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***In Vitro* Characterization and Micromechanics of Tumor Cell Chemotactic Protrusion, Locomotion, and Extravasation**

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Abstract

The objective of this paper is to introduce some novel *in vitro* applications in characterizing human melanoma cell protrusion and migration in response to soluble extracellular matrix protein stimulation. Specifically, we describe two assay systems: (1) dual-micropipette manipulation and (2) flow-migration chamber. Applications of the dual-micropipet technique provided kinetic measure of cell movement, cyclic pseudopod protrusion, and subsequent cell locomotion governed by chemotactic molecular transport dynamics. Chemotactic concentration gradient was found to influence significantly pseudopod protrusion frequency and locomotion speed, but not the protrusion extension. To further characterize active tumor cell extravasation, a process that involves dynamic tumor cell adhesion to vascular endothelium under flow conditions and subsequent transendothelial migration in response to chemotactic signals from the interstitial space, we developed a flow-migration chemotaxis system. This assay enabled characterization of tumor cell transcellular migration in terms of chemotactic signal gradients, shear forces, and cell-substrate adhesion. Results suggest that shear flow plays significant roles in tumor cell extravasation that is regulated by both tumor cell motility and tumor cell adhesion to endothelial molecules in a cooperative process.

Keywords

Cell adhesion; Migration; Melanoma; Endothelium; Type IV collagen; Micropipette manipulation; Shear flow; Chemotaxis

INTRODUCTION

Active tumor cell adhesion and migration have long been recognized as necessary for invasion and metastasis.^{9,12} The process of metastasis begins when tumor cells enter into the blood stream either directly or indirectly by lymphatic drainage. The tumor cells then must survive in the circulation, arrest on vascular endothelium and finally migrate through the vessel wall (extravasation) to proliferate in the secondary target organs (Fig. 1). Among these steps, tumor cell adhesion and migration play a key role in tumor metastasis. However, the precise behavior and mechanisms by which tumor cells adhere to and subsequently migrate through endothelium remain unclear.

Metastasizing tumor cells are likely to encounter both soluble and insoluble extracellular matrix (ECM) components as they traverse vascular walls and basement membranes. Some

chemotactic factors, including ECM molecules, have been shown to stimulate the intrinsic motility of tumor cells *in vitro*.^{1,4} These factors are believed to influence both the extent and the direction of tumor cell movement *in vivo* to specific target organs. Tumor cells exhibit an amoeboid movement similar to that of human polymorphonuclear leukocytes, characterized by pseudopod protrusion at the leading edge of the cell, followed by directional locomotion toward a chemotactic source derived from the target tissue. *In vitro* Boyden, Zigmond, and Dunn chambers^{17,18} have been used routinely to characterize cellular chemotaxis. However, those systems have some limitations in characterizing the active kinetic or dynamic process of cellular locomotion during chemotaxis. A dual-micropipette technique has recently been developed to overcome those limitations and study chemotaxis of individual human melanoma cells in response to a soluble type IV collagen (CIV), a component of ECM proteins.¹⁵ A micropipette with a focal chemotactic source constitutes a practical experimental assay simulating a biological barrier where a tumor cell has to crawl through the blood vessel wall.

Some recent *in vivo* studies^{2,16} have suggested that the arrest of tumor cells on vascular endothelium occurs by quite different means from which leukocytes are arrested. Leukocytes pass through blood capillaries and are arrested by adhesion to walls of venules much larger than the cell diameter, rolling along the endothelium before forming shear-resistant adhesion bonds. Cancer cells from solid tumors (~15 μm in average diameter), however, are much larger than leukocytes (~8 μm in average diameter) and are less able to pass through capillary dimensions to reach the venules. One observation from the *in vivo* video microscopy has indicated that tumor cells only become arrested on the basis of capillary-size restriction in the microcirculation.² However, another *in vivo* study has then reported that cytokine-mediated signaling could enable tumor cells to arrest by an adhesion-mediated mechanism to endothelial walls of vessels twice the cell diameter.¹¹ This study thus suggested the possibility that release of cytokines into the blood stream could arrest cancer cells in portal venules, rather than by a size-restriction mechanism. The actual extravasation mechanism still remains unclear.

To further simulate tumor cell migration within the circulation *in vitro*, micropipette techniques are not applicable. Therefore, we have developed a novel *in vitro* flow-incorporated chemotaxis system. It consists of a 48-well baseplate that contains soluble CIV proteins as chemoattractant. The top plate, that includes a flow inlet and outlet, is separated from the bottom wells in the baseplate by a gasket and a micropore filter. A cell monolayer can be cultured directly on this 8 μm pore sized polycarbonate filter to model an endothelial cell barrier. Using a peristaltic pump, circulating tumor cells can then be introduced into the channel space between the top plate and the substrate cell monolayer in a well-defined flow field. The total number of transcellular-migrated tumor cells can be microscopically quantified in terms of various parameters including shear stress, chemoattractant concentration and modulated cell adhesion.

MATERIALS AND METHODS

Materials

Soluble CIV was obtained from Collaborative Research (Bedford, MA). For chemotactic assays, the collagen was diluted to various concentrations in Dulbecco's modified Eagle's medium (DMEM) (Biofluids, Gaithersburg, MD) with 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO), and the solution was brought to a pH of 7.4 and an osmolality of 300 mmol/kg. Mouse monoclonal antibodies to human CD11a (LFA-1), CD11b (Mac-1), and CD-54 (ICAM-1) were purchased from CalTag Laboratories (Burlingame, CA). A mouse fibroblast L-cell line transfected with both human E selectin and ICAM-1 molecules ("EI cells")⁵ was provided by Dr. Simon at UC Davis, CA.

Cell Culture

A2058 and C8161 human melanoma cell lines were provided by the National Cancer Institute (MD) and Penn State Hershey Medical Center (PA), respectively. Cells were cultured in DMEM (for A2058 cells) or DMEM-F12 (for C8161 cells) supplemented with 10% fetal bovine serum (FBS) (Biofluids) within an atmosphere of 5% CO₂/95% air at 37 °C. Cells for assays were detached while subconfluent by brief exposure to 0.05% trypsin/0.02% EDTA and allowed to recover for 1 h in a tissue culture medium containing DMEM/10% FBS.

T-leukemic Jurkat cells (provided by Dr. Lawrence, University of Virginia, VA) were cultured in suspension of RPMI 1640 medium supplemented with 10% FBS, L-glutamine (0.292 mg/ml), penicillin (100 U/ml), and streptomycin sulfate (100 µg/ml) (Gibco Laboratories, Grand Island, NY). For assays, Jurkat cells were resuspended in serum-free medium with 0.1% BSA. To characterize the effect of an increase in LFA-1 avidity on Jurkat cells, cells were pretreated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) for 20 min at 37 °C.

Dual Micropipette Assay

Experiments were performed with individual A2058 tumor cells. Pipettes were pulled to desired dimensions using a pipette puller (Narishige, Japan) and a micro-forge (Stoelting, Wood Dale, IL). A single A2058 cell (15 µm in diameter ±1.0 µm) was retained in a larger pipette [14 µm inner diameter (i.d.) ±1.5 µm] containing FBS-free DMEM/0.1% BSA. The chemotactic solution with CIV was prepared to desired concentrations (100–200 µg/ml) and filled into a smaller pipette, in which the pressure gradient at the pipette tip was controlled at zero. This small pipette (10 µm outer diameter ±1.5 µm) was then inserted into the larger pipette at a controlled distance (δ) between the tip of the small pipette and the cell front surface (Fig. 2). An initial concentration of the chemoattractant C_0 and δ provided a gradient to influence cell chemotaxis. Cell images were taken by a video camera and recorded for off-line analyses. Measurements on cell pseudopod protrusion length (L_p ; a two-dimensional projected distance from the pseudopod tip to the base) and locomotion velocity (dL_c/dt ; velocity of the cell center) were made at a later time. The pseudopod protrusion frequency (f_p), defined as the total number of peaks in protrusion length per given time period, was also measured.

Chemotactic Diffusion

The CIV chemotactic source (at $x=0$) was assumed to be constant (C_0) (for all time $t \geq 0$). CIV diffuses from $x=0$ toward the cell surface at $x=L(t)$ when $t > 0$. Let $C(x,t)$ be the spatial-temporal distribution of the CIV concentration between the tip of the small pipette ($x=0$) and the moving cell surface [$x=L(t)$; Fig. 2], the diffusion equation for $C(x,t)$ was given by

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2}, \quad 0 < x < L(t), \quad (1)$$

where D is the diffusion constant for macromolecules within the medium. A steady chemotactic source was assumed at the small pipette tip with a constant concentration C_0 , such that the initial condition became

$$C=0, \quad 0 < x \leq \delta, \quad t=0 \quad (2)$$

and the boundary conditions were

$$C=C_0, \quad x=0, \quad t \geq 0, \quad (3)$$

$$-D \frac{\partial C}{\partial x} = K_r C, \quad x = L(t), \quad t \geq 0 \quad (4)$$

in which K_r is a constant, representing the binding rate of the CIV molecules bound to the melanoma cell surface receptors. A moving boundary $L(t) = \delta - L_C(t)$ was assumed to satisfy the following condition:

$$\frac{d[\delta - L(t)]}{dt} = K_g \frac{\partial C}{\partial x}, \quad x = L(t), \quad t \geq 0 \quad (5)$$

in which K_g is a constant correlating the speed of a moving boundary with the chemotactic concentration gradient. Due to the moving boundary at $x = L(t)$, the concentration $C\{L(t), t\}$ became a key variable in determining the actual chemotactic process of cells at subsequent times.

Collagen IV Binding to Melanoma Cells

Detached A2058 cells were incubated in DMEM/0.1% BSA at 37 °C with a chosen concentration of CIV. At the end of a chosen time, cold medium (DMEM/0.1% BSA; 1–2 °C) was immediately added to the cell suspension to minimize the CIV reaction with the cells, followed by a centrifugation at 200 g for 3 min (1–2 °C). After decanting the supernatant, the cells were resuspended at 1×10^7 cells/ml in PBS/CS/GS (phosphate buffered saline/5% calf serum/2% goat serum/0.1% N_aN_3). Primary antibody, 1 µg of antimouse CIV was added per 1×10^6 cells and incubated for 25 min at 4 °C. Cells were washed and tagged with phycoerythrin-conjugated antirabbit immunoglobulin antibody (1 µg/ 1×10^6 cells/100 µl) (Jackson Immuno Research Laboratories, West Grove, PA) for 25 min at 4 °C. The cells were rewashed and fixed in PBS containing 1% paraformaldehyde. The number of cells binding detectable levels of CIV was determined by flow cytometry. Appropriate controls of cells incubated with secondary antibody alone or cells incubated with primary and secondary antibodies but without CIV were included. Under saturating antibody conditions, approximately 100% of the cells which had been incubated with CIV were detected. To determine the number of CIV molecules bound per cell absolute fluorescent values were calculated from a standard curve generated by running Sphero Rainbow 30-5 calibration particles (SpheroTech, Libertyville, IL), a mix of five beads with known fluorescence values. In different experiments, the conditions were varied such that the concentrations of CIV ranged from 0 to 200 µg/ml and the time of incubation with collagen ranged from 1 to 50 min. However, because the washes took at least 3 min, we were unable to test actual binding times below about 3 min.

Adhesion Molecule Detection

The cells of interest were detached and prepared as described earlier. Samples were treated with murine anti-human CD Marker primary antibodies (LFA-1, Mac-1, ICAM-1; CalTag Laboratories) for 25 min at 4 °C (1 µg mAb/ 10^6 cells). The cells were then treated with secondary antibody, FITC-conjugated goat antimouse IgG F(ab)₂ fragment, for 25 min at 4 °C (Jackson ImmunoResearch, 1 µg/ 10^6 cells). The samples were fixed with 2% formaldehyde (Sigma) and at least 10,000 cells were analyzed per trial using Coulter EPICS XL (Coulter Corp.) flow cytometer. The data are represented as the mean fluorescence divided by the background fluorescence with the error bar representing the standard error of the mean (SEM). Each data point represents at least five trials.

Flow-Migration Chamber Assay

In order to simulate the *in vivo* tumor extravasation process, a chemotaxis flow-migration chamber (Fig. 3) was developed to model a capillary and to characterize tumor cell adhesion to a cultured substrate-cell monolayer representing binding to a vessel wall. Under the influence of shear flow and cell-substrate adhesion, tumor-cell extravasation from the top compartment into the bottom compartment simulated cell migration from the capillary lumen into interstitial tissue space.

This *in vitro* flow-migration device is a modified-48 well chemotactic chamber. It was fabricated from polycarbonate [Fig. 3(A)]. The gasket, 0.02 in. PharmElast silicon (SF Medical, Hudson, MA), is an 11 cm×5.5 cm rectangle with a 7 cm×2 cm opening cut from the center separating the top and base plates, which creates a rectangular cross-sectional flow field with a large width (~2 cm)-to-height (~0.051 cm) aspect ratio [Fig. 3(B)]. Applying a prescribed shear rate ($\dot{\gamma}$, s⁻¹) under a given volumetric flow rate Q :

$$\dot{\gamma}=6Q/wh^2, \quad (6)$$

where w and h are the width and height of the flow channel, respectively. The wall shear stress (τ_w ; dyn/cm²) exerted on an adherent cell was calculated by

$$\tau_w=\mu\dot{\gamma}, \quad (7)$$

where μ is the viscosity of the suspension fluid. A flow loop consisting of two reservoirs and the chamber top plate (with inlets and outlets) was connected using Nalgene PVC tubing (~1/16 in. i.d.). A peristaltic pump (Cole Parmer) drove a constant flow through the flow loop. A sealed reservoir was added within the loop to eliminate pulsatility from the flow. The flow loop was housed in a self-designed incubator that maintained a 5% CO₂ and 37 °C environment.

EI cells were cultured on a fibronectin-coated polycarbonate filter (8 μm pore size) to form an “endothelial” monolayer. Prior to cell culture, the filter was first sterilized in 95% ethanol and exposed to ultraviolet light. The topside of the filter was coated with fibronectin (Collaborative Biomedical Products, MD) at a concentration 30 μg/ml in medium and incubated for 3 h. The sterilized filter was then transferred to a sterile Petri dish with the fibronectin-coated side facing up. A sterile glass block was placed on top of the filter and EI cells were seeded in the center section of the glass block, to ensure controlled cell growth on the topside of the filter [Fig. 3 (C)]. Approximately 5×10^5 cells were seeded per filter. A monolayer of EI cells was grown to 100% confluence on a filter 36–48 h after being placed onto the base plate of the chamber [Fig. 3(D)].

To stimulate cell migration, the center 12 wells (in the baseplate below the filter) were filled with soluble CIV as the chemoattractant, with a selected concentration (50–200 μg/ml) in RPMI 1640/0.1% BSA (Collaborative Biomedical Products, MD). All other wells were filled with non-stimulatory 0.1% BSA medium as a control [Fig. 3(B)].

Tumor cells were introduced to the flow channel, after which the chamber was assembled and connected to the flow loop. Initially a very slow flow (2 ml/min) was pumped through the system. The flow rate was then slowly increased to the desired rate (0–20 ml/min). Experiments were run at wall shear stresses in a range of 0–4 dyn/cm². After a selected time duration (1–4 h), the flow-migration chamber was removed from the flow loop and disassembled. The filter was gently removed and stained with Diff-Quik (Dade Behring Inc.). The C8161 cells on the bottom side of the filter were imaged using an inverted microscope and captured via NIH Image

versus $\beta 4.0.2$ with a PC. Three images of each migration filter were quantified and averaged for each data point. For each data point, a minimum of three filters was analyzed.

Statistical significance between cases tested in the flow-migration chamber were determined by the unpaired t test on Sigma Plot Version 4.01 (SPSS, Chicago, IL) with the corresponding P value reported for each case. All error bars on flow migration data histograms represent the SEM.

RESULTS

Directed Tumor Cell Migration

A series of experiments was performed with A2058 tumor cells to examine pseudopod protrusion and subsequent locomotion in response to soluble CIV stimulation under various concentration gradients. The initial chemotactic gradient was determined by C_0/δ (Fig. 2). There was no movement if the initial concentration of the chemoattractant was set zero or if the source pipette was filled with DMEM culture medium only. Measurements of pseudopod protrusion (L_p) and subsequent cell locomotion (L_c) for each cell were made as a function of time from the video replay. The time was displayed in “hours: minutes: seconds/tenths seconds,” with “00:00:00.0” being the time at which the cell was initially located in the pipette. The magnification of the system was calibrated before each experiment using a micrometer ($50\times 2\ \mu\text{m}$) (Leica, Deerfield, IL).

In general, L_p has an initial lengthening phase over a short period of time (~ 3 min) followed by a fast shortening phase that seems to pull the whole cell forward by a distance L_c . These cyclic changes in L_p were found to be persistent, while L_c remained relatively idle during the lengthening phase of pseudopod protrusion in each cycle and increased only after every significant L_p lengthening peak. Pseudopod protrusion was, therefore, characterized by the protrusion frequency f_p as a function of time, defined by the total number of peaks in L_p divided by the time period. To obtain an average behavior of A2058 tumor cell locomotion, variables in dL_c/dt , L_p , and f_p were averaged (mean $\pm$ SEM) over the total number of cells assayed at specified time points (Fig. 4). It is apparent that dL_c/dt , L_p , and f_p all increase with time during an early phase of cell crawling, followed by a relatively rapid decrease as a cell moves close to the chemotactic source.

Figure 5 represents time averages (mean $\pm$ SEM) in $\overline{dL_c/dt}$, $\overline{L_p}$, and $\overline{f_p}$ from the data shown in Fig. 4, respectively, in response to three different initial chemotactic concentration gradients. The $\overline{dL_c/dt}$ showed a greater magnitude under a higher initial concentration gradient, where the average cell locomotion velocities ($\mu\text{m}/\text{min}$) were 0.231 ± 0.016 for G200:5 ($C_0=200\ \mu\text{g}/\text{ml}$ and $\delta=5\ \mu\text{m}$); 0.140 ± 0.017 for G100:5 ($C_0=100\ \mu\text{g}/\text{ml}$ and $\delta=5\ \mu\text{m}$); and 0.133 ± 0.016 for G200:10 ($C_0=200\ \mu\text{g}/\text{ml}$ and $\delta=10\ \mu\text{m}$), respectively [Fig. 5(A)]. In comparison, $\overline{L_p}$ did not appear to correlate consistently with the changes in initial concentration gradient [Fig. 5(B)] where the average peak pseudopod protrusion lengths (μm) were 1.886 ± 0.158 for G200:5; 1.337 ± 0.127 for G100:5; and 1.710 ± 0.132 for G200:10, respectively. However, $\overline{f_p}$ showed a strong correlation with the initial concentration gradient [Fig. 5(C)]. The mean peak pseudopod protrusion frequencies (1/min) were found to be 0.642 ± 0.053 for G200:5; 0.533 ± 0.025 for G100:5; and 0.517 ± 0.020 for G200:10, respectively.

It should be noted that C_0/δ only represents the initial concentration gradient for a fixed diffusion domain ($0 < x < \delta$) (Fig. 2). Any subsequent cell locomotion generates a moving boundary at $x=L(t)=\delta-L_c(t)$, that will modify the attractant diffusion domain and concentration profile. Using Eqs. (1)–(5) with a set of model parameters, the spatial and temporal distributions in $C(x,t)$ can be calculated. For example, if the attractant concentration at the cell front surface

is denoted by $C_d = C\{L(t), t\}$, the actual chemotactic concentration gradient at a later time, t , will become $\{C_0 - C_d\}/L(t)$. Apparently, C_d increases while $L(t)$ decreases. As a result of these temporal changes in $L(t)$, the actual concentration C_d and gradient $\{C_0 - C_d\}/L(t)$ at the cell front surface could be solved [Figs. 6(A) and 6(B)], where T is the total locomotion time for a cell to crawl over a given distance δ . The model parameters were determined based on the best agreement between the experimental measurements on $\overline{dL_c/dt}$ and the calculated cell locomotion velocity dL/dt [Fig. 6(C)]. From Eqs. (1)–(5), two dimensionless parameters $\{k_r = \delta K_r/D$ and $k_g = C_0 K_g/D\}$ were derived and found to be $\{3.42$ and $1.18 \times 10^{-10}\}$ for G200:5; $\{3.34$ and $0.53 \times 10^{-10}\}$ for G(100:5); and $\{2.89$ and $0.45 \times 10^{-10}\}$ for G200:10, respectively. Those kinetic parameters correlate with the concentration gradients of stimulus.

The dependence of CIV binding to A2058 cells on the local concentration of free CIV molecules was assayed by receptor staining and flow cytometry (Fig. 7). We found that CIV bound to cell surface receptors in a concentration-dependent manner [Fig. 7(A)]. However, the total amount of CIV bound to A2058 cells was apparently not influenced by time at least on the order of minutes [Fig. 7(B)]. We also performed CIV binding assay at a concentration up to 200 $\mu\text{g/ml}$ [Fig. 7(A)] without seeing obvious saturation for concentration until about 100–200 $\mu\text{g/ml}$.

Dynamic Tumor Cell Extravasation

Experiments were performed to characterize *in vivo* tumor cell extravasation, a process of tumor cell dynamic adhesion to vascular endothelium under flow conditions and subsequent transendothelial migration in response to chemotactic stimulation from interstitial space (Fig. 1). To model this extravasation process, a novel *in vitro* flow-migration assay was developed (Fig. 3). C8161 cells were selected to characterize this new extravasation assay because they are invasive and metastatic melanoma tumor cells, which have been known to be highly motile.

Tumor cell extravasation would normally occur in the microvasculature where fluid flows are steady and relatively slow. To model these *in vivo* conditions, flow-migration assays were run at a range of low shear stresses from 0 to 4 dyn/cm^2 . Figure 8 shows that shear stresses can modulate the extent of C8161 cell extravasation. There was a significant initial drop ($p=0.008$) from the static migration case (0 dyn/cm^2 ; 431.8 ± 56.1 cells per mm^2) to 1 dyn/cm^2 (234.83 ± 25.1 cells per mm^2), and a second decrease ($p=0.0015$) in migration from 1 to 2 dyn/cm^2 (122.2 ± 12.8 cells per mm^2). Subsequently, there was not a significant drop ($p=0.86$) in migration between 2 and 4 dyn/cm^2 (125.86 ± 14.9 cells per mm^2).

Standard static migration assays are commonly performed for the duration of 4 h.¹ It was necessary to determine if, under shear conditions, 4 h were sufficient for complete migration of tumor cells. To do this, a time curve of migration over time points from 1 to 4 h was assembled. It was found that, under 4 dyn/cm^2 shear stress, C8161 cell migration to CIV (100 $\mu\text{g/ml}$) increased as assay duration lengthened until about 3 h ($p=0.006$). Figure 9 demonstrates that migration between the time points of 3 and 4 h is not significantly different ($p=0.99$) and migration has reached a plateau by 4 h. This guaranteed that migration assays were run to completion within 4 h and determined a critical parameter in developing the flow-migration protocol.

The concentration gradient of the chemoattractant is also an important parameter in characterizing cell migration. The concentration of CIV in the bottom wells (Fig. 3) was varied from $C_0=50$ –200 $\mu\text{g/ml}$, and allowed to diffuse through the polycarbonate filter pores with an average filter thickness of $\delta \sim 10 \mu\text{m}$. Therefore, an initial chemotactic gradient was determined by C_0/δ , similar to that defined in the dual-pipette assay. A concentration of 100 $\mu\text{g/ml}$ CIV was previously determined to be sufficient for maximum stimulation of A2058 tumor cell

migration under static conditions.¹ In the flow-migration assay (under 4 dyn/cm² shear stress and 4 h migration time), a gradient G50:10 ($C_0=50\text{ }\mu\text{g/ml}$ and $\delta=10\text{ }\mu\text{m}$) was not sufficient for significant migration of the C8161 cells, as the migration was significantly below the case with a concentration gradient G100:10 ($C_0=100\text{ }\mu\text{g/ml}$ and $\delta=10\text{ }\mu\text{m}$) (Fig. 10). Further increasing chemotactic gradient to G200:10 ($C_0=200\text{ }\mu\text{g/ml}$ and $\delta=10\text{ }\mu\text{m}$) did not significantly increase migration above the case with G100:10.

The EI cell monolayer cultured on the flow-migration chamber substrate constitutively expresses ICAM-1 molecules.⁵ The level of ICAM-1 expression on EI cells (with an average mean fluorescence intensity 2.81 ± 0.39) was comparable to that expressed on human umbilical vein endothelial cells (with an average fluorescence intensity 3.28 ± 0.35) stimulated with cytokine interleukin IL-1 β for 4 h.⁵ In order to extravasate, C8161 tumor cells need to adhere to this monolayer firmly enough to resist the shear stress imposed by the given fluid shear forces. A significant drop ($p=0.002$) in melanoma cell extravasation was found from a static case to the flow case under 4 dyn/cm² shear stress [Fig. 11(A)]. Flow cytometry analysis indicated that unstimulated-C8161 cells expressed relatively low level of Mac-1 and LFA-1, compared with its ICAM-1 expression [Fig. 11(B)]. In order to characterize the C8161 migration and elucidate which adhesion molecules might make contributions to tumor cell adhesion to the endothelium, monoclonal antibodies were used to selectively block Mac-1 and LFA-1 on C8161 cells, and ICAM-1 on EI cells, respectively. Antibody against Mac-1 or ICAM-1 apparently blocked C8161 extravasation in shear flow under 4 dyn/cm² shear stress, whereas blocking LFA-1 did not impact C8161 migration [Fig. 11(A)]. Figure 11 also illustrates that melanoma cells expressed much lower efficiency in extravasation under the flow conditions than the static conditions ($p=0.002$), which was correlated with a limited amount of functional Mac-1 (not much from LFA-1) on C8161 binding to ICAM-1 molecules on the substrate EI cell monolayer.

Figure 12 (A) illustrates that under the 4 dyn/cm² shear stress, C8161 extravasation was significantly enhanced ($p<0.01$) in the presence of PMA-activated Jurkat cells (1:1 tumor/Jurkat cell ratio), although the total migration of C8161 under flow conditions was still less than that under the static case. Adhesion of single C8161 cells or C8161/Jurkat cells emboli to ICAM-1-expressed EI cell monolayer was further examined over a range of increasing shear stresses [Fig. 12(B)]. By their ability to remain adhered at higher shear stresses, C8161/Jurkat pairs demonstrated stronger adhesion to ICAM-1 substrate compared to single C8161 cells. There was no evidence that Jurkat cells migrate toward CIV. This leads to an interesting model to study tumor-endothelium interactions in a shear flow in the presence of leukocytes.

DISCUSSION

In vitro models often offer several advantages for defining cell behavior at both cellular and molecular levels. The objectives of the present paper for the special issue on “*Cell Mechanics*” are to introduce new *in vitro* techniques to characterize *in vivo* tumor cell extravasation.

The dual-micropipette assay provides a direct measure of tumor cell chemotaxis in terms of cellular pseudopod protrusion and subsequent locomotion toward a chemoattractant gradient under no-flow conditions. A micropipette with a focal chemotactic source constitutes a practical experimental assay for the simulation of a biological barrier where a tumor cell has to crawl through. In a small pore or capillary, protrusions were seen in some-what different patterns with either an irregular pseudopod⁴ or piston-like projection.¹³ Micropipette probes have been used previously for patch clamp analysis, cell deformability studies,^{3,7,10} and recently for measurements on leukocyte locomotion forces.¹³

A2058 human melanoma cells were investigated *in vitro* as a model for tumor cell protrusion and locomotion (Fig. 2). The kinetics of chemotaxis for individual A2058 cells in response to the soluble CIV was studied (Fig. 4), in which three initial chemotactic concentration gradients (C_0/δ) were defined. The chemotactic field influences cell locomotion patterns generated by tumor cells. Time dependencies of the cell locomotion velocity (dL_c/dt), the peak length in pseudopod protrusion (L_p), and the frequency in pseudopod protrusion (f_p) are not uniform, showing a general biphasic behavior (Fig. 4) due to temporal changes in chemotactic concentration gradients (Fig. 6).

The temporal average in locomotion velocity ($\overline{dL_c/dt}$) strongly depends on the gradient of chemoattractant concentration (C_0/δ) [Fig. 5(A)]. Figure 5 (B) illustrates the temporal average in peak pseudopod protrusion length ($\overline{L_p}$) in response to the concentration gradient change. As C_0/δ changed from G200:5 to G100:5, $\overline{L_p}$ showed a less significant decrease ($p=0.05$) compared with $\overline{dL_c/dt}$. Similarly, as C_0/δ decreased from G200:5 to G200:10, $\overline{L_p}$ responded with an insignificant change ($p=0.29$). In comparison, the temporal average in peak pseudopod protrusion frequency ($\overline{f_p}$) [Fig. 5(C)] demonstrates a strong dependency on C_0/δ . These results indicate that cell locomotion may directly correlate with the pseudopod protrusion frequency (not the protrusion length), which in turn is affected by the chemotactic concentration gradient. No significant correlation between L_p and f_p was found. The mean values displayed in Fig. 5 over time were affected by the events at the initial and endpoints and by the initial distance, and would not be expected to follow a normal distribution. Instead of using the student's T test, a Wilcoxon Mann-Whitney (WMW) test was applied. The WMW test is a two-sample rank test for the difference between two population medians and does not require the assumption that the differences between the two samples are normally distributed.

Calculations of $\{C_0 - C_d\}/L(t)$ have indicated a temporal change in concentration gradient with an initially gradual increase followed by a rapid decrease [Fig. 6(A)]. As a cell moves toward the chemotactic source, $C_d \rightarrow C_0$ [Fig. 6(B)] and $L(t) \rightarrow 0$. However, the diffusion time constant is $\sim D/L^2$, where D is the diffusivity and L is the distance. When $L(t) \rightarrow 0$, the diffusion process within $0 < x < L(t)$ is very fast ($\sim 1/L^2$), which makes $C_d \rightarrow C_0$ faster than $L(t) \rightarrow 0$. This explains a rapid gradient decrease in concentration gradient without having any steady plateau [Fig. 6 (A)], as well as the biphasic characteristics in cell locomotion [Fig. 4 and Fig. 6(C)]. Predicated results have shown higher averages and faster increases in $\{C_0 - C_d\}/L(t)$ with respect to time t/T for G200:5 compared with the other two C_0/δ cases. This prediction correlates with the greater magnitudes in $\overline{dL_c/dt}$ and $\overline{f_p}$ for G200:5 (compared with G100:5 or G200:10) [Fig. 5 (A) and 5(C)]. To obtain the results shown in Fig. 6(A), two dimensionless model parameters were derived based on the best agreement between computed and measured indices on $\overline{dL_c/dt}$ [Fig. 6(C)]. The parameter $k_r = (K_r \delta)/D$ reflects a ratio of the binding rate of CIV molecules bound onto cell surface receptors to the CIV diffusion, while the other parameter $k_g = (K_g C_0)/D$ represents a ratio of cell movement to the CIV diffusion. For example, under the concentration gradient G100:5, it was found that: $k_r = 3.34$ and $k_g = 0.53 \times 10^{-10}$. The k_r value (with $k_r > 1.0$) has indicated that the binding rate of CIV molecules to melanoma cells might be faster than the diffusion rate of molecules to the cell surface from a source point over a given distance. However, the diffusion rate is apparently much faster than the speed of cell locomotion (with $k_g < 1.0$). From the results of calculations, it appeared that k_r was not significantly influenced by the chemotactic concentration gradient C_0/δ , indicating that the binding rate of CIV molecules to melanoma cells could be independent of the diffusion gradient. In contrast, k_g expressed almost a 2.5-fold increase when the gradient changed from G100:5 (or G200:10) to G200:5, suggesting that an increase in diffusion gradient would increase the local concentration of free CIV molecules near the cell surface at $x=L(t)$, and therefore, affect the velocity of cell locomotion.

CIV is one of the ECM proteins, which has been shown its chemotactic influence on melanoma cell pseudopod protrusion⁴ and migration.⁸ Melanoma cells express CIV receptors, mainly the $\alpha_2\beta_1$ integrin.⁸ The amount of CIV bound to melanoma cells depends on the local concentration of free CIV molecules (Fig. 7). These results suggest a mechanism in which the chemotactic concentration gradient affects the total amount of attractant molecules bound to the cells, but not necessarily the binding rate. There was no direct concern in the integrin expression on the tumor cells, since the collagen concentrations used in all assays were under the full saturation dose.

While micropipette technique offers a direct measure of tumor cell protrusion and locomotion kinetics, it is hard to elucidate the effect of cell-substrate adhesion on cell migration, especially under the influence of a shear flow. A common *in vitro* method used to characterize cell-surface adhesion under flow conditions has been the parallel-plate flow chamber,⁹ in which a cover slip is covered with either confluent endothelial cells or coated with single type adhesion molecules. A gasket separating the cover slip from the deck plate of the flow chamber results in a channel gap from which the flow can be introduced via a perfusion pump. The strength of cell-surface adhesion can be characterized by the wall shear stress that is needed to detach the cell from its adhered substrate in terms of various adhesion molecules. However, such a flow chamber cannot provide any mechanism to study cell extravasation, although the adhesion aspect of cell interaction with the vessel wall can be simulated.

Studies in static cell migration have also employed the Boyden chemotaxis chamber.¹ The Boyden chamber consists of two wells separated by a micropore filter. Cells are placed in an upper well and are allowed to migrate through the filter pores to the opposite side over a standard period of time in response to different attractants with a chosen concentration in the lower well. Therefore, such a chamber can simulate *in vivo* migration to obtain dose-response data by staining and counting the cells migrated through the filter pores to the lower well. However, it is only a static assay that is not able to account for the flow influence on the process of cell extravasation.

The novel flow-migration system (Fig. 3) that we developed reserves all the features from the conventional parallel-plate flow chamber for characterizing cell adhesion to a cell monolayer under well-defined shear stresses. In addition, the flow-migration chamber combines the capability from the standard Boyden chamber from which static cell chemotactic migration has been assayed. Cell adhesion and cell motility can have decisive effects on whether an invasive tumor cell can successfully metastasize. This flow-migration system characterizes some of the most key events involved in tumor cell extravasation: tumor cell adhesion to vascular substrate under dynamic flow conditions, and subsequent tumor cell chemotactic protrusion and migration through the vascular wall barriers (Fig. 1).

Flow forces dramatically reduced C8161 melanoma cell migration (Fig. 8), indicating cell-substrate adhesion plays a significant role in the cell extravasation process within the circulation. The time scales of the cells extracirculatory residence may likely determine whether β_2 integrin-ICAM-1 may or may not effectively modulate C8161 tumor cells extravasation process in response to a defined chemotactic gradient under a given shear stress. A time curve of migration for a duration of 1–4 h was assembled (Fig. 9), showing a significant difference from 1 to 3 h, but little change in migration after 3 h. Residence time prior to migration would depend on the shear stress levels as well as the chemotactic diffusion gradients (data not shown), which indicate distinct roles for tumor cell adhesion and tumor cell motility in the extravasation process. As shown in Fig. 10, chemotactic diffusion gradients influence the total number of extravasated tumor cells through the barrier when gradients changed from G50:10 to G100:10, which correlate with earlier results in tumor cell protrusion frequency and cell locomotion speed.

Figure 11 (A) shows that the regulation of adhesion between C8161 and an ICAM-1 expressed monolayer is influenced by Mac-1, an $\alpha_m\beta_2$ integrin, and not necessarily by LFA-1 ($\alpha_L\beta_2$) integrin molecules. Some recent studies⁶ have suggested that at low shear, β_2 integrins adhesion to ICAM-1 appears to be a cooperative and sequential process of LFA-1-dependent capture followed by Mac-1-mediated stabilization. Those findings might explain why LFA-1 did not significantly influence C8161 adhesion to ICAM-expressed EI cells [Fig. 11(A)]. Melanoma cells [with relatively low β_2 integrins; Fig. 11(B)] appeared to be able to extravasate to ECM through EI cell monolayer under static conditions, but significantly lower ($p=0.002$) under dynamic flow conditions [Fig. 11(A)]. We used EI cells as an endothelial model during the development of this flow-migration system, because EI cells express stable ICAM-1 adhesion molecule.⁵ We found that human melanoma cells express relatively low β_2 integrins, but high ICAM-1 molecules [Fig. 11(B)]. Low levels of Mac-1 and LFA-1 expression on C8161 cells would make high affinity or receptor-mediated firm adhesion of C8161 to an ICAM-1-expressed endothelium unlikely, especially under dynamic flow conditions.

The question is how ICAM-1-positive melanoma cells attach to or arrest on an ICAM-1-expressing endothelium under dynamic flow conditions prior to extravasation. Do melanoma cells possibly attach to endothelium via other β_2 integrin-expressed myeloid cells within the circulation, e.g., leukocytes?

Leukocytes that express β_2 integrins could potentially enhance C8161 melanoma adhesion to endothelium by acting as a bridge between the two cells. For this reason, Jurkat cells, a human leukemic T-cell line (expressing LFA-1 integrins), were used in this study as a model for leukocytes. Results from Fig. 12(B) indicate that emboli formation of a melanoma cell to a β_2 integrin-rich cell, such as leukocytes, would allow melanoma cells to maintain a stronger contact with an endothelial surface, hence, subsequent extravasation under the dynamic flow conditions [Fig. 12(A)].

Although this paper has initially focused on ICAM-1 and β_2 integrin interaction as an important step in establishing tumor cell adhesion to the endothelium under flow conditions, VCAM-1 and cadherins are very important as well in tumor cell extravasation through the endothelial junction. Such investigations are currently undertaking in our laboratory. Studies on many cell types have shown that chemotactic signals can elicit a series of biochemical events, which are regulated in a coordinated manner to result in a controlled cell motion. At the biochemical level, the mechanism of migration used by tumor cells may parallel, or be similar to, that used by many nonmalignant motile cells. Such studies are currently being developed in our laboratory to provide insights into the ability and effectiveness of tumor cell extravasation, and respective mechanisms that underlie the diversity of the tumor metastatic processes.

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FIGURE 1.

Steps in the process of metastasis, illustrating the spread of cancer from the primary site to the secondary site. Tumor cells are shown crossing the wall of the blood vessel.

FIGURE 2.

Sequence of video images showing progressive pseudopod protrusion and subsequent cell forward motion of a representative human A2058 melanoma cell in response to soluble type IV collagen (100 $\mu\text{g/ml}$; from the left-side pipette). A schematic diagram of the dual-micropipette set-up is shown on the right. C_0 is the chemotactic source concentration. δ is the initial distance between the chemotactic source and the cell front surface. L_p and L_c are cell pseudopod protrusion length and forward distance, respectively. The coordinate $x=L(t)$ represents the moving boundary due to the cell movement.

FIGURE 3.

Schematic of the flow-migration chemotaxis chamber. (A) Side view of a chamber prior to assembly with both the top and base plates. The gasket is 0.02 in. silicone sheet and the filter is 8 μm pore polycarbonate film of approximately 10 μm thick. (B) Top view of an assembled chamber. (C) A glass block was used during the tissue culture. Within the space of this block, cells are seeded on the polycarbonate filter to ensure cell growth only on the topside of the filter. (D) A micrograph shows a monolayer of transfected cells has formed on the polycarbonate filter as a model of endothelium.

FIGURE 4.

Curves representing mean values for (A) cell forward speed, (B) pseudopod peak length, and (C) pseudopod peak frequency bracketed by their SEM, respectively, as a function of time. All the curves were generated from eight independent experiments (without specific adhesion proteins coated on the pipette substrate) under three initial gradients in chemotactic concentration: G200:5 (open squares; $C_0=200 \mu g/ml$ and $\delta=5 \mu m$), G100:5 (closed circles; $C_0=100 \mu g/ml$ and $\delta=5 \mu m$), and G200:10 (open triangles; $C_0=200 \mu g/ml$ and $\delta=10 \mu m$). SEM for abscissa values is also indicated, except where they are smaller than the size of the symbols.

FIGURE 5.

Shown are temporal averages for (A) forward speed $\overline{dL_c/dt}$; (B) pseudopod peak length $\overline{L_p}$; and (C) pseudopod peak frequency $\overline{f_p}$; bracketed by their SEM. The data were generated from Fig. 4 in response to initial chemotactic concentration gradients of G200:5, G100:5, and G200:10, respectively. p values were obtained using the Wilcoxon Mann-Whitney test.

FIGURE 6.

(A) The concentration gradient of type IV collagen over a distance $x=L(t)$, with three different initial C_0/δ : G200:5 (solid line); G100:5 (dashed line); and G200:10 (dotted line), respectively. T is the total migration time (averaged from eight observations in each C_0/δ) for a cell to crawl over the chosen distance δ . (B) Calculations of type IV collagen concentrations $C_d=C\{x=L(t),t\}$ on the cell front surface. T is the total migration time (average from eight observations in each C_0/δ) as in A. Curves, G200:5 (solid), G100:5 (dashed line), and G200:10 (dotted line), were obtained with $k_f=3.42$, $k_g=1.18 \times 10^{-10}$ (G200:5); $k_f=3.34$, $k_g=0.53 \times 10^{-10}$ (G100:5); and $k_f=2.89$, $k_g=0.45 \times 10^{-10}$ (G200:10), respectively. (C) Comparisons of experimental measurements (symbols) and theoretical calculations (curves) of cell forward speed as a

function of time. Mean $\pm$ SEM are shown for G200:5 (open squares); G100:5 (closed circles); and G200:10 (open triangles). Theoretical curves were obtained with the same parameters used in calculating B.

FIGURE 7.

Flow cytometry data showing the total amount of collagen IV molecules bound to A2058 human melanoma cells as a function of (A) molecule concentrations (at a given reaction time of 25 min), and (B) reaction time (at a given collagen IV concentration of 100 $\mu\text{g/ml}$).

FIGURE 8.

The results of migration assays under static and flow conditions. C8161 cells migrated under the influence of CIV (100 μ g/ml) over 4 h. Each bar represents at least four trials and the error bar represents the SEM.

FIGURE 9.

Time curve of migration assay results demonstrating that 4 h is sufficient for saturated cell migration. C8161 cells were allowed to migrate under 4 dyn/cm² shear stress to CIV (100 µg/ml) for the indicated lengths of time. Each bar represents at least three trials and the error bar represents the SEM.

FIGURE 10.

Functional curve of migration assay results demonstrating that varying chemoattractant concentration gradient (C_0/δ =G50:10, G100:10, and G200:10, respectively) can modulate cell migration. Conditions for each case are under 4 dyn/cm² shear stress over 4 h. Each bar represents at least three trials and the error bar represents the SEM.

FIGURE 11.

(A) Functional curve of migration assay results demonstrating that antibody blocking β_2 -integrins can characterize C8161 adhesion mechanisms. Blocking Mac-1 on C8161s or blocking ICAM-1 on EI cells inhibits C8161 migration through an EI-cell monolayer, whereas blocking LFA-1 does not affect C8161 migration. C8161 cells (treated or untreated) are assayed under 4 dyn/cm² shear stress in response to CIV (100 μ g/ml) over 4 h. Each bar represents at least three trials and the error bar represents the SEM. (B) Flow cytometry data showing LFA-1, Mac-1 and ICAM-1 molecule expressions on human C8161 melanoma cells. ICAM-1 expression was further increased when C8161 cells were activated by TNF- α (10 U/ml). There

was no significant increase in LFA-1 or Mac-1 expressions when C8161 cells were activated by PMA (50 ng/ml) or IL-8 (1 μ g/ml), respectively (data not shown).

FIGURE 12.

(A) Flow migration data showing that PMA-treated Jurkat cells were able to significantly recover tumor cell migration under shear stress. (B) C8161/PMA-treated Jurkat doublet adhesion to an EI cell monolayer as tested using a flow chamber assay. More doublets were able to adhere to the EI monolayer as compared to single C8161 cells under increasing shear stress, thus indicating improved adhesion.