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Adhesion of nonmetastatic and highly metastatic breast cancer cells to endothelial cells exposed to shear stress

Melissa Summers Moss [a], Betty Sisken [b], Steve Zimmer [c] and Kimberly W. Anderson [a,*]
[a] *Department of Chemical and Materials Engineering, University of Kentucky, Lexington, KY 40506, USA*
[b] *Center for Biomedical Engineering, University of Kentucky, Lexington, KY 40506, USA*
[c] *Department of Microbiology and Immunology, University of Kentucky, Lexington, KY 40506, USA*

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Abstract. The mechanical stimulus of shear stress has to date been neglected when studying the adhesion of cancer cells to the endothelium. Confluent monolayers of endothelial cells were subjected to either 4 or 15 hours of arterial shear stress. Adhesion of nonmetastatic (MCF-7) and highly metastatic (MDA-MB-435) human breast cancer cells was then quantified using a detachment assay carried out inside the parallel plate flow chamber. Four hours of shear stress exposure had no effect on adhesion. However, 15 hours of shear stress exposure led to marked changes in the ability of the endothelial monolayer to bind human breast cancer cells. An increase in adhesive strength was observed for nonmetastatic MCF-7 cells, while a decrease in adhesive strength was observed for highly metastatic MDA-MB-435 cells. Hence, endothelial shear stress stimulation does influence the adhesion of cancer cells to the endothelium and can have different effects on the adhesion of cancer cells with different metastatic potentials. Furthermore, adhesion of nonmetastatic and highly metastatic human breast cancer cells may be controlled by two different endothelial cell adhesion molecules that are differentially regulated by shear stress. Immunohistochemistry confirmed that shear stress did in fact differentially regulate endothelial cell adhesion molecule expression.

Keywords: Metastasis, adhesion, parallel-plate flow chamber, shear stress, adhesion molecules

1. Introduction

For most cancer patients, the ultimate cause of death is not the primary tumour itself, but metastasis, or the spread of cancer from the primary tumour throughout the body. Consequently, an important step in the treatment of cancer patients is understanding the metastatic cascade. Metastasis, or the formation of tumour foci at sites distant from the primary tumour, is a multistep process. To metastasize in the microcirculation, cancer cells must detach from the primary tumour, 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. It has been proposed in the literature that adhesion of circulating tumour cells to the endothelium is an essential prerequisite for extravasation to occur [7]. Many cancers metastasize preferentially to certain organs. For example, prostate cancers metastasize most frequently to bone and small cell lung carcinomas most often to the brain. This organ-specific nature of

*Corresponding author. Fax: +1 606 323 1929.

metastasis has been attributed to interactions between endothelial ligands and their specific receptors expressed on cancer cells [19]. Such interactions are responsible for the adhesion of circulating cancer cells to the endothelium that lines the vessel walls. Consequently, factors that alter the expression of endothelial ligands are likely to impact adhesion and therefore extravasation and metastasis.

Inside the body, the endothelium is continuously exposed to shear stress as a result of blood circulation. When an endothelial monolayer is subjected to shear stress, stress fibers within the cells align in the direction of flow. Consequently, shear stress leads to morphological changes in the endothelium [22]. In addition, several cell functions, including cell division, protein synthesis and excretion, and membrane protein organization are altered [3,25]. Thus, the mechanical stimulus of shear stress can be translated into a biochemical signal that can trigger morphological, transcriptional, and translational changes in the endothelial cell.

The ability of shear stress to induce biochemical signals equips the mechanical shear stress stimulus with the ability to alter the surface expression of adhesion molecules and, therefore, impact the endothelial monolayer's ability to bind tumour cells. Several studies have shown that exposure of an endothelial monolayer to laminar shear stress leads to alterations in the expression of endothelial cell adhesion molecules [1,2,15–17,21,26]. To date, *in vitro* studies of cancer cell adhesion fail to account for the role of endothelial shear stress stimulation.

In this study, the effect of exposing endothelial cells to shear stress on the adhesion of human breast cancer cells to endothelial monolayers was examined. A parallel plate flow chamber was employed to subject confluent monolayers of human umbilical vein endothelial cells (HUVECs) to an arterial shear stress of 15 dyn/cm^2 for either 4 or 15 hours. In parallel control experiments, the endothelial monolayer was maintained inside the parallel plate flow chamber under static conditions for an equivalent period of time. Following shear stress or static control treatment, a detachment assay, carried out inside the parallel plate flow chamber, was used to quantify changes in adhesive strength with shear stress exposure. The adhesive properties of two different human 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 shear stress exposure are a function of metastatic potential. Previous studies have indicated that endothelial cell surface expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1 or E-selectin) may be important for cancer cell adhesion in the vasculature [9,11,13,18, 23]. Immunohistochemistry was used to examine changes in the surface expression of these adhesion molecules following both static control and shear stress treatment to help explain changes in cancer cell adhesion that occurred following endothelial shear stress stimulation.

2. Materials and methods

2.1. 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 cancer cells 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). This cell line has been shown to be nonmetastatic in a nude mouse model [12]. The MDA-MB-435 human breast cancer cell line was obtained from the laboratory of Janet Price (M.D. Anderson Cancer Center, Houston, TX). This cell line has been shown to be highly metastatic in both spontaneous and experimental metastasis models [20,28]. Both human breast cancer cell lines were grown as monolayers in 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 penicillin (Gibco), 10 μg/ml 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.

2.2. Preparation of the HUVEC monolayers

HUVECs were seeded onto permanox slides 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 confluence (3–4 days) on the permanox slide prior to assembly of the flow chamber.

2.3. Preparation of human breast cancer cells

Prior to experiments, confluent monolayers of human breast cancer cells were trypsinized and resuspended at a concentration of 1.25×10^6 cells/ml in a solution of dextran (Sigma) dissolved in CSC Complete Medium. A calibrated amount of dextran was added to this media so that the viscosity of the cell suspension would match that of blood (3.9 cp). This cell suspension was maintained at 37°C.

2.4. Flow chamber and related equipment

A parallel plate flow chamber was employed both to subject endothelial monolayers to shear stress and to assess adhesive strength between human breast cancer cells and endothelial monolayers. The parallel plate flow chamber, depicted in Fig. 1, consisted of a polycarbonate plate and a permanox slide separated by a silastic gasket which gave the chamber height. The polycarbonate plate and permonox slide were held together by a vacuum to ensure a constant height, and therefore a constant shear stress, along the length of the flow chamber. A confluent endothelial monolayer was grown on the permanox slide prior to assembly of the flow chamber and, once assembled, this surface composed the bottom of the flow chamber. Two pressure ports located in the polycarbonate base enabled measurement of the pressure drop across 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 imparted 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

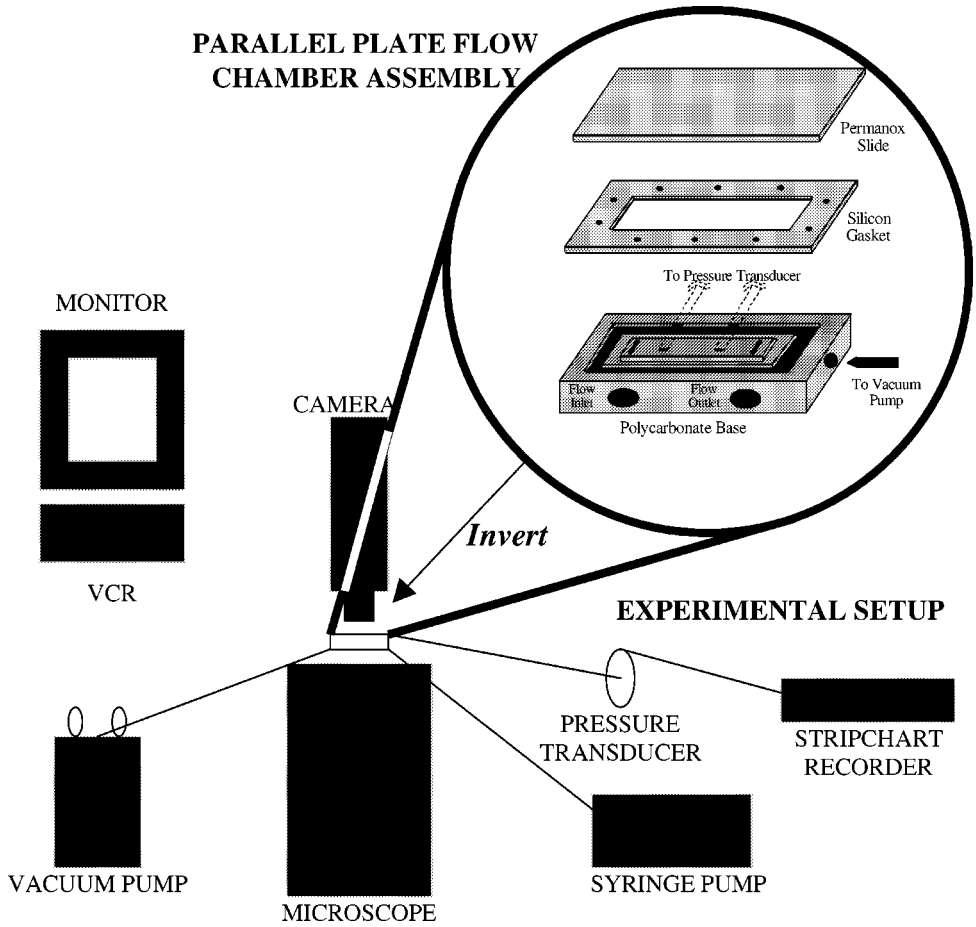

Fig. 1. Schematic of experiment setup employed in the detachment assay. The parallel plate flow chamber is inverted on the microscope stage such that the endothelial monolayer composes the bottom of the chamber.

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 was dissolved in this media 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 unknown shear stress in dyn/cm^2, ΔP is the measured pressure drop in mmHg, and L is the distance between the pressure ports (2.33 cm). The height of the flow chamber, d, in cm, was calculated by:

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

Here, μ is the fluid viscosity (3.9 cp), Q is the flow rate in ml/min controlled by the syringe pump, b is the chamber width (1.45 cm), and ΔP is the measured pressure drop in dyn/cm^2.

Once assembled, the flow chamber was 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 Fig. 1.

2.5. Endothelial shear stress exposure

Following assembly of the flow chamber, confluent HUVEC monolayers were subjected to an arterial shear stress of 15 dyn/cm^2 for either 4 or 15 hours. A solution of dextran dissolved in CSC Complete Medium with a viscosity of 3.9 cp was used for shear stress stimulation so that the flowing fluid would have a viscosity matching that of blood. Because the monolayer was exposed to shear stress outside of the incubator, parallel control experiments were performed where monolayers were maintained inside the closed parallel plate flow chamber under static conditions for an equal amount of time (4 or 15 hours). Maintaining the monolayer under static conditions simulated any changes in the endothelial monolayer which may occur during shear stress exposure as a result of removing the monolayer from ideal culture conditions but that are unrelated to the shear forces themselves.

2.6. Detachment assay

To quantify the adhesive strength between human breast cancer cells and HUVEC monolayers, a detachment assay was carried out inside the parallel plate flow chamber. A suspension of 1.25×10^6 human breast cancer cells/ml was introduced at a flow rate of 1 ml/min using the secondary line. 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. A 30 minute settling time was chosen to assure that the cells had plenty of time to reach the monolayer and form receptor/ligand interactions. At the end of the settling time, a shear stress of 5 dyn/cm^2 was imposed upon the monolayer. 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 measure the adhesive strength of both MCF-7 and MDA-MB-435 human breast cancer cells to the HUVEC monolayers in the control studies and to the HUVEC monolayers exposed to shear for 4 and 15 hours. All experiments were performed with 6 repetitions.

2.7. Data analysis

The 9 minute detachment time was analyzed by counting the total number of cells present on the endothelial monolayer at the end of the 30 minute settling time. This initial number of cells ranged from 50–100. The total number of cells detached following the onset of the 5 dyn/cm^2 shear stress at 5, 30, 60, 180, 300, 420, 480, and 540 seconds was then assessed. Raw numbers were converted to percentage values and results were plotted as percent detachment versus time. All results exhibited a leveling off of percent detachment values by the end of the 9 minute detachment time, indicating that an extinction value

of percent detachment 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 comparison between control and shear stress studies for time periods of both 4 and 15 hours.

2.8. Statistics

Statistical significance was assessed using a three-way ANOVA followed by Student–Newman–Keuls Post-Hoc tests to compare the effect and interaction of cell type, shear stress, and shear stress exposure time. Statistical analysis was carried out using the SigmaStat statistical software package.

2.9. Immunohistochemistry

Following exposure to either 15 hours of a 15 dyn/cm^2 shear stress or 15 hours of static conditions within the parallel plate flow chamber, confluent endothelial monolayers were carefully removed from the flow chamber apparatus, washed with 0.1 M PBS (Gibco), and fixed in 4% paraformaldehyde in 0.1 M PBS for 30 minutes at room temperature. The monolayers were then stored under 0.1 M PBS at 4°C until time for the assay.

At the time of the assay, monolayers were covered with the desired primary antibody: mouse IgG_1 anti-human VCAM-1, clone P3C4 (Chemicon, Temecula, CA), mouse IgG_1 anti-human ICAM-1, clone P2A4 (Chemicon), or mouse IgG_1 anti-human E-selectin, clone P2H3 (Chemicon). The primary antibody was added at a concentration of 10 μg/ml in 0.1 M PBS in a quantity of 2 ml. The monolayer and primary antibody were allowed to incubate for 2 days at 4°C. Following the 2 day incubation, the antibody solution was removed and monolayers were washed in 0.1 M PBS and blocked with Diluted Normal Blocking Serum (Vector Laboratories, Burlingame, CA) for 20 minutes at room temperature. The monolayers were then washed in 0.1 M PBS and covered with the secondary antibody, a biotinylated anti-mouse IgG (Vector Laboratories) diluted 1 : 200 in 0.1 M PBS. Following 30 minutes of incubation with the secondary antibody at room temperature, monolayers were again washed in 0.1 M PBS and the ABC Reagent (Vector Laboratories) containing an avidin-biotinylated peroxidase complex (4.5 μl/ml) was added to the cells. The ABC reagent was allowed to contact the cells for 30 minutes. The monolayers were again washed in 0.1 M PBS and 1 ml of the DAB Reagent (Vector Laboratories) containing 3,3′-diaminobenzidine-HCl (0.4 mg/ml) was added. Incubation with the DAB reagent for 8–10 minutes yielded a visible gray-brown precipitate on the cell surface where the primary antibody of interest was present. Monolayers were then washed, dried, and preserved in immersion oil. Pictures were taken at a magnification of 100× using a microscope equipped with a camera. Immunohistochemistry studies were performed once for each adhesion molecule for endothelial cells exposed to both 15 hours of shear and 15 hours of static conditions.

3. Results

3.1. Detachment assay

Confluent monolayers of HUVEC cells were exposed to either static conditions or a 15 dyn/cm^2 shear stress for a period of 4 hours. Results for detachment from 4 hour static control and shear stress treated endothelial monolayers are shown in Fig. 2 for both the nonmetastatic MCF-7 human breast cancer cell line and the highly metastatic MDA-MB-435 human breast cancer cell line. Four hours of endothelial

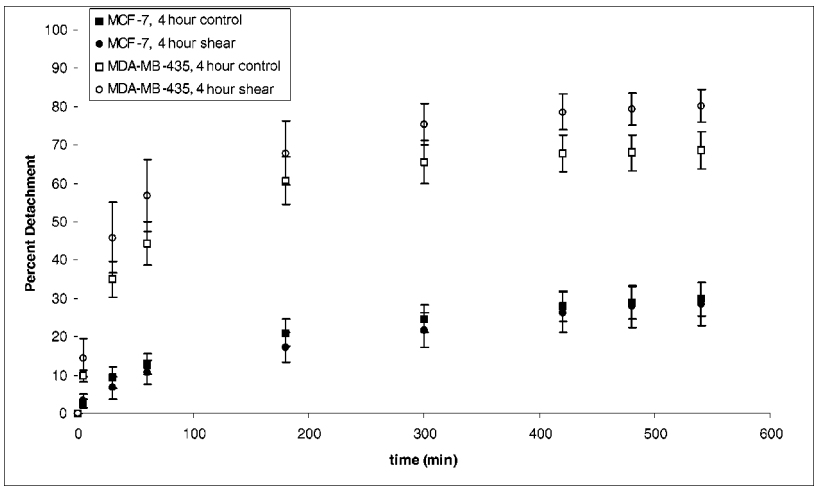

Fig. 2. Percent detachment vs time of the nonmetastatic MCF-7 human breast cancer cell line and the highly metastatic MDA-MB-435 human breast cancer cell line from HUVEC monolayers exposed to either static conditions or 15 dyn/cm^2 shear stress for 4 hours. Error bars indicate standard error.

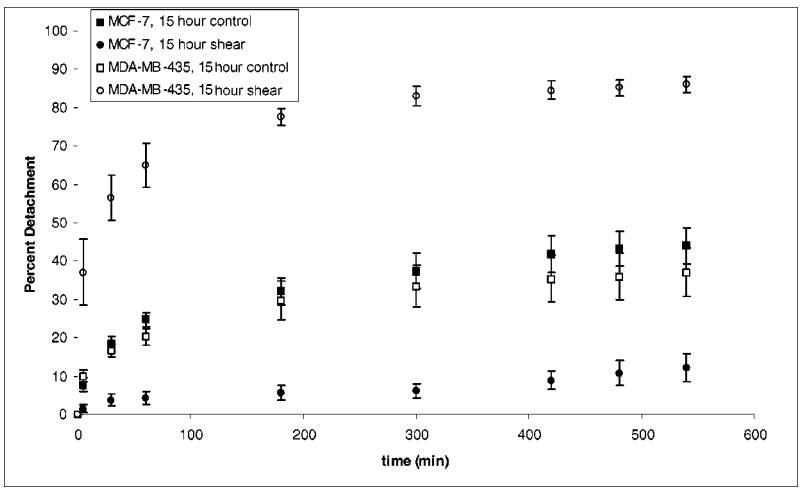

Fig. 3. Percent detachment vs time of the nonmetastatic MCF-7 human breast cancer cell line and the highly metastatic MDA-MB-435 human breast cancer cell line from HUVEC monolayers exposed to either static conditions or 15 dyn/cm^2 shear stress for 15 hours. Error bars indicate standard error.

shear stress exposure did not significantly effect percent detachment ($p > 0.05$). This was true for both the nonmetastatic MCF-7 human breast cancer cell line and the highly metastatic MDA-MB-435 human breast cancer cell line. These results indicate that 4 hours of endothelial shear stress exposure did not significantly alter adhesive strength for either human breast cancer cell line.

When the exposure time was increased to 15 hours, however, differences in adhesive strength with endothelial shear stress exposure became apparent. Results for detachment from 15 hour static control and shear stress treated endothelial monolayers are shown in Fig. 3 for both the nonmetastatic MCF-7 human breast cancer cell line and the highly metastatic MDA-MB-435 human breast cancer cell line. Here, extinction values for percent detachment of the nonmetastatic MCF-7 cell line decreased signif-

icantly ($p < 0.05$) from 44.0 $\pm$ 4.6% (SE) to 12.1 $\pm$ 3.7% (SE) following exposure of the endothelial monolayer to 15 hours of shear stress. These results indicate that exposure of the endothelial monolayer to 15 hours of arterial shear stress increased the adhesive strength between the nonmetastatic human breast cancer cell line and the endothelial monolayer. In contrast, 15 hours of endothelial shear stress exposure significantly increased ($p < 0.05$) extinction values for percent detachment of the highly metastatic MDA-MB-435 cell line from 37.2 $\pm$ 6.3% (SE) for control monolayers to 86.2 $\pm$ 2.1% (SE) for shear stress stimulated monolayers. These results indicate that exposure of the endothelial monolayer to 15 hours of arterial shear stress decreased the adhesive strength between the highly metastatic human breast cancer cell line and the endothelial monolayer.

3.2. Immunohistochemistry

Because four hours of endothelial shear stress exposure did not significantly alter adhesion of either the nonmetastatic human breast cancer cell line or the highly metastatic human breast cancer cell line, immunohistochemistry was performed only for monolayers exposed to either 15 hours of static control or 15 hours of shear stress treatment at 15 dyn/cm^2.

Immunohistochemistry detection of ICAM-1 expression is shown in Figs 4a and 4b for 15 hour static control and 15 hour shear stress stimulated monolayers, respectively. Staining of the static monolayer revealed a very low level of ICAM-1 expression on the monolayer surface. In contrast, staining appears significantly darker for the shear stress stimulated monolayer indicating that exposure of the endothelial monolayer to 15 hours of shear stress at a level of 15 dyn/cm^2 resulted in an increase in the surface expression of ICAM-1.

Immunohistochemistry detection of VCAM-1 expression is shown in Figs 4c and 4d for 15 hour static control and 15 hour shear stress stimulated monolayers, respectively. In contrast to ICAM-1, staining of the static monolayer for the VCAM-1 antibody revealed significant cell surface expression. Following exposure to shear stress, VCAM-1 staining was significantly lighter showing that exposure of the endothelial monolayer to 15 hours of shear stress at a level of 15 dyn/cm^2 decreased endothelial VCAM-1 expression.

Immunohistochemistry detection of E-selectin expression is shown in Figs 4e and 4f for 15 hour static control and 15 hour shear stress stimulated monolayers, respectively. Staining of the static monolayer reveals a low level of E-selectin expression on the monolayer surface. Following exposure to shear stress, staining for E-selectin was even lighter than on the static monolayer. This result indicates that exposure of the endothelial monolayer to 15 hours of shear stress at a level of 15 dyn/cm^2 decreased expression of E-selectin on the endothelial cell surface.

4. Discussion

The endothelium within the body is continuously exposed to shear stress as a result of blood circulation. The mechanical stimulus of shear stress has been shown to induce a variety of changes in endothelial cells. Such changes include immediate responses that occur within minutes of shear stress exposure, rapid responses that take place over a period of hours, and sustained responses that are permanent, adaptive alterations in response to the shear stress [24].

To date, studies of cancer cell adhesion to endothelial monolayers have failed to account for the effects of shear stress on the adhesive strength between circulating cancer cells and the endothelium. In light of the numerous changes that shear stress is known to invoke within endothelial cells, the mechanical

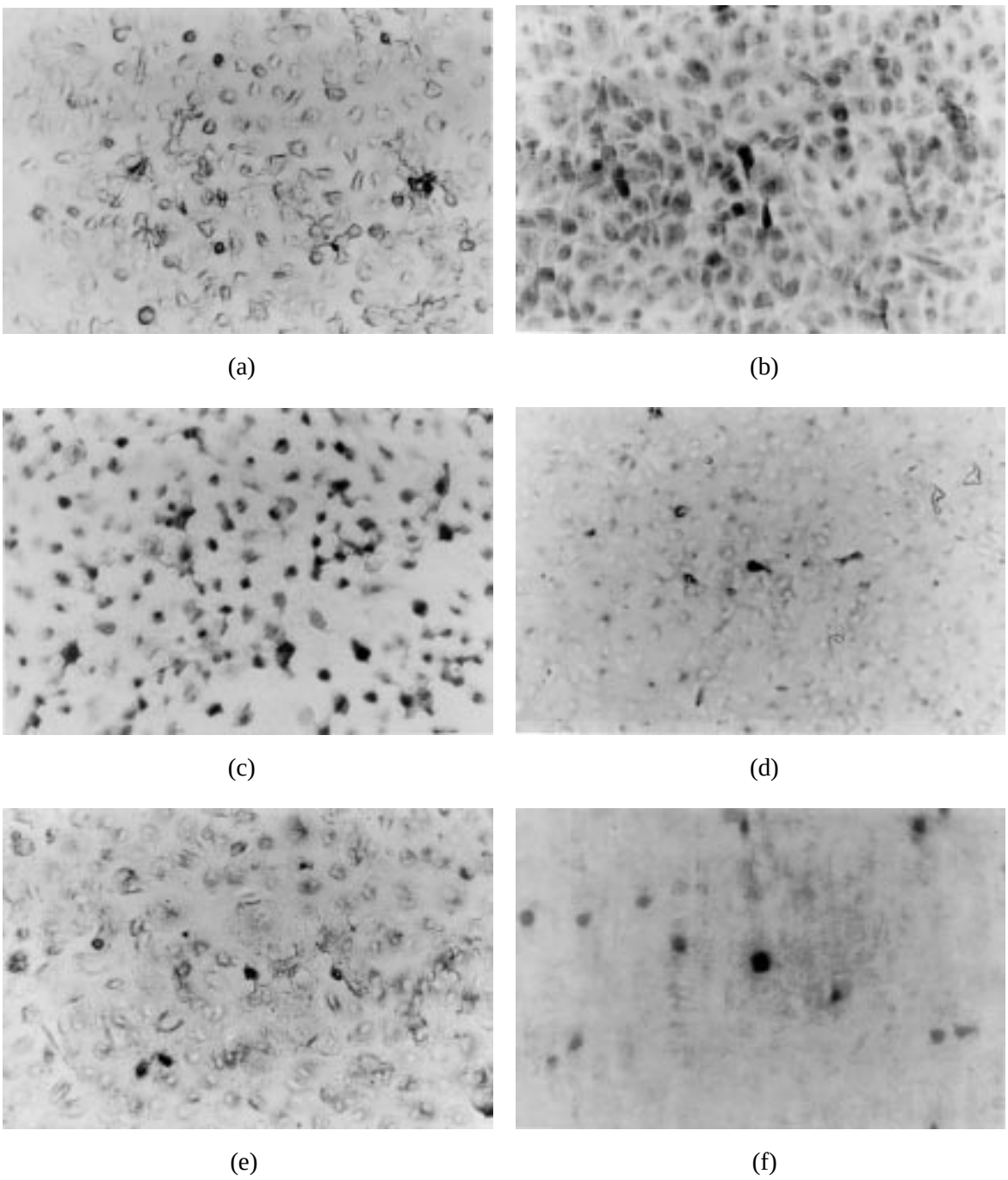

Fig. 4. (a) Immunohistochemistry staining for ICAM-1 on a HUVEC monolayer exposed to 15 hours of static conditions within the parallel plate flow chamber. (b) Immunohistochemistry staining for ICAM-1 on a HUVEC monolayer exposed to 15 hours of shear stress at 15 dyn/cm^2. (c) Immunohistochemistry staining for VCAM-1 on a HUVEC monolayer exposed to 15 hours of static conditions within the parallel plate flow chamber. (d) Immunohistochemistry staining for VCAM-1 on a HUVEC monolayer exposed to 15 hours of shear stress at 15 dyn/cm^2. (e) Immunohistochemistry staining for E-selectin on a HUVEC monolayer exposed to 15 hours of static conditions within the parallel plate flow chamber. (f) Immunohistochemistry staining for E-selectin on a HUVEC monolayer exposed to 15 hours of shear stress at 15 dyn/cm^2.

stimulus of shear stress is one which cannot be overlooked. In fact, exposure of endothelial cells to shear stress is known to alter the surface expression of adhesion molecules suspected to be involved in the adhesion of cancer cells to the endothelium [1,2,15–17,21,26]. In this study, the effect of endothelial shear stress stimulation on the adhesion of human breast cancer cells to endothelial monolayers was investigated *in vitro* using the parallel plate flow chamber. The effects of both shear stress exposure time and the metastatic capability of the cancer cell were examined.

Exposure of confluent endothelial monolayers to an arterial shear stress of 15 dyn/cm^2 for a period of 4 hours did not result in any differences in adhesive strength between either the nonmetastatic human breast cancer cell line or the highly metastatic human breast cancer cell line and the endothelial monolayer. In contrast, when the time of shear stress exposure was increased to 15 hours, endothelial shear stress stimulation did in fact present marked alterations in the adhesive strength between human breast cancer cells and the endothelial monolayer. For the nonmetastatic human breast cancer cell line, 15 hours of endothelial shear stress exposure led to a significant increase in adhesive strength. In contrast, 15 hours of endothelial shear stress exposure significantly decreased the adhesive strength between the highly metastatic human breast cancer cell line and the endothelial monolayer.

Thus, exposure of confluent endothelial monolayers to 15 hours of arterial shear stress led to opposing changes in adhesion for the nonmetastatic human breast cancer cell line and the highly metastatic human breast cancer cell line. Such opposing changes could result in novel conclusions concerning the relative adhesiveness of different cell types. In fact, when observing adhesion to 15 hour static monolayers, no significant differences in adhesion between the two cell lines and the endothelial monolayer were noted. However, following 15 hours of shear stress exposure, adhesion of the highly metastatic human breast cancer cell line to the endothelial monolayer was much less than adhesion of the nonmetastatic human breast cancer cell line. It has been suggested that cancer cell adhesion to endothelial cells correlates with metastatic potential, and hence, adhesion may play a key role in metastasis of these cell lines. The results presented here suggest that when endothelial cells are exposed to shear, the opposite occurs for these specific breast cancer cell lines.

These results also indicate that adhesion of these two cell lines to the endothelium may be controlled by two different adhesion molecule mechanisms. It has been suggested in the literature that expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1 or E-selectin) may play a role in cancer cell adhesion to endothelial cells [9,11,13,18,23]. Immunohistochemistry staining of 15 hour static control and 15 hour shear stress treated monolayers was performed to gain insight into these adhesion mechanisms. Endothelial cell surface expression of ICAM-1 was found to be upregulated following shear stress stimulation. In contrast, endothelial cell surface expression of VCAM-1 was found to decrease following shear stress stimulation of the endothelial monolayer. Our results obtained on ICAM-1 expression are in agreement with previous studies which have shown that ICAM-1 is selectively upregulated following exposure of endothelial monolayers to laminar shear stress both *in vitro* [5,15,16,21,26] and *in vivo* [27]. ICAM-1 upregulation has been shown to be a direct result of the mechanical shear stress stimulus as supernatants from HUVEC cells conditioned under laminar shear flow have failed to produce an increase in ICAM-1 expression [15].

Previous results on VCAM-1 have been contradictory. Sampath et al. [21] reported a decrease in VCAM RNA levels within an hour after exposing HUVECs to a shear stress of 25 dyn/cm^2. Three separate studies reported no change in VCAM-1 surface expression when exposing HUVECs to a shear stress of 15 dyn/cm^2 for 4 hours [26], 30 dyn/cm^2 for 2–6 hours [6], and 46 dyn/cm^2 for 48 hours [16]. While previous results have either reported a decrease or no change in VCAM-1 expression at high

shear stresses (15–46 dyn/cm^2), other results have shown an increase in VCAM-1 expression on human endothelial cells when exposing the cells to low shear stresses ranging from 2–5 dyn/cm^2 [4,6,14]. In contrast, others have reported a decrease in VCAM-1 expression on mouse endothelial cells when exposing the cells to a shear stress of 1.5 dyn/cm^2 for 6 and 24 hours [1,10,17]. These results seem to indicate that the effect of shear may depend on the origin of the endothelial cells. Contradictory results could also be due to the fact that VCAM-1 expression is a function of the magnitude of the shear stress and length of application.

Our results showed that the expression of E-selectin was decreased following endothelial shear stress stimulation. Previous results on E-selectin expression after exposing endothelial cells to shear stress have been limited. Sampath et al. [21] also reported a slight decrease in E-selectin after one hour of flow at 25 dyn/cm^2. Morigi et al. [15] reported no change in E-selectin when exposing HUVECs to 8 dyn/cm^2 for 6 and 15 hours. Nagel et al. [16] exposed HUVECs to shear stresses ranging from 2.5–46 dyn/cm^2 for 2 hours and also reported no change. Again, discrepancies in these reports could be due to the magnitudes of shear stresses applied and also the length of application.

Comparison of alterations in adhesion molecule expression following endothelial shear stress stimulation with changes in adhesion of the two human breast cancer cell lines reveals two separate but oppositely controlled adhesion mechanisms. Adhesion of the nonmetastatic human breast cancer cell line to the endothelial monolayer, which is increased with endothelial shear stress stimulation, might be controlled by an ICAM-1 mechanism since the expression of this adhesion molecule is increased by endothelial shear stress stimulation. In contrast, adhesion of the highly metastatic human breast cancer cell line to the endothelial monolayer, which is decreased with endothelial shear stress stimulation, might be controlled via a VCAM-1 and/or E-selectin mechanism since expression of these adhesion molecules is decreased with endothelial shear stress exposure. It is important to remember that when cancer cells form receptor/ligand interactions with endothelial cells, they first role along the monolayer forming initial attachments and then form firm adhesions. In addition, adhesion molecules involved in these two distinct steps may be different. Previous studies have suggested that E-selectin is important in mediating cell rolling along the endothelial cell monolayer [8]. In this study, we focused on firm adhesion and not initial attachment. Alternatively, adhesion might be controlled by multiple surface proteins, some for which the effects of shear stress have not yet been investigated. Studies involving antibody blocking of the suspected adhesion molecules are needed to confirm the exact mechanisms involved in the adhesion of these nonmetastatic and highly metastatic human breast cancer cell lines to the endothelium. It is interesting to note that following exposure of the endothelial cells to shear stress, the immunohistochemistry shows almost no expression of VCAM-1 or E-selectin. However, approximately 15% of the MDA-MB-435 cells are still adhering. It is possible that these cells are heterogeneous and the 15% remaining on the monolayer are adhering by other surface proteins. Such possibilities remain to be explored.

This study has investigated the effects of endothelial shear stress exposure on the adhesion of human breast cancer cells to endothelial monolayers when shear stress exposure occurred at an arterial rate (15 dyn/cm^2). The effects of shear stresses experienced by endothelial cells in other vessel types remain to be elucidated. Lower shear stresses that occur in venules are of particular interest as low levels of shear stress have been found to increase VCAM-1 expression as opposed to the decrease in VCAM-1 expression observed at higher shear stresses as previously discussed. In addition, it is possible that secondary metastasis occurs in the venular system where the shear stress in less than 15 dyn/cm^2. Studying the effect of endothelial shear stress exposure on the adhesion of cancer cells to the endothelium at various shear stress magnitudes seen in different vessel sizes might lend insight into cancer cell extravasation occurring preferentially in certain vessel types. In addition, blood flow is not purely laminar but

also pulsatile in nature. The increase in VCAM-1 expression at low shear stresses has been shown to be magnified when pulsatile flow is superimposed upon laminar shear stress [14]. Thus, pulsatile flow could lead to further alterations in adhesive strength between cancer cells and the endothelium. Finally, the impact of oscillatory shear stress on cancer cell adhesion should be studied to elucidate adhesion at special sites such as vessel bifurcations where shear stresses are low and flow is primarily oscillatory. In one study, oscillatory shear stress was found to increase endothelial expression of ICAM-1, VCAM-1, and E-selectin and these increases were accompanied by an increase in monocyte binding [4].

In this study, control experiments were performed where the endothelial cells were exposed to static conditions inside the closed flow chamber for 4 and 15 hours. The results show that the percent detachments were modified for both the MCF-7 and MDA-MB-435 cells due to the endothelial cells sitting outside of their natural environment. These results suggest that expression of adhesion molecules can change not only due to the applied shear stress but also due to the environmental conditions. This emphasizes the importance of comparing the shear stress results to static controls where the endothelial cells are exposed to the same environment as in the shear stress experiments.

To quantify the adhesive strength between cancer cells and endothelial cells, we allowed the cancer cells to settle onto the treated monolayer for 30 minutes prior to applying a shear stress. The 30 minutes settling time was chosen to assure that the cells had enough time to interact with the monolayer and form receptor/ligand interactions. This is clearly not representative of what occurs physiologically where the cells roll along the monolayer and then form firm adhesion. However, the information obtained using this methodology does provide valuable information on the differences in firm adhesion as a function of metastatic potential and endothelial cell exposure to pre-shear. Studies to investigate the initial attachment of theses cell lines to endothelial cells are the focus of future work.

In summary, this study shows that shear stress does influence the ability of endothelial cells to bind cancer cells and that endothelial shear stress stimulation can have different effects on the binding of cancer cells with different metastatic capabilities. These results also indicate that adhesion of the non-metastatic and highly metastatic human breast cancer cell lines studied is controlled via two different adhesion molecule mechanisms involving endothelial cell adhesion molecules that are differentially regulated by shear stress. The results suggest that ICAM-1, VCAM-1 and E-selectin may play a role in the adhesion. Future studies using antibody blocking are needed to determine the possible mechanisms. A complete investigation of the effects of shear stress on the adhesion of cancer cells to the endothelium will require examination of various shear stress magnitudes, pulsatile flow, oscillatory shear stress and antibody blocking studies to gain further insight into the adhesion molecules involved.

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