAM-1 expression was found, especially on C8161 and WM9 cells (Table 1). The expression level usually remains stable over at least 4 hours (data not shown). Stable expressions of E-selectin and ICAM-1 were also confirmed on EI cells.

3.2 PMN-facilitated Melanoma Cell Extravasation under Flow Conditions

Melanoma cell chemotactic migration in response to type IV collagen (CIV) ($100\ \mu\text{g}/\text{ml}$) was characterized using the flow-migration chamber under both static and flow conditions. As a negative control, non-metastatic melanocyte migration was first tested under the static condition toward CIV and found to be near or at a background level (Fig. 2A, bottom). In comparison, highly-metastatic C8161 and WM9 cells were more actively migratory under the no-flow condition than low-metastatic WM35 cells (Fig. 2A, "Static"). When exposed to a shear flow ($\sim 0.4\ \text{dyn}/\text{cm}^2$), extravasations of C8161, WM9 and WM35 cells toward CIV were all significantly less than those under static conditions (Fig. 2A), at a level similar to the melanocyte case. Addition of PMN to the melanoma cell suspension significantly enhanced tumor cell extravasation under shear stress compared with melanoma cells only (all P values < 0.05). Obviously, tumor metastatic potential correlates with melanoma cell extravasation behavior.

Figure 2B-C indicates that each binding step between the triad of the endothelium, PMN and melanoma cell affects tumor cell extravasation under flow conditions. ICAM-1 was functionally blocked on the C8161 cells and EI cells, respectively. Both anti-ICAM/C8161 and anti-ICAM/EI cases significantly reduced melanoma extravasations. Blocking E-selectin on the endothelial cells reduced C8161 melanoma cell migration by 80% compared with the C8161 + IL-8/PMN case (Fig. 2B). However, blocking E-selectin on the EI cells without PMN did not really change melanoma extravasation (Fig. 2B, bottom).

Receptor-ligand binding via β_2 integrins (on PMN) and ICAM-1 (on melanoma cells) has been implicated in mediating PMN-melanoma adhesion under flow conditions (Fig. 1). To investigate this mechanism further, melanoma cell extravasations were assayed in the presence of PMN, in which LFA-1 and Mac-1 on PMN were inhibited with functional blocking antibodies. Blocking

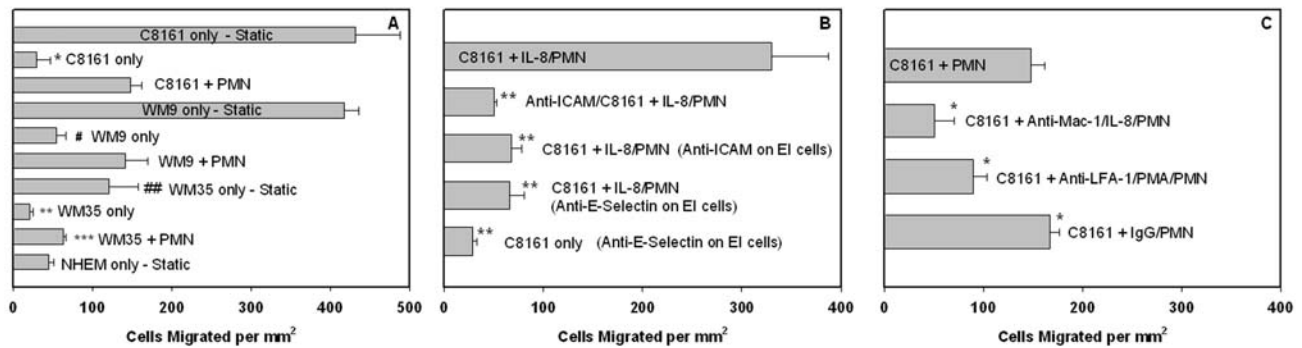


Figure 2 : PMN facilitate melanoma cell migration through an adhesion-mediated mechanism. (A) PMN affect C8161, WM9 and WM35 cell migration under shear conditions. NHEM, which are non-cancerous melanocytes used as a negative control, migrate at a low-threshold level even under the static condition. All cases were under a flow shear stress at 0.4 dyn/cm^2 , unless labeled “Static” (* $P < 0.01$ with respect to C8161 static case; # $P < 0.05$ with respect to WM9 static case; ** $P < 0.01$ with respect to WM35 static case; ## $P < 0.05$ with respect to both C8161 and WM9 static cases; and *** $P < 0.02$ with respect to both C8161+PMN and WM9+PMN cases). (B) ICAM plays an important role in PMN-facilitated melanoma cell migration. The second bar shows disrupting C8161-PMN aggregation by blocking ICAM-1 on melanoma significantly reduces melanoma cell migration (PMN were activated by IL-8). The third and fourth bars show inhibiting IL-8-stimulated PMN adhesion to the endothelium by blocking E-selectin or ICAM-1 on the monolayer also decreases C8161 cell migration (** $P < 0.01$ with respect to C8161+IL-8/PMN case at 0.4 dyn/cm^2). (C) Differential role of LFA-1 and Mac-1 on PMN. Both Mac-1 and LFA-1 (to lesser degree) contribute to PMN-mediated melanoma extravasation. The last bar shows migration with isotype antibody-treated PMN, as a control (* $P < 0.05$ with respect to C8161+PMN case at 0.4 dyn/cm^2). All the values are mean \pm S.E.M. for $N \geq 3$.

CD11b resulted in the greatest reduction in C8161 migration (66% reduction as compared to C8161+PMN under 0.4 dyn/cm^2 shear stress; Fig. 2C). Similar results are shown in Figure 2C; migration of melanoma cells was decreased by 40% in the presence of CD11a-blocked PMN.

Collectively, results shown in Figure 2 provide new evidence for the complex role of hemodynamic forces and PMN in the recruitment of metastatic cancer cells to the endothelium. PMN-melanoma adhesion and PMN-endothelium adhesion differentially affect PMN-facilitated melanoma migration under flow conditions, specifically via interactions of ICAM-1 and β_2 -integrins.

3.3 Relative Role of PMN-EC and PMN-Melanoma Adhesion in Melanoma Cell Arrest

EI cells which express endothelial E-selectin and ICAM-1 adhesion molecules were treated with mouse anti-human E-selectin and anti-ICAM-1 blocking mAbs, respectively. Blocking E-selectin and ICAM-1 on the monolayer significantly reduced PMN tethering to the EC (Fig. 3A) and subsequent melanoma cell adhesion

efficiency (Fig. 3B), compared with the respective controls. Blocking ICAM-1 on WM9 significantly reduced melanoma adhesion efficiency compared with the control (Fig. 3B), which indicates PMN-melanoma binding via β_2 integrins/ICAM-1 is important for PMN-facilitated melanoma cell arrest on the EC.

Functional blocking mAbs against CD11a and CD11b were used to elucidate how PMN-melanoma aggregates were arrested on the endothelial monolayer under shear conditions. Blocking CD11a and CD11b, respectively, inhibited melanoma adhesion efficiency partially under all shear conditions tested. Results indicate that both LFA-1 and Mac-1 are required for melanoma cells to be maintained on the endothelium via melanoma-PMN aggregation, but may have different roles (Fig. 3C). Figure 3D shows that blocking CD11b did not significantly alter the rate of aggregation between entering melanoma cells and tethered PMN, and LFA-1 alone supported melanoma aggregation with tethered PMN on the EC initially. However, after an initial period of ~ 3 minutes, disaggregation of WM9-PMN aggregates on the monolayer surface proceeded more rapidly in the presence of

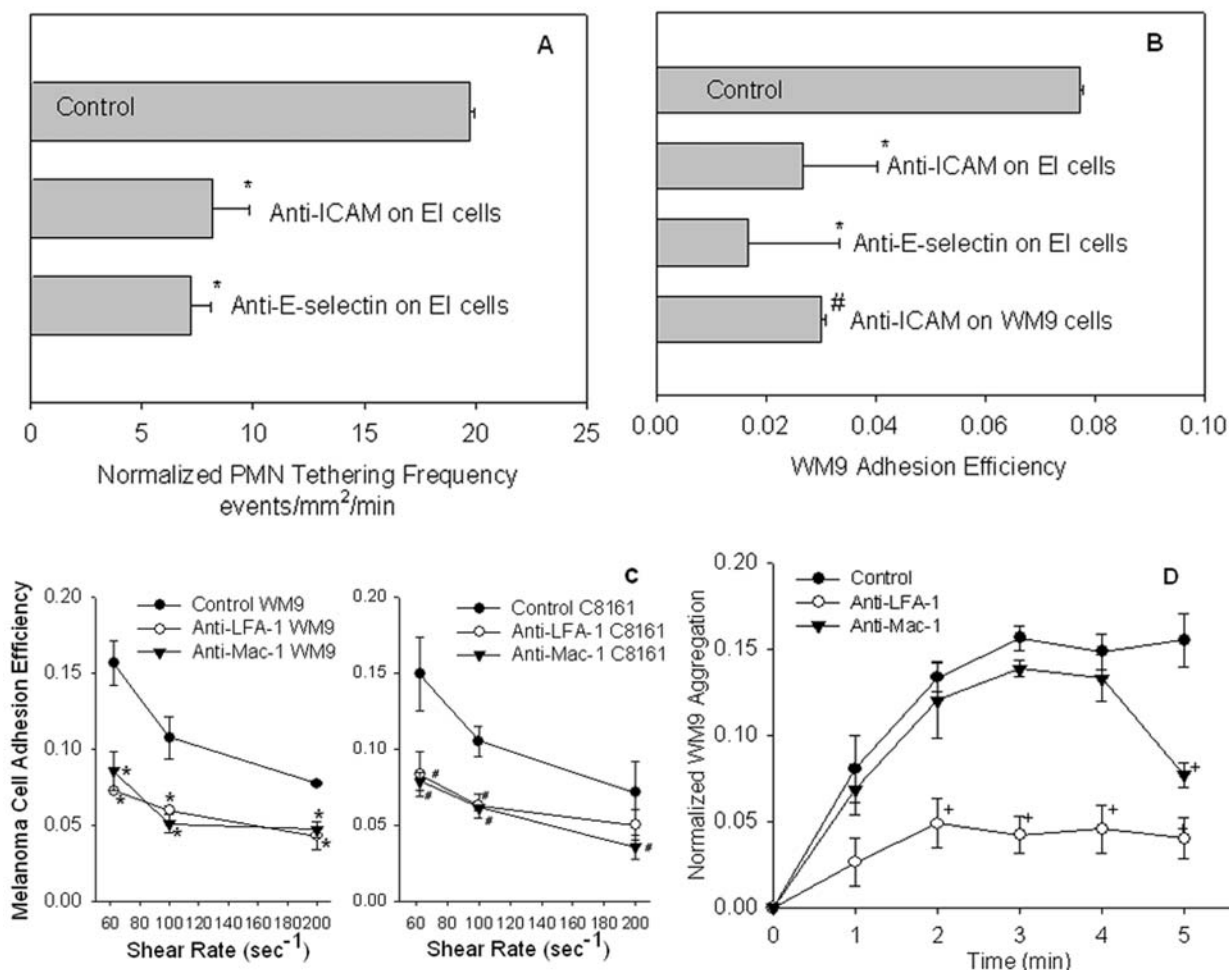


Figure 3 : Tethered PMN facilitates melanoma cell migration in close proximity to the endothelium under shear conditions. (A) PMN tethering frequency shows that the number of PMN that adhered to the endothelium per unit time and area is significantly reduced under the shear by blocking ICAM-1 or E-selectin on the monolayer ($*P < 0.05$ with respect to Control: PMN tethering on untreated monolayer). Shear stress was 2 dyn/cm². (B) Melanoma cell adhesion efficiency shows that the number of WM9 cells arrested on the monolayer as a result of WM9-PMN collisions is significantly decreased by blocking either PMN-EC adhesion or WM9-PMN aggregation ($*P < 0.05$ compared with control samples; $\#P < 0.05$ compared with the control). Shear stress was 2 dyn/cm². (C) WM9 and C8161 adhesion efficiency at different shear rates (media viscosity 1.0 cP) over a period of 5 min. Antibody blocked cases (anti-LFA-1 and anti-Mac-1) are statistically significant ($P < 0.05$) compared with control at the same shear rate and cell type. (D) Relative contributions of LFA-1 and Mac-1 to binding of PMN-WM9 heterotypic aggregates on the monolayer. Blocking LFA-1 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 tethered PMN on the monolayer at each time point. Error bars are mean \pm S.E.M. for $N \geq 3$.

anti-CD11b mAb than in the control (Fig. 3D). These results suggest that LFA-1 alone is necessary and sufficient for the initial formation of melanoma-PMN aggregates and Mac-1 maintains the stability of formed melanoma-PMN aggregates on the endothelial surface after the ini-

tial melanoma cell capture by PMN, which ultimately affects melanoma cell extravasation (Fig. 2C).

Therefore, we have found PMN increase melanoma cell arrest on the endothelium, which involves initial PMN tethering on the EC, subsequent PMN capture of

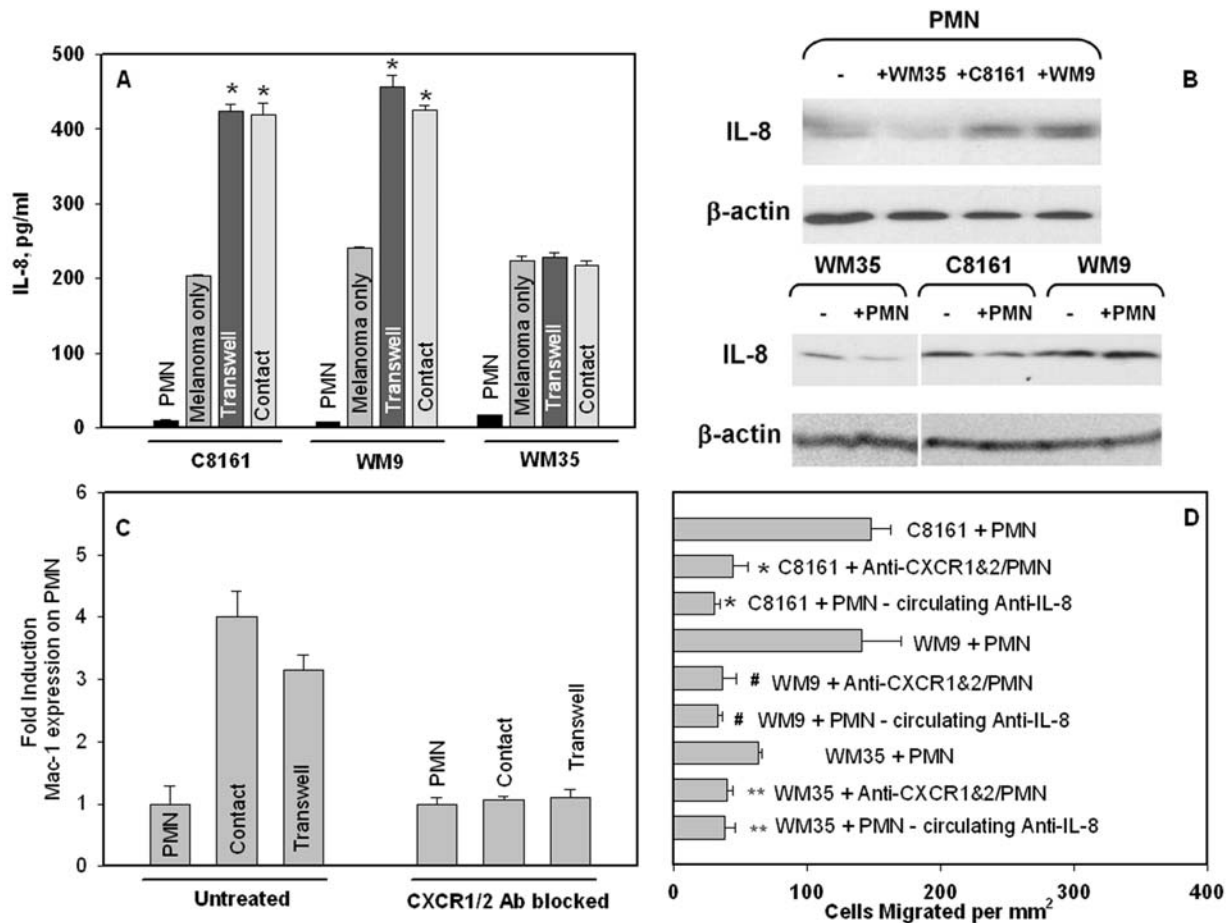


Figure 4 : PMN-facilitated melanoma cell arrest on the endothelium under flow conditions is modulated by endogenously produced IL-8. (A) ELISA detected increased secretion of IL-8 after PMN co-cultured with melanoma cells (C8161 and WM9, respectively), either in contact with each other or Transwell (not in contact but sharing the same medium). * $P < 0.05$ compared with the sum of IL-8 expression from PMN and melanoma only in the respective group. (B) Immunoblot results show that increased IL-8 is from PMN after their Transwell co-culture with respective melanoma cells. (C) Fold induction of Mac-1 expression on PMN after co-culture with C8161 and WM9 melanoma cells, respectively; either in contact or Transwell. Blocking the IL-8 receptors (CXCR1/2) resulted in no increase in Mac-1 expression compared with the control case. (D) Effects of IL-8 on PMN-facilitated melanoma migration. Isotype control cases were run for all the cases and were found to be statistically the same as the C8161+PMN case (* $P < 0.04$ with respect to C8161+PMN case; # $P < 0.05$ with respect to the WM9+PMN case; and ** $P < 0.05$ with respect to WM35+PMN cases). All co-culture cases were performed in 4 hours, and all flow experiments were under a flow shear stress 0.4 dyn/cm^2 .

melanoma cells and maintenance of their close proximity to the EC. LFA-1 influences the capture phase of PMN binding to both melanoma cells and the endothelium, while Mac-1 affects prolonged PMN-melanoma aggregation. Results also show that although PMN tethering on the EC is necessary for PMN-facilitated melanoma coming into and maintaining close contact with the EC, it is not sufficient.

3.4 Melanoma Cell Increases IL-8 Production in PMN

ELISA and Western blot were used to assess chemotactic cytokine production by melanoma cells and PMN as a stimulus, which might be responsible for cell-cell signaling. Figure 4A shows that supernatant of PMN co-cultured with selected-type melanoma cells, either via Transwell (non-contact) or direct contact, contained

different amounts of IL-8 production compared with summed background levels by PMN and melanoma cultured separately. There were significant increases in IL-8 from the supernatant of PMN co-cultured with highly-metastatic C8161 or WM9 cells compared with low-metastatic WM35 cells.

To more specifically identify which cells produced increased amounts of IL-8, cell lysates were then analyzed using immunoblot. Obviously, low-metastatic WM35 melanoma cells had less endogenous effect on PMN in increasing IL-8 production (Fig. 4B, top second lane). In comparison, PMN that had been Transwell co-cultured with highly-metastatic C8161 or WM9 cells (Fig. 4B, top third or fourth lane) contained significantly increased amount of intracellular IL-8 compared to PMN cultured alone without melanomas (Fig. 4B, top first lane). No melanoma cells from Transwell co-culture showed any detectable changes in intracellular IL-8 amount, with (+) or without (-) PMN (Fig. 4B, bottom).

Results suggest that PMN-melanoma communication creates a microenvironment that is potentially self-stimulatory, which causes changes in IL-8 production. We have found a trend of increasing amounts of IL-8 in PMN during co-culture versus nearly constant expression by the melanoma cells. Soluble IL-8 as released by melanoma cells and PMN would possibly have environmental effects of stimulating or immunoediting nearby PMN.

3.5 IL-8 Influences PMN Recruitment in Melanoma Cell

Untreated PMN or PMN treated with blocking antibodies for CXCR1 and CXCR2 were co-cultured with C8161 melanoma cells, either in contact (referred to as "Co-culture" case) or separated by a Transwell insert (referred to as "Transwell" case). Figure 4C indicates that non-CXCR1/2-blocked PMN co-cultured with melanoma cells experienced a nearly 4-fold increase in Mac-1 expression levels over those cultured alone for 4 hours. PMN with antibody blocked CXCR1 and CXCR2 showed no change in Mac-1 levels after co-cultured with C8161 cells compared with PMN cultured alone (Fig. 4C).

To elucidate the possibility that secreted chemokines could be a stimulus responsible for melanoma-PMN communication, the IL-8 receptors (CXCR1 and CXCR2) on PMN were antibody blocked and tested in

the migration assay. Melanoma cell extravasations dramatically decreased under 0.4 dyn/cm² shear stress in the presence of CXCR1/CXCR2-blocked PMN compared with unblocked PMN (~70% decrease with C8161; ~74% decrease in WM9; ~38% decrease in WM35) as shown in Figure 4D. Neutralizing anti-IL-8 antibody (1µg/ml) was also used to bind soluble IL-8 secreted by melanoma cells and PMN, which showed similar inhibitory effects on melanoma migration as those CXCR1/2-receptor blocked PMN cases (Fig. 4D).

Collectively, these results show PMN-facilitated melanoma cell arrest on the EC is modulated by endogenously produced IL-8. The influence of IL-8 in melanoma cell extravasation will be amplified under higher shear-rate conditions.

4 Discussion

The advancement of experimental assays to characterize cellular adhesion and migration are in a period of rapid development. For example, PMN-EC adhesion has been widely examined using various *in vitro* experimental systems. *In vitro* parallel-plate flow chambers are common assays for characterizing PMN-EC adhesion in shear flow (Lawrence *et al.*, 1987). This type of flow chamber consists of a glass substrate, on which a monolayer of endothelial cells or a monolayer of transfected cells expressing endothelial adhesion molecules (Gopalan *et al.*, 1997), or simply immobilized purified adhesion molecules (Lawrence and Springer, 1991; Cao *et al.*, 1998; Lei *et al.*, 1999) can be introduced to simulate an *in vivo* vascular wall. The strength of cell-substrate adhesion is determined by the shear stress needed to detach the cells from the surface. The *limitation* from this type of assay is that although the interactions between cells and vessel walls can be simulated, it is hard to study cell extravasation under flow conditions, even using phase-contrast videomicroscopy (Smith *et al.*, 1989). For *in vitro* cell migration or invasion studies, Boyden-chemotaxis chambers are often employed, consisting of two wells separated by a micro-pore filter (Aznavoorian *et al.*, 1990; Hodgson and Dong, 2001). Cells are placed in an upper well and allowed to migrate through the porous filter to the opposite side, in response to a chemoattractant source placed in the lower well. However, the *limitation* of this assay is that it is restricted to modeling only static conditions and does not permit investigating how dynamic flow conditions alter

cell extravasation. None of these methods permits investigating how dynamic flow conditions alter cell extravasation. One motivation of this study is to apply a new approach to studying cell adhesion and extravasation using a novel *in vitro* flow-migration assay, which will allow us to investigate heterotypical cell-cell adhesion involved in melanoma cell extravasation under dynamic shear-flow conditions.

Significant progress has been made in the past decades toward understanding how PMN roll along the EC before forming shear-resistant bonds (Hentzen *et al.*, 2000; Neelamegham *et al.*, 1998). But, how do tumor cells bind the endothelium? One observation from *in vivo* videomicroscopy has indicated that tumor cells are trapped in capillaries and only arrested on the EC on the basis of vessel-size restriction in the microcirculation of whatever organ or tissue they extravasate (Chambers *et al.*, 2000). It has been suggested that initial microvascular arrest of metastasizing tumor cells (from cell lines of 6 different histological origins) does not exhibit “leukocyte-like rolling” adhesive interaction with the EC (Thorlacius *et al.*, 1997). In contrast, another *in vivo* study (Scherbarth and Orr, 1997) has discovered that the B16F1 melanoma cells could become arrested by shear flow-resisted adhesion to the walls of presinusoidal vessels in mice pretreated with interleukin 1 α (IL-1 α). This suggests that release of cytokines into the bloodstream could bring arrest of melanoma cells in portal venules, by a chemoattraction and adhesion-mediated mechanism, rather than by size-restriction-only mechanism. Clearly, those studies are somewhat contradictory and additional work is needed to characterize the event in tumor cell adhesion to the EC. Several new studies have been launched recently in investigating whether PMN play any roles in tumor cell migration through the EC (Wu *et al.*, 2001), especially under flow conditions (Jadhav *et al.*, 2001; Slattery and Dong, 2003).

Hydrodynamic forces play an important role in regulating melanoma cell adhesion and extravasation in the microcirculation. Without an ability of selectin- or β_2 integrin-mediated binding by melanoma cells alone to the endothelial surface under shear conditions, tumor cells may recruit PMN to aid in binding to the blood vessel wall. Results from Figure 2A show PMN influence melanoma cell extravasations under dynamic flow conditions, strongly correlated with tumor metastatic potentials. PMN-facilitated melanoma adhesion to the EC

is a multi-step process. Blocking either ICAM-1 or E-selectin adhesion molecules on the endothelial monolayer reduces melanoma migration (Fig. 2B), which suggests PMN adhesion to the EC is necessary for melanoma cells arrest on the EC. Blocking the ICAM-1 molecules on C8161 cells also inhibits melanoma migration (Fig. 2B), which further indicates that a β_2 integrins/ICAM-1 binding mechanism is involved in PMN-EC and melanoma-PMN adhesion. Down-regulation in LFA-1 or Mac-1 shows significant impact on the melanoma cell migration through the EC (Fig. 2C).

To understand whether changes in melanoma cell migration as shown in Figure 2 are due to melanoma-PMN adhesion or due to PMN-EC adhesion, the parallel-plate flow assay elucidates the distinct roles of PMN in melanoma cell interactions with the EC. Previous studies have shown that endothelial E-selectin initiates leukocyte capture to the EC and facilitates rolling by binding sialylated ligands expressed on PMN (Crutchfield *et al.*, 2000; Kansas, 1996). Figure 3A shows blocking E-selectin and ICAM-1 on the EI monolayer inhibits the capture and rolling of PMN on the surface, resulting in decreased WM9 adhesion efficiency (Fig. 3B). 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. Blocking either LFA-1 or Mac-1 function abrogates melanoma cell binding to tethered PMN under shear conditions (Fig. 3C). LFA-1 and Mac-1, the β_2 integrins, have sequential roles in binding leukocytes to ICAM-1. Studies have shown that adhesion begins with LFA-1-dependant capture and is stabilized and maintained by Mac-1 (Neelamegham *et al.*, 1998; Hentzen *et al.*, 2000). Clearly from the results in adhesion assays (Fig. 3D), LFA-1 affects adhesion over the entire time course, whereas the effect of Mac-1 is only seen after 3 minutes. 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 melanoma migration under shear, but Mac-1 and LFA-1 are both necessary and neither is sufficient to allow PMN to bind melanoma cells and the EC.

Recently, it has been demonstrated that melanoma cells produce soluble interleukin 8 (IL-8/CXCL8), which pos-

sesses PMN chemotactic and activating capacities (Smith *et al.*, 1991; Yamamura *et al.*, 1993) in addition to T-lymphocyte chemotactic activity (Larsen, 1989). PMN-melanoma cell attachment to the EC creates a microenvironment that is potentially self-stimulatory, which could alter PMN immuno-responses. Analysis of cytokine production by each of the two cell types during coculture shows that melanoma-PMN communication induces changes in IL-8 expression. ELISA assays indicate that highly-metastatic melanoma cells alone produce relatively high amounts of IL-8 compared with low-metastatic cells; but in contrast PMN do not constitutively express high level IL-8 (Fig. 4A). IL-8 production increases significantly in supernatant of PMN cocultured with melanoma cells, especially with highly metastatic cells. As determined by Western blot (Fig. 4B), endogenous IL-8 arises more significantly from PMN in response to highly-metastatic melanoma cells. There are no apparent changes in IL-8 secretion from various melanoma cells in the presence of PMN. A dramatic inhibition of Mac-1 expression on CXCR1/2-blocked PMN (Fig. 4C) suggests that melanoma-induced IL-8 from PMN could be responsible for the Mac-1 up-regulation on PMN. Significant inhibition in melanoma extravasation was found when neutralizing IL-8 antibody was present in the media, which clearly identifies the role of endogenously produced IL-8 in PMN-mediated melanoma cell extravasation (Fig. 4D).

Melanoma cells overly express GRO- α (growth-related oncogene- α ; CXCL1) and GRO- γ (CXCL3), which promote PMN chemotaxis (Baggiolini *et al.*, 1994) as well as melanoma growth both *in vitro* and *in vivo* (Balentien *et al.*, 1991; Owen *et al.*, 1997). CXCR2 is a major receptor for GRO, which was found in melanoma cells (Schadendorf *et al.*, 1993). Because melanoma cells have not been found to express CXCR1 (Müller *et al.*, 2001), a main receptor for IL-8, soluble IL-8 released by melanoma cells and PMN would probably have an environmental effect mainly on stimulating nearby endothelium or an autocrine effect on leukocytes. In addition to IL-8 and GRO receptors, Müller *et al.* (2001) detected significant levels of chemokine receptors CXCR4 and CCR7 in melanoma cells. The ligand for CXCR4 is SDF-1 α (stromal cell-derived factor-1 α ; CXCL12), which is broadly expressed and has been demonstrated to arrest lymphocyte rolling on the EC under flow conditions (Campbell *et al.*, 1998). Monocyte inflammatory pro-

tein (MIP-3 β ; CCL19), which is produced by activated PMN, is the ligand for CCR7 (Kasama *et al.*, 1995). Recent study also shows that MCP-1 (monocyte chemoattractant protein-1) is expressed by melanoma cells, which could recruit PMN under chronic inflammatory conditions (Brent *et al.*, 1999). These different chemokines and cytokines may initiate additional communication between melanoma cells and PMN. Such studies are currently being conducted in our laboratory.

Our studies present a novel finding that PMN-facilitated melanoma cell arrest on the EC within the circulation is modulated by endogenously produced IL-8. Therefore, understanding cancer immunoediting via intercellular communications between tumor cells and leukocytes will foster new approaches to cancer treatment through anti-inflammatory therapeutics.

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