Flow shear stress regulates endothelial barrier function and expression of angiogenic factors in a 3D microfluidic tumor vascular model

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Endothelial cells lining blood vessels are exposed to various hemodynamic forces associated with blood flow. These include fluid shear, the tangential force derived from the friction of blood flowing across the luminal cell surface, tensile stress due to deformation of the vessel wall by transvascular flow, and normal stress caused by the hydrodynamic pressure differential across the vessel wall. While it is well known that these fluid forces induce changes in endothelial morphology, cytoskeletal remodeling, and altered gene expression, the effect of flow on endothelial organization within the context of the tumor microenvironment is largely unknown. Using a previously established microfluidic tumor vascular model, the objective of this study was to investigate the effect of normal (4 dyn/cm²), low (1 dyn/cm²), and high (10 dyn/cm²) microvascular wall shear stress (WSS) on tumor-endothelial paracrine signaling associated with angiogenesis. It is hypothesized that high WSS will alter the endothelial phenotype such that vascular permeability and tumor-expressed angiogenic factors are reduced. Results demonstrate that endothelial permeability decreases as a function of increasing WSS, while co-culture with tumor cells increases permeability relative to mono-cultures. This response is likely due to shear stress-mediated endothelial cell alignment and tumor-VEGF-induced permeability. In addition, gene expression analysis revealed that high WSS (10 dyn/cm²) significantly down-regulates tumor-expressed MMP9, HIF1, VEGFA, ANG1, and ANG2, all of which are important factors implicated in tumor angiogenesis. This result was not observed in tumor mono-cultures or static conditioned media experiments, suggesting a flow-mediated paracrine signaling mechanism exists with surrounding tumor cells that elicits a change in expression of angiogenic factors. Findings from this work have significant implications regarding low blood velocities commonly seen in the tumor vasculature, suggesting high shear stress-regulation of angiogenic activity is lacking in many vessels, thereby driving tumor angiogenesis.

Introduction

Solid tumors are comprised of a porous interstitium and an irregular vascular network characterized by highly permeable vessels. The parenchyma and stromal cells that reside in this microenvironment are exposed to interstitial fluid flow, pressure gradients, and hemodynamic shear that can significantly influence tumor progression. These hydrodynamic stresses are generated by osmotic and hydrostatic pressure gradients that arise from tumor pathophysiology; including tumor growth-induced compressive strain, increased extravasation of macromolecules from leaky tumor vessels, and elevated interstitial pressure due to lack of functional lymphatics. For the last decade, investigation of flow through the vasculature and interstitium of tumors has been motivated by the desire to understand the role of fluid convection in the delivery of therapeutic drugs to treat cancer.1-7 However, recent studies have revealed an underlying importance of fluid dynamics in regulating molecular signaling, gene expression, cell cycle progression and tumor development.8-12 Emerging evidence suggests that interstitial flow plays a significant role in promoting tumor cell migration and metastasis. Such studies have shown that interstitial flow not only influences the direction and magnitude of cell migration, but also generates transcellular chemokine gradients that guide metastasis.9,11 Clinically significant findings have also demonstrated that interstitial fluid pressure is correlated with survival of cervical cancer patients13 and lowering interstitial fluid pressure reduces tumor cell proliferation in vivo.12 Although few studies have explored the effect of flow shear stress on tumor cell phenotype, experiments by Chang et al. have characterized a mechanism by which high shear stress (12 dynes/cm²) induces a G2/M cell cycle arrest in tumor cells.8 These findings suggest that tumor cells in regions subjected to low flow shear stress may not become growth arrested and continue to undergo mitosis. Thus, different fluid flow patterns may have a regulatory affect on tumor cell

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proliferation and invasion, thereby influencing region-specificity for tumor metastasis. Collectively, these data imply that tumor response to fluid shear stress should be considered in the treatment and management of metastatic disease. However, no experimental studies have considered the downstream effects of flow shear stress on other stromal cells of the tumor microenvironment, which may consequently direct tumor progression.

It is well known that endothelial cells lining blood vessels respond to shear stress, a fluid mechanical force generated by blood flowing tangential to the luminal surface, thereby maintaining homeostasis of the circulatory system. Many reports have documented the effect of wall shear stress (WSS) on endothelial cell-matrix interactions, junction molecules, gene expression, barrier transport properties, and angiogenesis.14-27 For example, reports have shown that shear stress causes upregulation of Tie2 and MMP-125 and increases vascular endothelial growth factor (VEGF) expression in endothelial cells.28 Similar studies have also shown that shear stress stimulates endothelial cell migration as well as supports blood vessel maintenance.29,30 These shear-mediated changes in vascular stability and endothelial barrier function can directly influence tumor angiogenesis. For example, loosening of tight vascular structures results in increased exposure of endothelial cells to VEGF, facilitating the initiation of angiogenesis through endothelial cell proliferation, sprouting, and tube formation.31,32 However, the exact mechanisms of endothelial reorganization in response to shear stress are not fully defined.33 Even less understood is the role of shear stress within the context of tumor angiogenesis. The average WSS in normal microvessels is around 4 dyn/cm²; however, geometrical resistance of the abnormal tumor vasculature can compromise tumor blood flow, resulting in overall WSS that are reduced relative to normal blood capillaries.34 While previous studies have demonstrated the importance of tumor-endothelial cross talk for tumor angiogenesis,35-37 it is not known to what extent shear forces interface with or regulate the activity of angiogenic signaling pathways within the tumor microenvironment.

The objective of this study was to characterize the role of flow shear stress on tumor-endothelial cross talk and vascular barrier function. Using a previously established 3D, in vitro microfluidic tumor vascular model,38 tumor and endothelial cells were co-cultured under a range of tumor-relevant hydrodynamic stresses. It was hypothesized that high flow shear stress will decrease vascular permeability and tumor cell expression of angiogenic growth factors. Permeability of the endothelium, secretion of pro-angiogenic factors, and tumor-expressed angiogenic genes were assessed as a function of normal microvascular WSS (4 dyn/cm²),24 as well as a low (1 dyn/cm²), and high (10 dyn/cm²) WSS to target a range of tumor-relevant fluid forces. This is the first study to utilize a 3D in vitro culture model to demonstrate tumor cell-mediated changes in endothelial barrier function, in addition to flow-mediated vascular permeability and gene expression. Understanding the significance of flow shear stress on tumor angiogenesis is important for overcoming challenges associated with therapeutic targeting of the tumor vasculature. In addition, defining a relationship between fluid flow and tumor angiogenesis may provide evidence that further supports the theory of tumor vascular normalization strategies to revert the malignant phenotype and halt tumor progression.

**Results**

**In vitro tumor vascular model**

Co-culture of breast tumor and endothelial cells within microfluidic type I collagen hydrogels was conducted using a previously developed tumor vascular model.38 Briefly, 1 × 10⁶ MDA-MB-231 cells were resuspended in 8 mg/ml neutralized collagen, poured into FEP tubing fit concentrically with a 22G needle (711 μm), and capped with PDMS sleeves (Fig. 1). After polymerization for 20 minutes at 37°C, the needle was removed and 10 × 10⁶ endothelial cells/ml were carefully injected into the central microchannel 2X at 10 min intervals and slowly rotated to completely cover the luminal surface. Flow was then immediately introduced through the endothelialized microchannel by perfusing EBM-2 growth media at a low flow rate of 3 μl/min. A graded increase in flow that subjected the endothelium to a low shear stress of τ = 0.01 dyne/cm² up to τ = 0.1 dyne/cm² over 72 hours was utilized to encourage the establishment of a viable and functional endothelialized channel.38 Following this initial preconditioning time period, the flow was increased to induce one
VEGF protein secretion

Total VEGF protein was isolated from the perfusate of endothelial mono-cultures compared to co-culture with tumor cells during the 72 hr preconditioning period. Analysis shows a statistically significant increase in VEGF secretion from day 1 to day 3. While a greater amount of VEGF protein was measured in co-cultures samples relative to endothelial mono-cultures, the difference was not significant. This may be due to matrix binding or endothelial VEGFR association of tumor-secreted VEGF, such that measurable changes in total VEGF secretions were undetected during co-culture. In addition, measured VEGF protein secretion from tumor-monocultures (data not shown) was slightly greater than that of endothelial mono-culture or co-culture, though the difference was not statistically different. While total VEGF secretion was significantly increased after 6 hr exposure to each $\tau_W$ relative to precondition conditions ($\tau_W < 0.01$ dyn/cm$^2$), no trends were observed as a function of increasing $\tau_W$ immediately post-shear, as VEGF concentration remained relatively constant for each flow condition (data not shown).

Our current findings indicate that under flow shear stress conditions ($0.1 < \tau_W < 10$ dyn/cm$^2$) and short culture durations ($<3$ days), VEGF protein secretion is slightly increased by co-culture conditions relative to tumor or endothelial mono-cultures. Additional studies investigating endothelial-originated VEGF in the microfluidic collagen hydrogel are needed to fully understand the role of flow shear stress on autocrine signaling mechanisms. The dynamic interplay between VEGF and its downstream signaling pathways has only recently been implicated in studies investigating shear stress-mediated angiogenesis. Although hypoxia is the most well characterized factor that causes VEGF upregulation, emerging mechanisms by which mechanical forces regulate VEGF expression are now being discovered. For example, a dynamic regulation pattern of VEGF gene and protein expression in endothelial cells was first demonstrated by Gan et al., in which high shear stress (25 dynes/cm$^2$) decreased VEGF gene expression after exposure for 1.5 hours, but increased VEGF gene expression after exposure for 3 hours. A later study by Conklin et al. showed that 24 hours of low shear stress (1.5 dynes/cm$^2$) significantly increased VEGF gene and protein expression in endothelial cells. In addition, we have previously shown that VEGF protein expression is enhanced in 2D, static tumor-endothelial co-cultures compared to monocultures, though this response was observed over long-term culture durations (up to 28 days).

Tumor-expressed angiogenic genes in response to WSS

Tumor co-cultures and tumor mono-cultures were maintained under the 72 hr low flow preconditioning scheme, followed by exposure to one of 3 target WSS ($\tau_W = 1, 4,$ or $10$ dynes/cm$^2$) for 6 hrs, after which tumor mRNA was isolated for gene expression analysis. qRT-PCR results indicate that in the presence of an endothelialized microchannel, high WSS ($\tau_W = 10$ dynes/cm$^2$) significantly down-regulates all MDA-MB-231-expressed angiogenic factors probed in this experiment (Fig. 3). In contrast, WSS did not have an effect on tumor mono-cultures, suggesting fluid forces acting on the endothelium regulate cross-talk with surrounding tumor cells, thus altering gene expression activity. Furthermore, the decreasing trend in gene expression as a function of increasing WSS was not observed in tumor-monocultures compared to tumor co-cultures, thereby suggesting that this phenomenon is not a function of flow-mediated transport of oxygen and nutrients through the collagen matrix.

A conditioned media experiment was conducted in which MDA-MB-231 cells were cultured in collagen hydrogels polymerized in 48-well plates under static conditions. Cells were grown for 3 days in media collected from the flow shear stress experiments to determine if down-regulation of angiogenic proteins was due to soluble factors present in the media.
Results showed no change in MDA-MB-231 gene expression after culture in conditioned media (data not shown). This suggests a physical, flow-mediated mechanism exists in the microfluidic collagen hydrogel, which plays a role in transcriptional regulation of the angiogenic factors probed in these experiments.

In a recent study by Song et al. investigating the role of VEGF gradients and shear stress on in vitro endothelial morphogenesis, results demonstrated that physiological shear stress (3 dyn/cm²) attenuates VEGF-driven endothelial sprouting.²² Even in the presence of high VEGF concentrations (50 ng/ml) endothelial invasion was minimal under flow relative to static conditions. These studies further confirmed that inhibition of VEGF-induced sprouting requires nitric oxide (NO) signaling, an important shear stress-responsive signaling molecule. Results from this work may provide an explanation by which high shear stress down-regulates tumor expressed angiogenic factors via NO secretion. For example, several studies have shown that NO can have inhibitory effects on tumorigenesis, in which DNA damage induced by NO causes tumor regression and inhibition of metastasis.⁴⁵,⁴⁶ Angiogenic factors such as VEGF and the angiopoietins have also been shown to activate eNOS in vascular endothelial cells.⁴⁷,⁴⁸ As such, co-culture conditions within the microfluidic tumor vascular model, which increase VEGF, may stimulate endothelial NO secretion. Flow shear stress may further increase NO signaling, such that the combined effect of flow shear stress and co-culture-induced VEGF decreases tumor angiogenic activity. Further analysis is needed to better understand the underlying flow-mediated effects.

**Endothelial barrier function**

The permeability of the endothelium was measured following the 72 hrs precondition period, and after 6 hrs exposure to one of 3 target WSS (τₚ = 1, 4, or 10 dyn/cm²). The measured effective permeability coefficient, Pₑ, of the endothelialized microchannel as a function of WSS indicates that Pₑ of the 70 kDa Oregon Green-conjugated dextran decreases with increasing WSS for both endothelial mono-culture and co-culture with tumor cells (Fig. 4A). This decreased permeability is likely due to increased endothelial alignment and spreading in the direction of flow,³⁸ in which high WSS (τₚ = 10 dyn/cm²) significantly decreased Pₑ relative to the preconditioned endothelium. These findings are consistent with others showing shear stress-mediated endothelial morphological adaptations,²²,³⁸,⁴⁹-⁵² and permeability.¹⁴,¹⁷,¹⁹,²¹,²³,⁵³ The presence of tumor cells also increased Pₑ relative to endothelial mono-cultures, with a significantly greater Pₑ under high WSS conditions. Images taken at the end of the permeability assay for the case of τₚ = 10 dyn/cm² qualitatively show greater distribution of dextran across the endothelium during co-culture with tumor cells, relative to

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**Figure 3.** High WSS down-regulates tumor-expressed angiogenic factors in the presence of an endothelium. Tumor mono-cultures (A) or co-cultures with endothelial cells (B) were cultured under the 72 hr precondition flow rate, after which the target WSS (τₚ = 1, 4, or 10 dyn/cm²) was introduced through the microchannel for a total 6 hrs. Total tumor mRNA was then isolated for gene expression analysis. Relative mRNA to GAPDH mRNA expressed as a fold induction ± standard deviation (n = 4) *P < 0.05. Scale bar 200 µm.
dextran distribution immediately post 72 hrs preconditioning (Fig. 4B). A more disorganized endothelium at the channel wall are also observed during co-culture, with initial signs of migration off the luminal surface, which may contribute to increased permeability.

Vessel destabilization and reduced endothelial barrier function increase endothelial cell exposure to angiogenic growth factors such as VEGF and the angiopoietins, and leads to vessels that are more amenable to sprouting and angiogenesis. Studies by Holash et al. in a rat glioma model have also demonstrated that mature vessels must first destabilize to allow for subsequent sprouting and vessel growth. Endothelial tight junctions and adherens junctions are known to play a role in the paracellular permeability of the endothelium; therefore, a more thorough investigation of tight junction protein distribution in response for flow shear stress and co-culture with tumor cells is needed to determine if down-regulated tight junction protein expression explains the decreased permeability observed in the current study. Several reports have provided evidence of flow shear stress-mediated distribution of tight junction proteins, in which exposure to shear stress leads to a transient decrease (<4 hrs post shear) in VE-cadherin or occludin protein expression, as endothelial cells migrate and align in the direction of flow. Studies investigating the effect of shear stress on permeability of macromolecules across the endothelium also revealed that endothelial permeability is acutely sensitive to shear stress in vitro. Exposure of both 1 and 10 dyne/cm² dramatically increased permeability of FITC-BSA after 30 and 60 minutes; however, permeability returned to pre-shear values after 120 minutes. Similar studies demonstrated that when subjected to low shear stress, endothelial cells cultured in collagen hydrogels were visibly more permeable to FITC-BSA. These findings are consistent with permeability results obtained from this study, suggesting that the endothelial cells migrate and align to varying extents in response to different magnitudes of flow shear stress.

**Discussion**

Endothelial response to abnormal shear stress impairs various vascular functions and can lead to diseases such as atherosclerosis, thrombosis or pathological angiogenesis commonly...
seen in tumors. However, the effect of fluid shear stress on the endothelial cell phenotype within the context of tumor progression is largely unknown. The relationships between blood flow, mechanotransduction, and local cell behavior can be discovered by the advancement of in vitro tissue culture models designed to quantitatively measure fluid mechanics coupled with cellular response.

This study has been designed to investigate the combinatorial effect of shear stress and tumor-endothelial cross-talk on tumor angiogenesis in a 3D microfluidic collagen hydrogel. We have previously provided evidence that tumor cells have an altered phenotype when cultured under 3D dynamic conditions compared to both 2D static and 2D mono-culture controls. Results presented here further support the idea that endothelial cells play an active role in regulating tumor angiogenic activity, in that flow shear stress influences tumor-endothelial cross-talk such that barrier function and expression of angiogenic factors are altered. Our findings demonstrate that increasing high WSS decreases tumor expressed angiogenic factors and decreases endothelial permeability, thereby improving vascular integrity such that tumor angiogenic potential is reduced. More importantly, this shear-mediated tumor cell response is only observed in the presence of an endothelium. To date, no experimental in vitro models have been previously designed to specifically probe the affect of tumor cells on endothelial barrier function under physiologically relevant flow conditions. A better understanding of the physical and chemical mechanisms by which endothelial cells convey instructive signals to promote tumor progression will provide a foundation for improved clinical studies to treat vascularized tumors.

**Methods**

**Cell culture**

A human breast carcinoma cell line (MDA-MB-231) (American Type Culture Collection (ATCC)) and a telomerase-immortalized human microvascular endothelial cell line (TIME) were used in this study. TIME cells were provided as a generous gift from Dr. Shay Soker at the Wake Forest Institute for Regenerative Medicine (Winston-Salem, NC). A lentiviral vector system was used to genetically modify the TIME cells to stably express a red fluorescent protein (RFP) for real-time visualization of endothelial morphology during culture. Imaging was performed using a fluorescence microscope (Leica AF6000) to monitor cell growth during culture.

MDA-MB-231 cells were cultured in Dulbecco’s Modified Eagle Medium: nutrient mixture F-12 (DMEM/F12) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). TIME cells were cultured in EBM-2 (Lonza, Rockland, ME) media supplemented with a growth factor BulletKit (Lonza CC-4176). All cells were maintained in a humidified 5% CO2/95% air atmosphere at 37°C within an incubator for all experiments.

**Enzyme-linked immunosorbent assay**

Protein levels of VEGF during the 72 hr preconditioning time period as well as post-shear were measured using an enzyme-linked immunosorbent assay (ELISA) (Quantikine Human Immunoassay kits (R & D Systems, Minneapolis, MN)) according to the manufacturer’s protocol. Samples were collected from conditioned perfusate of tumor-endothelial co-cultures and compared to tumor and endothelial mono-cultures at 24, 48 and 72 hrs (n = 4). Samples were also collected after the 6 hr exposure to each τ_W to assess flow-mediated translation of VEGF.

**Quantitative RT-PCR**

To determine the effect of WSS on tumor-endothelial cross-talk, expression levels of target genes in MDA-MB-231 during co-culture or mono-culture after the 6 hr exposure to each τ_W were determined quantitatively by real-time RT-PCR. To isolate tumor cell mRNA from co-cultures, the endothelialized microchannel was de-nuded by carefully scraping the cells off the luminal surface and subsequently washing with PBS. Total RNA from the microfluidic collagen scaffolds was then isolated by the phenol-chloroform extraction method as described previously. Reverse transcription and PCR amplification of gene-specific TaqMan PCR primers: VEGF-A (NM_001025366.2), ANG-1 (NM_001146.3), ANG-2 (NM_001118887), HIF1-α (NM_001243084.1) and MMP-9 (NM_004994.2) (Applied Biosystems, Foster City, CA) were then conducted. Evaluation of 2^−ΔΔ_C_T indicates the fold change in gene expression, normalized to GAPDH (NM_002046.3) housekeeping gene and relative to the control group (n = 4).

**Endothelial permeability assay**

A permeability assay to quantify barrier function of the endothelialized microchannel was conducted as described previously. Briefly, 70 kDa Oregon green-conjugated dextran (10 μg/ml in serum-free medium) was perfused through the endothelialized microchannel at 26 μl/min (τ = 0.1 dyn/cm²) for 1 hr, as selectively permeable mature capillaries are known to be impermeable to dextrans over a molecular weight of 65 kDa. Images were captured every 4 minutes using an inverted fluorescence microscope (Leica AF6000). ImageJ (National Institutes of Health, Bethesda, MD) was then used to measure average fluorescence intensity in a ROI that spanned the width of the collagen, including the endothelialized microchannel. The effective permeability coefficient, P_d, which describes the ability of a solute to escape uniformly from the vascular lumen, was calculated using the following equation:

\[
P_d = \frac{1}{I_1-I_b} \left( \frac{I_2-I_1}{\Delta t} \right) \frac{d}{4}
\]

where d is the microchannel diameter, I_b is the average background intensity, I_1 is the average initial intensity, and I_2 is the average intensity after the recovery time Δt. P_d was calculated as a function of each target WSS (τ_W = 1, 4, or 10...
Statistical analysis
Experimental groups were tested and analyzed and the data are expressed as mean ± standard deviation. Significance of the RT-PCR results, ELISA results and permeability assay were verified using Student’s t-test. A 95% confidence criterion will be used to determine statistically significant differences between experimental groups.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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