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LITTLE CHANNELS, BIG DISEASE: USING MICROFLUIDICS TO INVESTIGATE CANCER METASTASIS

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The leading cause of death in human patients with malignant cancer is the dissemination of the primary tumor to secondary sites throughout the body. It is well known that cancers metastasize to certain tissues (e.g. breast cancer typically spreads to the lungs. brain and bone), in a pattern that cannot be explained by blood flow from the primary tumor or simple mechanical arrest. Circulating tumor cells usually arrest in the microvasculature of target tissues. At these sites, they must adhere to the endothelium, survive, proliferate and extravasate in order to form a secondary tumor. In vitro tools that appropriately mimic the microvasculature in which cancer metastasis occurs have been largely unavailable. With the advent of microfluidic and nanotechnology, we can now more accurately model the complexity of the microvascular environment, in terms of representative endothelial cells, geometry, shear stress and exposure to organ-specific environmental cues. This talk will focus on the use of microfluidic devices to explore mechanisms involved in tumor-endothelial cell interactions that govern cancer metastasis to organ specific sites.

Keywords: Microfluidic, model, shear, endothelium, cancer, metastasis

CANCER METASTASIS

Cancer is one of the leading causes of death in humans and, due to enhanced longevity, incidence rates continue to increase incrementally each year [1]. More than 90% of patients with malignant cancer eventually die from metastatic disease [2]. Metastasis is a complex process, consisting of genetic mutations resulting in the evolution of a neoplastic clone, survival and growth of the clone, stimulation of tumor angiogenesis, invasion through the surrounding extracellular matrix and into lymphatic or blood vessels (intravasation), survival within the circulation, adhesion in the vasculature of secondary organs, followed by survival, growth and angiogenesis to form a secondary tumor [3-6].

Metastasis is a relatively inefficient process. Millions of cells can be released from the primary tumor, but only a few are able to evade destruction by mechanical and immunologic defenses once within the circulation. Those cells that do survive must physically arrest then adhere to the vasculature of distant target organs of metastasis. The mechanisms by which tumor cells form secondary growths differ depending on the type of tumor (e.g. carcinoma, sarcoma), the nature of the vascular bed, and the metastatic target organ [3,4,7]. Tumor cells can arrest by simple mechanical size restriction. This appears to be the main mechanism of arrest for tumors that metastasize to the brain (e.g. pulmonary carcinoma, breast carcinoma) [8], which is lined by continuous endothelium characterized by large numbers of tight junctions [9,10]. However, for many tumors and organs (such as the lungs, which is a common metastatic site), cancer cells arrest in the microvasculature, specifically in precapillary arterioles and post-capillary venules, vessels whose diameter is larger than that of the tumor cells [11-13]. In these organs, arrest is thought to be mediated by the binding of tumor cells to adhesion molecules (e.g. integrins, selectins, immunoglobulin superfamily cellular adhesion receptors) expressed on the vascular endothelium (for continuous endothelium, e.g. lungs) or the subendothelial matrix (for discontinuous sinusoidal endothelium, e.g. liver) [5,9,10]. Adherent tumor cells must then survive and grow to form a secondary tumor.

There are currently two prevailing theories on how adherent tumor cells form secondary growths within these distant organs: extravasation through the endothelium or proliferation intravascularly to rupture through the endothelium [3,4,11]. Which mechanism is operative likely depends on a combination of the type of tumor and the organ microenvironment. Once extravasated, tumor cells can die, remain dormant, or proliferate to form a secondary tumor [8,14]. Host cells in the organ parenchyma respond to infiltrating tumor cells and, through direct contact or indirectly through secretion of soluble growth factors and chemokines, can promote tumor survival and growth. The factors that govern these host-tumor cell interactions are just beginning to be understood, with evidence that tumor-induced host responses and host-induced tumor responses are both important for evolution of cancer, successful metastasis and even resistance to chemotherapy [8,14-16].

Cancer metastasis is also non-random. Certain tumors metastasize to specific organs, even if that organ is not in the natural path of blood flow from the primary site. Breast carcinomas frequently metastasize to the lungs, brain and bone, prostatic carcinomas to the bone and colonic cancer to the liver [3,7]. This site-specific nature of metastasis has been attributed to expression of endothelial receptors or ligands in specific vascular beds (which encounter the appropriate counter-ligand or receptor on tumor cells) and to localized secretion or availability of organ-derived growth factors or chemokines. This “seed and soil” hypothesis, which was originally coined by Paget in 1889, is undergoing a major resurgence [6,17,18]. Genetic profiles of metastasis have been established for various tumors, which correspond to organ-specific homing. These studies have uncovered hitherto unidentified molecular pathways that may affect metastasis and, potentially, new therapeutic targets [6].

MODELS FOR EXPLORATION OF TUMOR-ENDOTHELIAL CELL INTERACTIONS

The mechanisms which govern circulating tumor-endothelial or tumor-host cell interactions in metastatic target organs are still the least understood aspect of the intricate metastatic cascade. This is primarily due to difficulties with accurately modeling the complex in vivo microvascular and tissue environment where cancer metastasis occurs. In vivo murine models of metastasis have provided a wealth of information on cancer metastasis, permitting examination of tumor-host cell interactions in real-time with intravital multiphoton microscopy [8,11-13,19]. However, some target organs may be physically inaccessible for visualization and the complexities of the biological environment cannot be controlled, which can mask subtle molecular mechanisms. The optimal in vitro model should ideally replicate the fluid dynamics of blood flow, the geometry and specific type of microvascular endothelium, and the presence of organ-derived chemokines or growth factors. The ability of in vitro models to temporally and spatially control defined factors in a precise manner affords exploration into the detail of mechanisms of metastasis that cannot be achieved with the inherent biological variability of in vivo models.

The parallel plate flow chamber has been the traditional in vitro model used to investigate tumor-endothelial cell interactions under conditions of physiologic flow [20]. It is vital to study these interactions under flow, because endothelial cells and tumor cells respond to shear stress (mechanosensing) which alters their behavior in ways that cannot be predicted from static condition [21]. The parallel plate flow chamber consists of two polymethylmethacrylate plates separated by a gasket, creating a 3 x 1 cm adhesion surface. Confluent

endothelial cells on coverslips are placed within a well in the bottom plate and tumor cells are perfused through the chamber at physiologic shear rates, using a syringe pump. Adhesive events are captured with video- or digital microscopy [20]. However, this instrument has a large flat interactive surface, which does not represent the physical geometry (curved surface) of the microvasculature or its size (< 100 um), and organ-derived chemokines or growth factors cannot be introduced into the system. Micro- and nanotechnology has ushered in a new era of device fabrication for studying microvascular biology [22]. There has been a surge of publications involving the generation of microfluidic models of the vasculature for tissue engineering and modeling of cancer metastasis [23-29]. Of these studies, the device engineered by Song and colleagues [24] has come the closest to mimicking all aspects of the microvasculature, including the use of primary microvascular endothelium (albeit from the dermis, which is not a typical site of cancer metastasis), incorporation of flow and addition of specific growth factors or chemokines in a spatially controlled manner. However, the microfluidic channel is still larger (60 um high by 800 um wide) than microvessels and rectangular, thus not completely duplicating the geometry of in vivo microvasculature.

A MICROFLUIDIC DEVICE TO STUDY ENDOTHELIAL CELL BIOLOGY AND TUMOR-ENDOTHELIAL CELL INTERACTIONS

At Cornell University, we have taken advantage of the unique resource, the Center Nanoscale Science and Technology facility, to generate a microfluidic device consisting of endothelialized channels. The device consists of 1-3 parallel channels that are of similar scale to microvessels (50 x 50 um), where cancer metastasis occurs. We can also create single and multiple branching channels that mimic the complexity of the in vivo vasculature. The channel pattern is etched onto a silicon mask and is then transferred by high temperature embossing into a polystyrene mold, which is used as a template for recreating the channels in the silicone-based elastomer, polydimethylsiloxane (PDMS). We chose PDMS because it is non-toxic, sturdy and transparent, permitting visualization of cells (endothelial- or tumor-derived) within the channels with fluorescent and phase-contrast microscopy. PDMS can be bonded to glass (after plasma oxidation), forming sealed channels, and can be sterilized, permitting long-term cell culture [30]. Since PDMS is hydrophobic, cells will not adhere to the native polymer. Thus, precoating of the PDMS channels with soluble extracellular matrix protein, such as fibronectin, is required to “endothelialize” the device (i.e. to coat the channel surface with a single layer of endothelium). An additional advantage of PDMS is that three-dimensional channels can be incorporated into the PDMS using femtosecond laser ablation [31].

We have used human umbilical vein endothelial cells (HUVEC) as our model endothelium. They are a well-accepted model endothelial cell, since they are readily available and relatively simple to grow in culture versus primary microvascular endothelium. We are moving towards using primary microvascular endothelium that is representative of the metastatic organs for studying cancer-endothelial interactions, e.g. primary human pulmonary microvascular endothelial cells. This is important, because endothelial cells are heterogeneous and microvascular-derived cells will express different receptors or ligands than HUVECs [9,10] and are likely to interact uniquely with infused tumor cells.

Effect of channel shape on endothelial structures

We are using the endothelialized device to answer specific bioengineering and biomedical questions related to cancer metastasis. The first question we asked was: What is the effect of channel shape (rectangular versus cylindrical) on endothelial cell structure? The rationale for this was that in vivo microvessels are curved not rectangular, but fabrication of rectangular, but not circular, channels is readily accomplished using standard lithographic techniques. To answer this question, we compared the structure of HUVECs grow in open (unsealed) and closed (sealed) circular and rectangular channels with in situ immunofluorescent microscopy (Esch et al., under revision). We focused on two aspects of endothelial structure: 1) The formation of a confluent monolayer, as assessed by unbroken expression of vascular endothelial (VE)-cadherin. VE-cadherin is a key component of adherens junctions in endothelial cells and is critical for barrier function [32]. A confluent monolayer and normal barrier function is crucial for studies of cancer metastasis because disruption of barrier function (e.g. by vasoactive agents) can promote metastasis by allowing cells to bind directly to exposed subendothelial matrix components, such as collagen and fibronectin [33]. 2) Formation of focal adhesions, as assessed by vinculin expression. Focal adhesions are important adhesive loci between cells and extracellular matrix proteins, allowing cells to respond (through cytoskeletal changes) to environmental stimuli, including shear stress [34]. Cells will undergo apoptosis (called anoikis) if such bonds are not formed [35]. Thus, both barrier function and focal adhesion formation is crucial for normal functioning of endothelial cells within the fabricated microfluidic channels.

We used a xenon-difluoride etching process to generate semi-cylindrical channels [36]. By creating duplicate channels in 2 separate sections of PDMS which are then bonded together, a completely circular channel was obtained. Open rectangular channels of similar size were generated using standard lithographic techniques, and were sealed with a glass coverslip to produce a closed channel. We then introduced HUVECs into the open channels under static conditions and into the closed channels under low shear stress. We then compared HUVEC structure within the open or closed circular or rectangular channels.

We found that HUVECs formed confluent monolayers when cultured under static conditions in open semi-circular or rectangular channels or under closed conditions of shear flow in circular or rectangular channels. Where we did observe a difference was in focal adhesion formation in open rectangular channels. In open semi-circular channels, numerous focal adhesions were seen on all aspects of the channel walls, indicating HUVECs conformed to the channel shape and were firmly adherent to the fibronectin coating of the channels. In contrast, no focal adhesions were observed on the bottom and sides of the open rectangular channels, suggesting that the HUVEC monolayer was suspended like a hammock in the channel and was not firmly adherent to the channel walls. This lack of adhesion to the fibronectin coating would affect endothelial behavior within the channels, since cells require firm adhesion to the subendothelial matrix in order to respond to various chemical and mechanical stimuli. Adhesion to the channel wall was re-established when HUVECs were cultured in closed rectangular channels under shear stress (Fig. 1), suggesting that the shear stress "forced" the cells into direct contact with the channel walls, permitting them to form focal adhesions. Our results indicate that standard fabrication of rectangular channels can be used to model the microvasculature, as long as endothelial cells are cultured under shear stress conditions. We are planning to test whether channel shape also affects endothelial behavior, e.g. response to vasoactive agents or inflammatory cytokines.

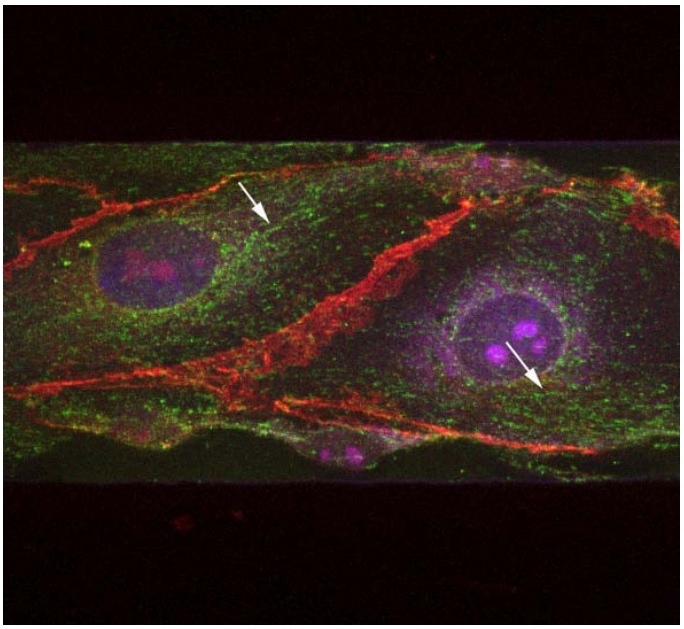

FIGURE 1: **IMMUNOSTAINING OF HUVECS WITHIN MICROFLUIDIC CHANNELS.**
HUVECs cultured under shear conditions form a confluent monolayer as shown by immunostaining for VE-cadherin (red unbroken lines that delineates cell borders) and are adherent to the fibronectin substrate coating the channels, as shown by immunostaining for vinculin (green dash-like structures, arrows). Nuclei are counterstained with TOPRO3 (purple).

Effect of channel shape on endothelial structure
Since we found that shear was important for establishing a confluent adherent HUVEC monolayer in our microfluidic channels, we then asked if infusing HUVECs into channels under different shear stresses affects their ability to endothelialize the channels. For this study, HUVECs were added to a channel reservoir then drawn into 50 x 50 um rectangular channels using reverse shear (suction). The length of endothelial coverage within the channel was then measured using phase contrast microscopy at various time intervals after shear was applied (Fig. 2A). We found that shear stress did promote endothelial coverage within the channels (Fig. 2B).

FIGURE 2: EFFECT OF SHEAR ON ENDOTHELIAL COVERAGE OF MICROFLUIDIC CHANNELS.

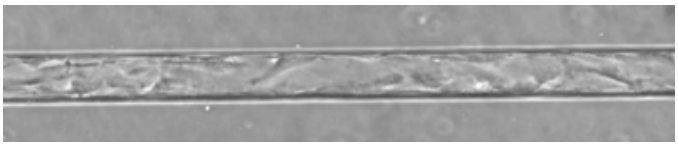

FIGURE 2A: Phase contrast image of HUVECs 2 days after being introduced into a microfluidic channel under shear conditions (0.9 Pa).

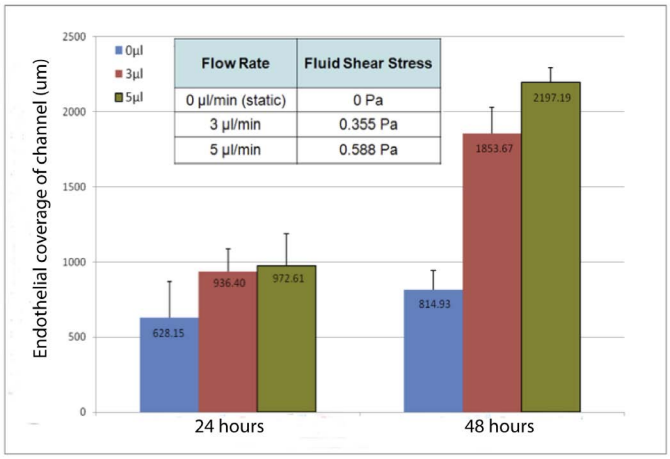

FIGURE 2B: Means ± standard deviation of length of channel coverage by HUVECs, 24(left columns) and 48 (right columns) hours after being introduced into the channels under different shear rates (0, 0.4 Pa, 0.9 Pa).

Inclusion of organ-derived soluble mediators through femtosecond laser-ablated micropores
Incorporation of metastatic target organ-derived growth factors or chemokines is desirable in order to mimic environmental cues that could affect tumor-endothelial interactions. These soluble mediators can be infused over the endothelialized surface through the channel lumen but cannot be introduced into our in vitro model system from the abluminal side of the endothelium. Since these mediators are derived from the organ parenchymal tissue, they would normally access circulating or adherent tumor cells from the abluminal endothelial surface. Indeed, abluminally applied chemokines are more effective at recruiting tumor cells

than those that are applied to the luminal surface [24]. Thus, we used femtosecond laser ablation to introduce micropores into the channels, so that soluble mediators could be introduced on the abluminal side of the endothelialized channels. Femtosecond laser ablation is a novel technique for creating micropores, in which micropore width and location can be finely controlled in any dimension. A femtosecond laser generates a localized intense energy source, which ionizes and ablates transparent material, like PDMS, in a small area that is directly within the focus of the laser. Thus, small holes and channels can be drilled in PDMS without affecting its surface. Micropores as small as 0.5 μm can be created in any direction in molded PDMS channels [31].

We fabricated two parallel channels with separate inlets and outlets, one for endothelialization, the other for mediator delivery. Micropores (10 um) were drilled with a femtosecond laser between the two channels (Fig. 3A). To demonstrate that soluble mediators can move from the mediator to the endothelialized channel, we introduced a fluorescent dye into the mediator channel and observed its passage through the micropores into the second (empty) channel (Fig. 3B). To ensure that HUVECs were not perturbed by the presence of micropores, we cultured HUVECs in the second channel and found that they formed confluent layers with focal adhesions that overlaid the micropores (Fig. 3C).

Thus, we have shown feasibility of using a microfluidic device of comparable size to the microvasculature for generating endothelialized channels with intact barrier and adhesive structure. We can also introduce soluble mediators into the endothelialized channels through micropores that access the abluminal endothelial surface, mimicking the exposure of endothelial cells to organ-derived chemokines or growth factors. Shear stress is important for attaining uniform endothelial coverage of the channels. We will continue to use the device to study molecular mechanisms that govern tumor-endothelial cell interactions during cancer metastasis.

FIGURE 3: INTRODUCTION OF A FLUORESCENT DYE INTO A MICROFLUIDIC CHANNEL THROUGH FEMTOSECOND-LASER DRILLED MICROPORES

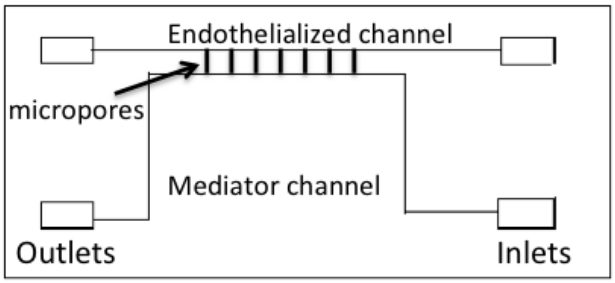

FIGURE 3A: Design of a microfluidic device containing endothelialized and mediator channels connected by femtosecond laser-ablated micropores.

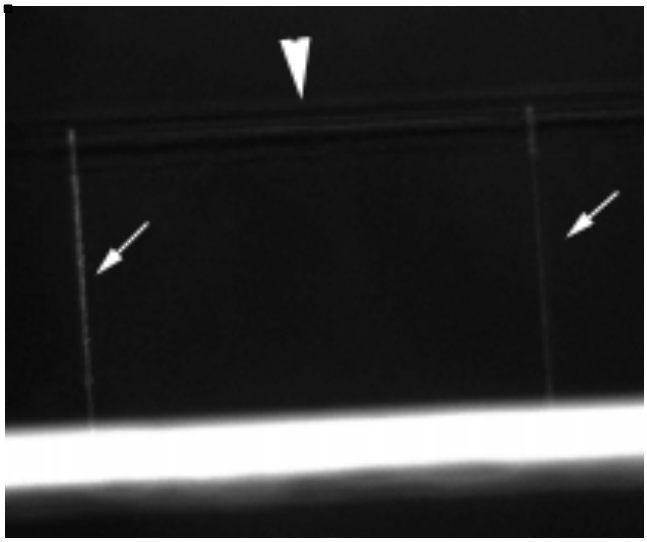

FIGURE 3B: Fluorescent dye introduced into the mediator channel (bottom) moves by diffusion through the micropores (arrows) into the second channel (arrowhead), which will be endothelialized.

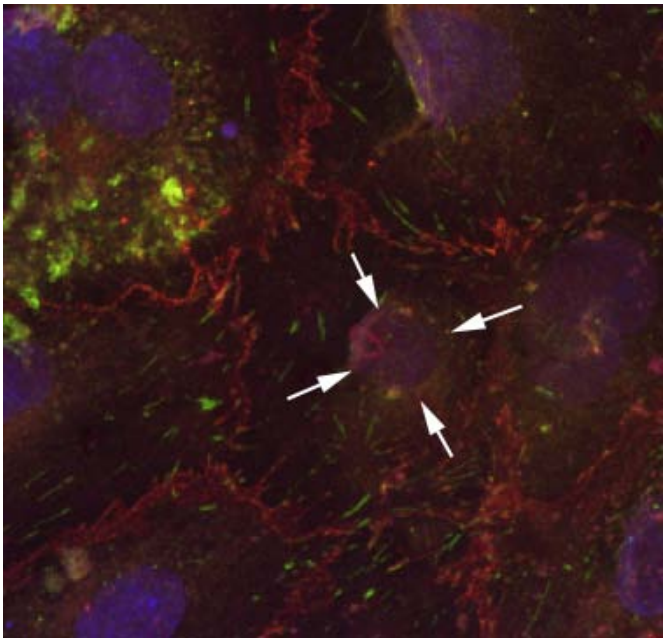

FIGURE 3C: HUVECs cultured within the endothelialized channel cover the femtosecond laser-ablated micropores (indicated by arrows) and form confluent adherent monolayers as shown by immunostaining for VE-cadherin (red) and vinculin (green), respectively. Nuclei are counterstained with TOPRO3 (blue).

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