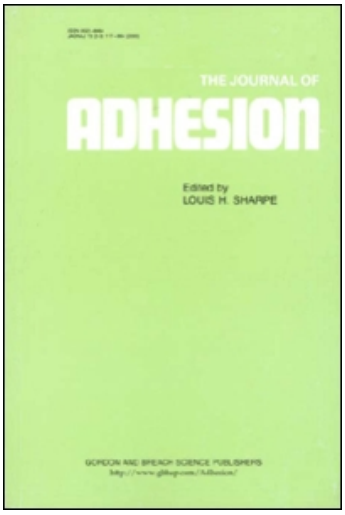

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Adhesion of Cancer Cells to Endothelial Monolayers: A Study of Initial Attachment *Versus* Firm Adhesion

Melissa A. Moss[a]; Kimberly W. Anderson[b]
[a] Department of Research, Mayo Clinic Jacksonville, Jacksonville, FL, USA [b] Department of Chemical and Materials Engineering, 177 Anderson Hall, University of Kentucky, Lexington, KY, USA

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Adhesion of Cancer Cells to Endothelial Monolayers: A Study of Initial Attachment *Versus* Firm Adhesion

MELISSA A. MOSS [a] and KIMBERLY W. ANDERSON [b,*]

[a]*Department of Research, Mayo Clinic Jacksonville, Jacksonville, FL 32224, USA;*
[b]*Department of Chemical and Materials Engineering, 177 Anderson Hall, University of Kentucky, Lexington, KY40506-0046, USA*

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For most cancer patients, the ultimate cause of death is not the primary tumor itself, but metastasis, or the spread of cancer from the primary tumor throughout the body. The formation of tumor foci at sites distant from the primary tumor is a multistep process which includes dissemination of the cancer cells through the blood stream and hence, interactions with the endothelium lining the blood vessels walls. At least two theories have been proposed for explaining the interaction between cancer cells and endothelium. According to one theory, the tumor cells roll along the endothelium and the rolling velocity decreases until the cells become firmly attached to the vessel wall. In another theory, the circulating cancer cells must first lodge inside small vessels before they attach to the endothelium. In the latter case, the cells would only metastasize in the smaller vessels where lodging can occur. To gain further insight into the process of metastasis, the adhesion of human breast cancer cells to human umbilical vein endothelial monolayers was investigated in terms of both initial attachment followed by firm adhesion and firm adhesion following incubation in a static environment. The parallel plate flow chamber was employed to perform two different adhesion assays that would simulate these two adhesion mechanisms. Adhesion assays were carried out at a variety of physiological shear stresses found in the microvasculature and both highly metastatic and nonmetastatic cells were investigated. Results showed that initial attachment was only observed at very low shear stresses whereas firm adhesion occurred at a number of physiological shear stresses. These results suggest that the adhesion of the human breast cancer cells used in this study to endothelium most likely takes place *via* a lodging-firm adhesion mechanism in the capillaries and venules. However, it is important to note that other factors such as pulsatility and vessel compliance may contribute to the attachment. It was also shown that,

* Corresponding author. Tel.: (606) 257-8059, Fax: (606) 323-1929, e-mail: kanderson@engr.uky.edu

for these specific breast cancer cells, adhesion did not correlate with metastatic potential. This suggests that while blocking the adhesion of highly metastatic cells may inhibit their ability to metastasize, adhesion properties alone do not provide an indication as to whether a cell is metastatic or nonmetastatic under the conditions studied here.

Keywords: Metastasis; Adhesion; Parallel-plate flow chamber; Lodging; Attachment

INTRODUCTION

Breast cancer is the most common cancer among women in the USA with one in eight women falling victim to the disease (Statistics from the National Cancer Institute). For most cancer patients, the ultimate cause of death is not the primary tumor itself, but metastasis, or the spread of cancer from the primary tumor throughout the body. Consequently, an important step in cancer research is understanding the metastatic cascade. Correlations between tumor microvessel density and degree of metastasis indicate that breast cancer metastasizes primarily *via* the blood vessels, rather than through the lymphatic system [1]. Metastasis, or the formation of tumor foci at sites distant from the primary tumor, is a multistep process. This process is depicted in Figure 1 for metastasis *via* the blood stream. To metastasize, cancer cells must detach from the primary tumor, intravasate through the vessel wall to enter the blood stream, disseminate through the blood stream, and extravasate back through the vessel wall to reestablish in the interstitial tissue. Many cancers will metastasize preferentially to certain organs. For example, prostate cancers metastasize most frequently to bone and small-cell lung

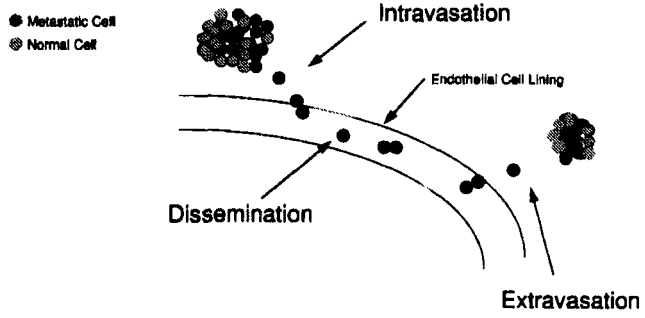

FIGURE 1 Schematic representing the various steps of the metastatic cascade in the microvasculature.

carcinomas most often to the brain. This organ-specific nature of metastasis has been attributed to interactions during the dissemination step between endothelial adhesion molecule ligands and their specific adhesion molecule receptors expressed on cancer cells [2]. Such interactions are involved in the attachment of circulating cancer cells to the endothelium that lines the vessel walls. Changes in adhesion molecule expression or binding affinity will enhance or decrease the adhesion potential of a receptor-ligand pair. Identification of adhesion molecules involved in the interaction of a cell pair is important to understanding adhesion. The adhesion between different cancer cell-endothelial cell pairs is likely to be mediated by different receptor-ligand combinations. In addition, more than one receptor-ligand pair may be responsible for a single cell-cell adhesion event. A review of the surface molecules responsible for adhesion can be found in [3].

At least two theories by which cancer cells adhere to the endothelium have been suggested in the literature. One hypothesis, set forth by Honn and Tang [4], is known as the 'docking and locking' hypothesis. According to this hypothesis, cancer cells first 'dock' and roll along the endothelium by rapidly forming and breaking adhesion molecule interactions with endothelial cells [5]. The rolling of tumor cells along the endothelium may also serve to activate additional adhesion molecules involved in the second step of cell attachment. As adhesion interactions form and break, the rolling velocity of the cancer cell decreases until the cell velocity is slow enough that more firm adhesion forms in the second 'locking' step of adhesion. These interactions are able to resist rheological forces and, thus, render the circulating cell stationary. In support of this hypothesis, cancer cell rolling followed by attachment has been observed *in vivo* for activated endothelium [6]. In addition, several researchers have employed *in vitro* dynamic adhesion assays to observe tumor cell rolling on endothelial monolayers [7–12].

In another theory, circulating cancer cells must first lodge inside small vessels before they adhere to the endothelium. When a circulating cancer cell encounters a vessel diameter smaller than that of the cancer cell itself, the cell will deform similar to noncancerous cells in the microvasculature in an attempt to pass through the vessel. If deformation of the cell does not exceed the elasticity limits of the cell membrane, the cancer cell will successfully pass through the vessel. If the

elasticity limits of the cell membrane are exceeded, however, the cell membrane will rupture and the cancer cell will be killed [13–15]. Those cells that are not killed can be trapped in the vasculature. Entrapment of deformed cancer cells has been observed in the microcirculation of model animals [13]. After the cancer cell has stopped as a result of lodging, firm adhesion is able to take place between the stationary cancer cell and the endothelium. These interactions serve to stabilize the arrested cell so that rheological forces fail to dislodge the cell and the cell remains adherent. *In vivo* observations of cancer cell arrest without stimulation of adhesion molecules on endothelial cells reveal an absence of cell rolling along the endothelium and attribute cancer cell arrest to lodging of the cells within the microvasculature [6, 16, 17]. It remains to be shown which of these hypotheses most accurately reflects the mechanism by which cancer cells adhere to the endothelium, an essential step in the metastatic cascade.

Many studies which have investigated the adhesion of cancer cells to endothelial monolayers or extracellular matrix proteins have employed a static adhesion assay to quantify adhesive strength [18–30]. In a static adhesion assay, cancer cells are allowed to settle and adhere to the endothelial monolayer for a given period of time and nonadherent cells are removed through a series of manual washes. Difficulties in quantification arise from the fact that forces imposed during manual washing usually are not measured and may not be uniform. As a result, static adhesion assays provide only qualitative comparisons of adhesive properties. Furthermore, this system permits only the measurement of firm adhesion under static conditions. The event of initial attachment of cancer cells in fluid flow to a stationary endothelial monolayer or extracellular matrix protein cannot be observed.

Studies of the adhesion of cancer cells to endothelial monolayers or extracellular matrix proteins have also employed flow chambers [8–12, 31–33]. The hydrodynamic forces acting upon the cells within these flow chamber systems can be both measured and controlled. Consequently, accurate quantitative measurements of cell adhesion are possible. Flow chambers also provide an adhesion environment that better mimics the conditions under which cancer cells attach to the endothelium *in vivo* by allowing observation of the attachment of cells under flow conditions. Because adhesion can differ under static and flow conditions, it is important to study adhesion under conditions that

best mimic the *in vivo* situation. For example, Felding-Habermann *et al.* [33] observed that while M21 melanoma cells adhere to a collagen matrix under static conditions, adhesion fails to occur under dynamic flow conditions, even at a low shear stress of 2 dynes cm^{-2}. Similarly, Kojima *et al.* [8] demonstrated that differences in the adhesion of B16 melanoma variant cells to endothelial monolayers were more readily observed in a dynamic assay than in a static assay. Furthermore, it was deduced that the mechanism responsible for adhesion differed in the two systems.

In any flow chamber system, both attachment and detachment assays may be used. In our specific detachment assay, cells are allowed to settle under zero flow for a given period of time. The cells are then removed with a measurable shear stress and the detached cells are quantified. In contrast, during an attachment assay, cell adhesion takes place as the cells are moving with the shearing fluid and the adherent cells are quantified.

To study the mechanism for adhesion in metastasis, we used the parallel plate flow chamber to investigate the adhesion of human breast cancer cells to human umbilical vein endothelial cell (HUVEC) monolayers using the attachment and detachment assay types to simulate initial attachment and post-lodging firm adhesion, respectively. These two mechanisms for adhesion were studied at a variety of shear stresses corresponding to the shear stresses that the cancer cells experience in different vessel types within the body. The adhesive properties of two different breast cancer cell lines with different metastatic potentials, MCF-7 (nonmetastatic) and MDA-MB-435 (highly metastatic), were studied to elucidate whether variations in adhesive strength with assay type and shear stress are dependent on metastatic potential. The rest of this manuscript will focus on the procedure for performing these studies and the results obtained.

MATERIALS AND METHODS

Cell Lines and Cell Culture Techniques

Endothelial monolayers were composed of human umbilical vein endothelial cells (HUVECs) obtained from Cell Systems (Kirkland, WA). Cells were purchased at passage 1 and used in adhesion studies

up to passage 8. Preliminary studies showed that adhesion of the breast cancer cells used in this study to HUVECs was consistent up to passage 10. HUVECs were grown in 75 cm^2 Costar tissue culture flasks coated with CSC attachment factor (Cell Systems). HUVECs were sustained in CSC Complete Medium (Cell Systems) and maintained in a humidified incubator which provided an atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 C.

The MCF-7 human breast cancer cell line was obtained from American Type Cell Culture (ATCC, Rockville, MD) at passages ranging between 120 and 180. This cell line has been shown to be nonmetastatic in a nude mouse model [34]. The MDA-MB-435 human breast cancer cell line was obtained from the laboratory of Janet Price (M. D. Anderson Cancer Center, Houston, TX) at passages ranging between 50 and 100. This cell line has been shown to be highly metastatic in both spontaneous and experimental metastasis models [35, 36]. Because the cells were received at high passage, they were checked periodically to confirm their metastatic potential. Both human breast cancer cell lines were grown as monolayers in either 75 cm^2 or 150 cm^2 Corning tissue culture flasks. Cells were maintained in Minimum Essential Media (MEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO), 10,000 units ml^{-1} penicillin (Gibco), 10 μg ml^{-1} streptomycin (Gibco), 1 mM glutamine (Gibco), 0.3% sodium bicarbonate (Gibco), and 2.5 mM nonessential amino acids (Gibco). Human breast cancer cells were maintained in a humidified incubator which provided an atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 C.

Preparation of the HUVEC Monolayer

HUVECs were seeded onto Permanox slides (Fisher) coated with 2% gelatin (Sigma) and endothelial cell attachment factor (Cell Systems). Seeded monolayers were sustained in CSC Complete Medium (Cell Systems) and maintained in a humidified incubator which provided an atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 C. HUVEC monolayers were allowed to reach confluency (3–4 days) under static conditions on the Permanox slides prior to assembly of the flow chamber. HUVEC monolayers were stimulated by replacing cell culture media with 240 U of tumor necrosis factor-alpha

(TNF-α) (Promega, Madison, WI) suspended in 2 ml of CSC Complete Medium 4 hours prior to flow chamber assembly. TNF-α is a 52 kDa protein and is named for its ability to induce necrosis in primary tumors [37]. It has been shown that exposure of endothelial cells to TNF-α leads to an increase in expression of adhesion molecules such as ICAM-1, VCAM-1, *E*-selectin, *P*-selectin, and hyaluronate [38–43]. We chose to stimulate our endothelial cells with TNF-α because several *in vivo* sources of TNF-α exist and previous results have shown that the cancer cells used in these studies do not attach to unstimulated endothelial cells under flow conditions [10, 44]. The monolayers were returned to the cell culture incubator for the 4-hour incubation period. Prior to experimentation, the TNF-α was removed and the endothelial cells were washed with media.

Preparation of Human Breast Cancer Cells

Prior to experiments, confluent monolayers of human breast cancer cells were trypsinized and resuspended at a concentration of either 5.0×10^5 cells ml^{-1} for attachment experiments or 1.25×10^6 cells ml^{-1} for detachment experiments. Resuspension took place in a solution of dextran (Sigma) dissolved in CSC Complete Medium. A calibrated amount of dextran was added to this medium so that the viscosity of the cell suspension would match that of blood (3.9 cp). The cell suspension was maintained at 37 C.

Flow Chamber and Related Equipment

To assess the adhesive strength between human breast cancer cells and endothelial monolayers, two different adhesion assay types, an initial attachment assay and a detachment assay, were carried out inside a parallel plate flow chamber. The parallel plate flow chamber, depicted in Figure 2, consisted of a polycarbonate plate and a Permanox slide with a confluent layer of endothelial cells. The two plates were separated by a Silastic gasket. The system was held together by a vacuum to ensure a constant height and, therefore, a constant wall shear stress, along the length of the flow chamber. The Permanox/cell surface composed the bottom of the flow chamber. Two pressure ports located in the polycarbonate base enabled measurement of the pressure drop across

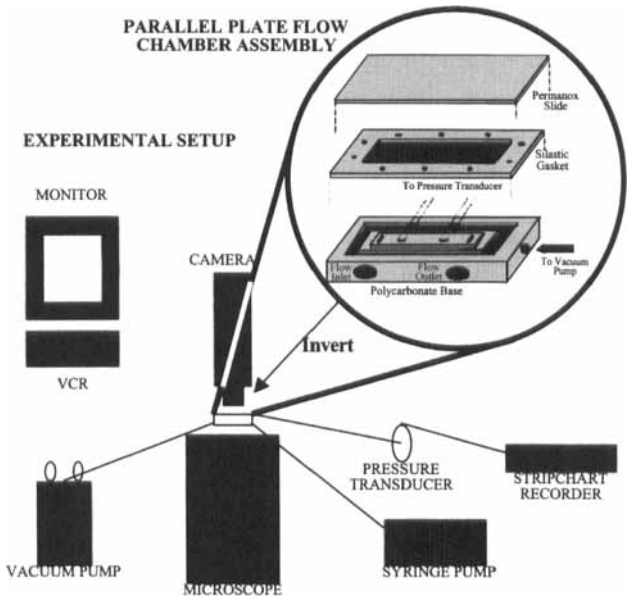

FIGURE 2 Schematic of experiment setup employed in the adhesion studies. The parallel-plate flow chamber is inverted on the microscope stage such that the endothelial monolayer comprises the bottom of the chamber.

the chamber. These pressure ports were connected *via* fluid filled tubing to a variable reluctance differential pressure transducer (Validyne Engineering, Northridge, CA). The transducer transmitted a signal to a digital indicator (Validyne Engineering) and stripchart recorder (Linseis, Princeton, NJ) so that the pressure drop across the monolayer could be continuously monitored and recorded. This pressure drop was related to the shear stress imposed upon the endothelial monolayer using the theory of plane Poiseuille flow, or flow between two infinite parallel plates.

Inlet and outlet ports within the flow chamber permitted the entrance and exit of media and cell suspensions. Inlet tubing consisted of both a primary and a secondary line. The primary line connected directly to the flow chamber itself, while the secondary line formed a T-connection with the primary tubing. During experimentation, the primary line was employed to introduce cell-free media into the flow chamber, and the secondary line was used to introduce the human breast cancer cell suspensions. All flow solutions contained endothelial

CSC Complete Medium, a native environment for the HUVEC monolayer. A calibrated amount of dextran (Sigma) was dissolved in this medium to increase the fluid viscosity to match that of blood (3.9 cp). All flow solutions were introduced using a Harvard syringe pump. Media and cell suspensions were maintained at 37 C.

The shear stress imposed upon the endothelial monolayer, or the shear stress at the wall, was calculated using:

$$\tau = \frac{\Delta P d}{2L}. \tag{1}$$

Here, τ is the shear stress in dynes cm^{-2}, ΔP is the measured pressure drop in mm Hg, and L is the distance between the pressure ports (2.33 cm). The height of the flow chamber, d, in cm, was calculated by:

$$d^3 = \frac{12\mu QL}{b\Delta P}. \tag{2}$$

Here, μ is the fluid viscosity (3.9 cp), Q is the flow rate in $ml\,min^{-1}$ controlled by the syringe pump, b is the chamber width (1.45 cm), and ΔP is the measured pressure drop in mm Hg. The height varied in each experiment depending on the quality of vacuum and the compressibility of the gasket. The range was 0.018 – 0.025 cm.

Once assembled, the flow chamber was inverted so that the endothelial monolayer served as the bottom of the chamber. The flow chamber was then mounted on the stage of an inverted, phase-contrast, light microscope (Zeiss, Batavia, IL). This microscope was equipped with a video camera (Phonic Microscopy, Oak Brooke, IL), black and white monitor (Sony, Teaneck, NJ), VCR (Panasonic, Secaucus, NJ), and time-date generator (Panasonic) so that experiments could be documented for analysis at a later time. A schematic of the complete system is shown in Figure 2.

Adhesion Assays

Initial Attachment

To quantify the initial attachment of human breast cancer cells to endothelial monolayers, an attachment assay was carried out inside

the parallel plate flow chamber. Following assembly of the flow chamber, the primary line was used to perfuse the monolayer at a flow rate of 1.0 ml min^{-1} (shear stress of approximately 8 dynes cm^{-2}) for 2 minutes to remove loose endothelial cells or cell fragments resting on the monolayer. The monolayer was then perfused at the flow rate required to impose the desired shear stress of 0.25, 1, 5, 10 or 15 dynes cm^{-2}. The monolayer was perfused for 3 minutes to ensure that the desired shear stress was obtained. The secondary line was then employed to pass human breast cancer cells at a concentration of 5.0×10^5 cells ml^{-1} across the monolayer. Attachment of human breast cancer cells was allowed to proceed at this desired shear stress for a period of 30 minutes and the experiment was terminated. This experiment simulated the initial attachment of cancer cells to the endothelium *in vivo*. Rolling cancer cells were observed to slow and eventually stop and adhere to the endothelial monolayer. The attachment period was recorded and the video was analyzed at a later time for total number of stationary cells at 0, 5, 10, 15, 20, 25 and 30 minutes. Details of the data analysis are given in the section below.

This attachment protocol was employed to evaluate the conditions of initial attachment between the HUVEC monolayer and both MCF-7 and MDA-MB-435 human breast cancer cells using 5 different shear stresses (0.25, 1, 5, 10 and 15 dynes cm^{-2}). All experiments in which attachment was observed were completed with 6 repetitions. Experiments where rolling but no attachment was observed were completed with 3 repetitions.

Detachment

A detachment assay carried out inside the parallel plate flow chamber was employed to assess the firm adhesion of human breast cancer cells to HUVEC monolayers. A suspension of 1.25×10^6 human breast cancer cells ml^{-1} was introduced at a low flow rate corresponding to a shear stress of 0.025 dynes cm^{-2} using the secondary line. Running at this low shear stress also permitted the removal of loose endothelial cells and cell fragments from the monolayer. The flow was then stopped and the cancer cells were allowed to settle and adhere to the endothelial monolayer for a period of 30 minutes. Because a cell that becomes trapped in a vessel probably occludes or partially occludes

the vessel, this stationary settling simulated the lodging of cancer cells within the vasculature. At the end of the settling time, the desired shear stress of 1, 5, 10 or 15 dynes cm^{-2} was imposed upon the monolayer to assess the strength of adhesion. Shear exposure continued for a period of 9 minutes, a duration that allowed detachment to reach an extinction value. The 9-minute detachment period was recorded and the video was analyzed at a later time.

This detachment assay was employed to evaluate the relative strength of adhesion between the HUVEC monolayer and both MCF-7 and MDA-MB-435 human breast cancer cells after a static 30-minute exposure period. Studies were performed at 4 different detachment shear stresses (1, 5, 10 and 15 dynes cm^{-2}). All experiments were completed with 6 repetitions.

Data Analysis

Initial Attachment

The attachment experiments were analyzed by counting the total number of stationary, adherent cells at 0, 5, 10, 15, 20, 25 and 30 minutes. The results showed a linear trend of attachment *vs* time and experimental results were reported as the total number of adherent cells at the end of the 30-minute attachment period. This value was used for statistical analysis of the effects of shear stress and metastatic potential on attachment.

Detachment

The detachment experiments were analyzed by counting the total number of cells present on the endothelial monolayer at the end of the 30-minute settling time and then counting the total number of cells remaining following the onset of the desired shear stress at 5, 30, 60, 180, 300, 420, 480 and 540 seconds. Results were plotted as fraction of cells retained *versus* time. The initial number of cells prior to shear stress ranged from 80–100. All results exhibited a leveling off of the value of fraction retained by the end of the 9-minute detachment time, indicating that an extinction value had been reached. Results were, thus, recorded as the extinction value at the end of the 9-minute

detachment period. These extinction values were used for statistical analysis of the effects of shear stress and metastatic potential on detachment.

For both attachment and detachment experiments, the final magnification on the video monitor was 200X. One field per plate was analyzed with an area of $0.012\,cm^2$. The area was chosen in the center of the plate to ensure that the cells were exposed to fully developed flow (entrance length = 0.003 cm).

Statistics

Statistical significance was assessed using a two-way ANOVA followed by Student-Newman-Keuls *post-hoc* tests to compare the effect and interaction of cell type and shear stress for both initial attachment and detachment experiments.

RESULTS

Initial Attachment

Results for the initial attachment of human breast cancer cells to stimulated endothelial monolayers under shear stresses of 0.25, 1, 5, 10, and 15 dynes cm^{-2} are shown in Figure 3 for both nonmetastatic MCF-7 cells and highly metastatic MDA-MB-435 cells. These results indicated that the initial attachment of highly-metastatic human breast cancer cells to endothelial monolayers was significant only at a shear stress of 0.25 dynes cm^{-2}. In addition, the number of highly metastatic cells adhering at this shear stress was significantly greater than the number of attached lowly-metastatic cells. Approximately 100 MDA-MB-435 cells had attached after 30 minutes of flow compared with 60 MCF-7 cells. At higher shear stresses, zero attachment of the highly-metastatic cells was observed. Initial attachment of nonmetastatic cells was significant at both 0.25 and 1.0 dynes cm^{-2} although the number of cells observed at 1 dyne cm^{-2} was 69% less than at 0.25 dynes cm^{-2}.

Attachment under higher shear stresses of 5, 10 and 15 dynes cm^{-2} yielded zero attachment to endothelial cell monolayers although

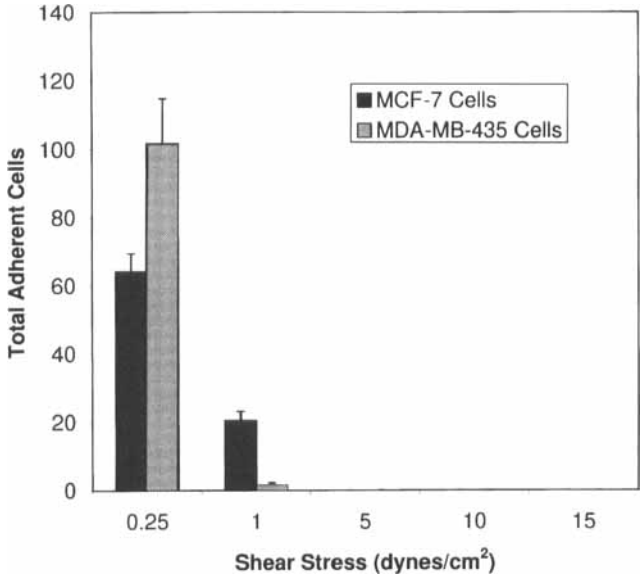

FIGURE 3 Total number of adherent cells *vs* shear stress applied in the attachment assay for both nonmetastatic (MCF-7) and highly-metastatic (MDA-MB-435) cells. Error bars indicate standard error. $N = 6$ for 0.25 and 1 dyne cm^{-2}. $N = 3$ for 5, 10 and 15 dyne cm^{-2}.

rolling along the monolayer was observed. This was true for both nonmetastatic MCF-7 cells and highly-metastatic MDA-MB-435 cells.

Detachment

Results for detachment of human breast cancer cells to endothelial monolayers under detachment shear stresses of 1, 5, 10 and 15 dynes cm^{-2} are shown in Figure 4 for nonmetastatic MCF-7 cells and highly metastatic MDA-435 cells. These results showed that at a shear stress of 15 dynes cm^{-2}, less than 20% of the cells remained attached to the endothelial monolayer. At detachment shear stresses of 10 dynes cm^{-2} or lower, however, more than 30% of cells remained attached to the endothelial cells. There was no significant difference between the nonmetastatic cells and highly-metastatic cells except at 10 dynes cm^{-2} where the nonmetastatic cells were slightly more adherent and at 5 dynes cm^{-2} where the highly-metastatic cells were slightly more adherent.

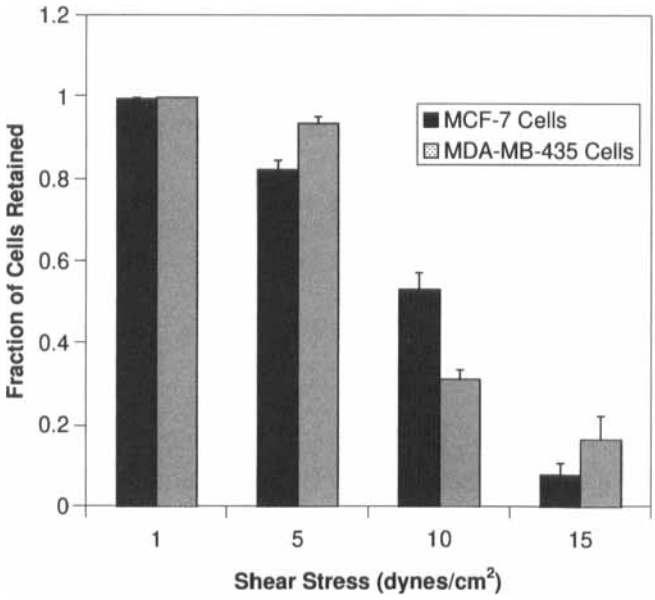

FIGURE 4 Fraction of cells retained *vs* shear stress applied in the detachment experiments for both nonmetastatic (MCF-7) and highly-metastatic (MDA-MB-435) cells. Error bars indicate standard error. $N = 6$ for all shear stresses.

DISCUSSION

Adhesion of cancer cells to vascular endothelium and its role in metastasis formation has been a focus of interest in cancer research for a long time. This research has led to at least two different theories concerning the means by which cancer cells adhere to the endothelium. Many of the studies that have investigated the adhesion of cancer cells to the endothelium have employed a static adhesion assay to evaluate adhesive strength [18, 20–22, 45]. In a static adhesion assay, cancer cells are allowed to settle and adhere to the endothelial monolayer for a given period of time and nonadherent cells are removed through a series of manual washes. Usually, the detachment forces imposed upon adherent cells are neither controlled nor measured. Furthermore, a static system permits only the evaluation of relative adhesion. The initial attachment of cancer cells in fluid flow to a stationary endothelial monolayer cannot be observed. The parallel-plate flow

chamber design overcomes these obstacles. Inside the parallel-plate flow chamber, fluid flow between two parallel plates can be observed. This flow is described by plane, Poiseuille flow, and fluid flow can be modeled, measured, and controlled. Consequently, cells suspended in the fluid can attach or adherent cells can be detached under a constant, designated shear stress.

A number of researchers have employed this flow chamber geometry to study the adhesion of cancer cells to endothelial monolayers, extracellular matrix proteins, and ligand coated surfaces [7–10, 12, 31, 33]. Aigner *et al.* [12] employed the parallel-plate flow chamber to study the rolling of breast carcinoma cells on *P*-selectin coated surfaces. Tozeren *et al.* [10] used the parallel-plate flow chamber to carry out both detachment and attachment assays at several shear stresses to study the adhesion of a variety of breast cancer cells to TNF-α stimulated endothelial monolayers. In a separate study, Tozeren and coworkers employed the parallel-plate flow chamber at a variety of shear stresses to demonstrate that the integrin $\alpha_6\beta_4$ was capable of supporting both stable and dynamic attachment of several tumor cell lines [32]. Giavazzi *et al.* [9] studied the dynamic interaction of colon carcinoma, ovarian carcinoma, and breast carcinoma cells with IL-1 activated endothelial monolayers under incrementally decreasing shear stresses using the parallel-plate flow chamber. Patton *et al.* [31] employed the parallel-plate flow chamber, along with computerized analysis, to observe the attachment and subsequent stabilization of tumor cells on a laminin matrix. Kojima *et al.* [7] used the parallel-plate flow chamber over a range of shear stresses to demonstrate that adhesion of HL60 leukemia cells to *E*-selectin coated surfaces is mediated by different ligands at low and high shear stresses. In a separate study, Kojima and coworkers [8] employed the parallel-plate flow chamber and a dynamic attachment assay to correlate the initial attachment of B16 melanoma cell variants to endothelial monolayers with their metastatic capability. Felding-Habermann and coworkers employed the parallel plate flow chamber to investigate the involvement of platelets in the adhesion of melanoma cells to collagen [33].

In this study, the parallel-plate flow chamber was employed to observe both the initial attachment of cells in fluid flow to an endothelial monolayer and the detachment of adherent cells from an endothelial monolayer, such that both the initial attachment under flow conditions

and detachment following exposure to a 30-minute settling time of human breast cancer cells to the endothelium could be evaluated.

Experiments carried out inside the parallel-plate flow chamber indicated that the initial attachment of highly metastatic human breast cancer cells to endothelial monolayers was significant only at a shear stress of 0.25 dynes cm^{-2}. At higher shear stresses, zero attachment was observed even though the cells did roll along the monolayer. Nonmetastatic cells were able to attach at 0.25 and 1.0 dynes cm^{-2}; however, the number of cells remaining attached at 1.0 dynes cm^{-2} was less than the number at 0.25 dynes cm^{-2}. Parallel detachment experiments showed that at a shear stress of 15 dynes cm^{-2}, less than 20% of the cells remained attached to the endothelial monolayer. At detachment shear stresses of 10 dynes cm^{-2} or lower, however, more than 30% of cells remained attached with 100% of the cells retained at 1 dyne cm^{-2}. These experimental results were compared with physiological parameters for several different human vessel types. Findings are summarized in Table I. Here, the average diameter and wall shear stress for each vessel type are given. The wall shear stress was calculated from the average blood velocity, the vessel diameter, and the viscosity of plasma, or the fluid viscosity at the wall [45]. The wall shear stress was compared with results from the initial attachment and detachment experiments at various shear stresses to determine whether initial attachment and/or detachment could occur in each vessel type. Finally, using the vessel diameter, the possibility of cell lodging was determined by comparing vessel diameter with cell size. An average tumor cell diameter of 20 μm was used [46] which corresponds to the average diameter of the cancer cells used in this study.

As indicated in Table I, our results support the hypothesis that initial attachment of human breast cancer cells to endothelium is not

TABLE I Comparison of physiological parameters in various human vessel types with results from initial attachment and firm adhesion studies. Average values for diameters and shear stresses in the vessels were obtained from Ref. [45]

Vessel type	*D (μm)*	τ_w *(d/cm²)*	*Initial attachment*	*Firm adhesion*	*Lodging*
Arteries	4000	9.9	–	+	–
Arterioles	50	88	–	–	–
Capillaries	8	11	–	+	+
Venules	20	8.8	–	+	+
Veins	5000	1.76	–	+	–

likely to occur at the average physiological shear stress reported in the vessel types shown. In contrast, firm adhesion can occur at the shear stresses observed in the arteries, capillaries, venules, and veins. While 88 dynes cm^{-2} is beyond the limit of our experimental system, a single firm adhesion experiment performed at a detachment shear stress of 30 dynes cm^{-2} for each cell line revealed that less than 5% of cells remained firmly adherent to the endothelial monolayer (data not shown). Thus, it is unlikely that significant cellular retention would occur at an even higher shear stress of 88 dynes cm^{-2}. Before firm adhesion can occur, cancer cells must first lodge inside the vessel. The size comparison revealed that lodging is likely to occur only in capillaries and venules. The other vessel types have diameters much larger than the diameters of the cells and, hence, lodging would be unlikely except in cases of bifurcations where stagnation points could occur. Thus, results from initial attachment and detachment experiments together with physiological parameters suggest that during the metastatic cascade, the adhesion of human breast cancer cells to the endothelium is most likely to occur *via* a lodging-firm adhesion mechanism, and this adhesion could take place in capillaries or venules. These results agree with previous experimental observations of extravasation occurring most often in capillaries [47,48]. In addition, Shioda *et al.* [49] reported entrapment and extravasation of melanoma cells in the capillary bed of a chick embryo chorioallantoic membrane.

It should be noted that other factors may also contribute to the attachment of human breast cancer cells to the endothelium. The flow of blood through the circulatory system is pulsatile, not constant. Consequently, shear stresses at the low end of this pulse may approach 0.25 dynes cm^{-2}, where initial attachment was seen to take place in the experiments reported here. Thus, initial attachment of human breast cancer cells to the endothelium cannot be entirely excluded. Pulsatile flow may also influence both the initial attachment and firm adhesion processes as cancer cells attempt to adhere under shear stresses which are continually changing. In addition, vessels are elastic and compliant. Vessel elasticity could allow breast cancer cells to pass though vessels where they were predicted to lodge. Alternatively, vessel contraction during circulation may enable cancer cells to lodge in vessel sizes where lodging was not predicted. In addition, the cancer

cell itself may deform and, thus, pass through vessels where it was predicted to lodge or deformation may lead to activation of the cell which could modify adhesion. A recent study by Shioda *et al.* [49] has shown entrapment and extravasation of melanoma cells in the chick embryo chorioallantoic membrane leads to alterations of gene expression. Many blood factors also have the potential to influence adhesion. For example, platelets present on either the cancer cell or the endothelium could enhance adhesion by presenting additional adhesion molecule interactions. Platelets could also cause cancer cell clumping, thus allowing clumps of cells to lodge in vessels where single cells would easily pass through [23].

At this point, it is unclear as to how important adhesive interactions are in lodging. Because the cell will probably initially occlude or partially occlude the vessel, the shear stress acting on the cell would be either zero or close to zero. In this case, the ability of the cell to extravasate becomes important and this process may require adhesive interactions that are distinct from interactions required to withstand shear stress. However, it is also possible that following the initial lodging, the cell adheres and spreads out on the endothelial layer. At this point, the vessel may no longer be occluded and the ability to withstand shear stress is important. Clearly, while these results from this paper provide a basis for understanding the role of initial attachment and firm adhesion on cancer cell interactions with the endothelium, much research must be done before the mechanism of adhesion between cancer cells and the endothelium can be conclusively determined.

In this study, the endothelial cell monolayer was activated with TNF-α prior to experimentation to increase expression of adhesion molecules on the surface. We chose to stimulate the cells with TNF-α because several *in vivo* sources of TNF-α exist. In addition, previous studies have shown that cancer cells do not initially attach to unstimulated endothelial cells [10,44] at shear stresses as low as $0.25\,\text{dynes}\,\text{cm}^{-2}$ and detachment results are similar regardless of activation (unpublished results). Hence, these results indicate that even if the endothelium are not activated, lodging followed by adhesion still seems to be the governing mechanism.

Another objective of this study was to elucidate whether variations in adhesive strength with assay type and shear stress were dependent

on metastatic potential. The attachment results showed that at a very low shear stress of 0.25 dynes cm^{-2}, approximately 100 highly-metastatic cells adhered to the monolayer compared with approximately 60 nonmetastatic cells. While this difference is significant, it can be concluded that a significant number of cells attached to the monolayer at this shear stress regardless of metastatic potential. In addition, it was shown that there was no significant difference between the detachment results when comparing the highly-metastatic cells with the nonmetastatic cells except at 10 dynes cm^{-2} where the nonmetastatic cells were approximately 50% more adherent and at 5 dynes cm^{-2} where the highly-metastatic cells approximately 15% more adherent. Even with these differences, the results suggest that with these specific breast cancer cells, a significant number of cells remained attached to the monolayer regardless of metastatic potential.

SUMMARY

In summary, use of the parallel-plate flow chamber as a controlled flow system allowed for the quantification of both initial attachment and firm adhesion of human breast cancer cells to endothelial monolayers at several relevant physiological shear stresses. A comparison of results from both initial attachment and detachment experiments with physiological parameters suggests that the adhesion of human breast cancer cells to endothelium most likely takes place *via* a lodging-firm adhesion mechanism in the capillaries or venules. However, it is important to note that other factors such as pulsatility may contribute to the attachment and, hence, initial attachment at low shear stresses in the absence of lodging cannot be eliminated as a possible mechanism. Further research is needed before the mechanism of adhesion between cancer cells and endothelium can be determined. Finally, it was shown that for these specific breast cancer cells, adhesion does not seem to correlate with metastatic potential. Both the initial attachment and firm adhesion results were similar for the highly-metastatic and nonmetastatic cell lines. Hence, while blocking the adhesion of highly metastatic cells may inhibit their ability to metastasize, adhesion properties alone do not provide an indication as to whether a cell is metastatic or nonmetastatic.

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