Recapitulating physiological and pathological shear stress and oxygen to model vasculature in health and disease

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Studying human vascular disease in conventional cell cultures and in animal models does not effectively mimic the complex vascular microenvironment and may not accurately predict vascular responses in humans. We utilized a microfluidic device to recapitulate both shear stress and O2 levels in health and disease, establishing a microfluidic vascular model (µVM). Maintaining human endothelial cells (ECs) in healthy-mimicking conditions resulted in conversion to a physiological phenotype namely cell elongation, reduced proliferation, lowered angiogenic gene expression and formation of actin cortical rim and continuous barrier. We next examined the responses of the healthy µVM to a vasotoxic cancer drug, 5-Fluorouracil (5-FU), in comparison with an in vivo mouse model. We found that 5-FU does not induce apoptosis rather vascular hyperpermeability, which can be alleviated by Resveratrol treatment. This effect was confirmed by in vivo findings identifying a vasoprotecting strategy by the adjunct therapy of 5-FU with Resveratrol. The µVM of ischemic disease demonstrated the transition of ECs from a quiescent to an activated state, with higher proliferation rate, upregulation of angiogenic genes, and impaired barrier integrity. The µVM offers opportunities to study and predict human ECs with physiologically relevant phenotypes in healthy, pathological and drug-treated environments.

Human vascular disease is commonly studied using preclinical animal models, which do not fully represent human endothelial characteristics, or through conventional in vitro cultures of human endothelial cells (ECs), which are typically maintained at static and atmospheric oxygen (O2) conditions. The EC microenvironment, however, is orchestrated by complex chemical and mechanical signaling making the achievement of physiological endothelial phenotype in conventional laboratory settings a difficult task. Shear stress, caused by blood flow, is one of the main regulators of EC phenotype and barrier integrity. Variations of shear stress levels in different regions of the arteries were shown to play a pivotal role in the formation of distinct phenotypes. Long-term exposure of cultured ECs to physiological levels of laminar shear stress leads to various cellular responses such as cellular alignment with the direction of flow, improvement of endothelial barrier integrity, decreased proliferation and upregulation of transcription factors such as Kruppel-like factor-2 (KLF2), all of which are associated with EC phenotype in vivo. On the other hand, ECs are also exposed to sub-atmospheric dissolved O2 (DO) levels ranging from 5-12% O2. It is well established that the hypoxic conditions alter endothelial phenotype and permeability. We and others have shown that cultured ECs respond differently when at physiological O2 (5-12%) compared to conventional atmospheric O2 (21% O2). Overall, while the individual effects of varying shear stress and O2 tension on angiogenesis have been studied, creating an in vitro system with control over both shear stress and O2 levels will advance our understanding and predictability of human endothelial functionality in healthy and diseased conditions.

Microfluidic technology offers an approach to precisely control the level, duration, and extent of various cues in the cellular microenvironment. In the last decade, EC maintenance in microfluidic systems has been established by a number of studies, as reviewed elsewhere. These systems were designed to mimic different physiological factors such as hemodynamic forces, O2 or co-culture in order to achieve an in vitro vascular model. However, none of these systems have focused on simultaneous control of shear stress and O2 tension. Furthermore, only a small number of studies thoroughly demonstrated the establishment of an in vivo mimicking EC phenotype in physiological/pathological states. In the first part of the study, we establish a microfluidic vascular model (µVM) recapitulating both physiological shear stress and O2 levels in a device that was recently developed in our lab. We begin by demonstrating time-dependent transition of human ECs from in vitro phenotype to...
physiologically relevant phenotypes. To confirm the phenotypic similarity of the µVM to *in vivo*, we study endothelial responses to a vasotoxic chemotherapeutic drug, 5-Fluorouracil (5-FU; (20)) and to a vasoprotective agent Resveratrol17 in the µVM and *in vivo*. In the past decade, long-term cardiotoxic adverse effects of chemotherapeutics including 5-FU have become a pivotal issue with the increased life span of cancer patients owing to progress in cancer treatment20. However, the mechanisms underlying direct effects of 5-FU on ECs is yet to be understood due to difficulties in clinical examinations and lack of physiologically relevant *in vitro* models.

Finally, we explore whether the µVM can also be used to create a vascular disease model. We propose that the µVM can accurately simulate atherosclerosis-associated ischemic conditions *in vitro* by simultaneously controlling shear stress and O2 levels. In ischemic tissues, shear stress and O2 tension reach low levels due to reduced or complete cessation of blood flow21. Stimulation of angiogenesis using proangiogenic recombinant proteins or adenoviral vectors containing proangiogenic transgenes has been used as a therapeutic approach to ischemic injury20,21. Although proangiogenic therapy has led to promising results in both preclinical and clinical trials, angiogenic responses to pathological microenvironments should be better understood in order to improve the therapeutic efficacy and to prevent commonly encountered complications such as vascular permeability and hypotension22. Therefore, *in vitro* models that allow for accurate simulation of ischemia by recapitulating the disturbed chemical and mechanical factors in vascular microenvironment are necessary.

**Results**

**Establishing a microfluidic vascular model (µVM): phenotypic adaptation from conventional culture to physiological conditions.** Using the capabilities of the microsystem previously developed in our laboratory16, we mimicked healthy conditions where cultured cells can be maintained at physiological shear stress and O2 tension (i.e. 12 dyn/cm² and 5% O2 (38 mmHg)) as well as ischemic conditions (i.e. 0.01 dyn/cm² and 1% O2 (7.6 mmHg)) compared to conventional culture conditions (i.e. 0.01 dyn/cm² and 21% O2 (159.6 mmHg)) as described in Figure 1A. The time points were initially chosen based on previous studies focused on the formation of continuous adherent junctions23–24. In addition, DO levels in the cell culture media were continuously monitored using O2 sensor patches at the inlet and outlet of the microchannel11,16,25 throughout the experiments at each condition (Figure 1B).

Using an improved microfluidic setup (Supplementary Figure 1), ECs were seeded and subjected to physiological shear stress and O2 for two days. Time dependent changes in morphology, F-actin organization, endothelial barrier integrity and localization of adherens junction proteins were examined to understand the phenotypic adaptation from conventional culture (i.e. 0.01 dyn/cm² and 21% O2) to physiological conditions. After 24 hours at physiological conditions, ECs were elongated and aligned with the direction of the flow, continuing their elongation after 48 hours (Figure 2A). F-actin staining of the ECs demonstrated that most of the actin fibers were localized at the cell borders in conventional culture conditions (Figure 2B (ai)). After 24 hours in physiological conditions, we observed the formation of abundant and thicker actin stress fibers that were organized throughout the cytoplasm and aligned with the direction of the flow (Figure 2B (bi)). As the duration of the physiological conditions was extended to 48 hours, the stress fibers disassembled into shorter actin filaments and reorganized peripherally to form the cortical actin rim, indicating a more stabilized state of cell-cell contact (Figure 2B (ci);26). The morphology and organization of actin filaments were found to be comparable to that of an intact EC inner lining layer of mouse aorta (Figure 2B (dl)). Intercellular gaps and localization of the vascular endothelial cadherin (VECAD) and platelet endothelial cell adhesion molecule 1 (PECAM1), which are adherens junctional proteins and responsible for mechanotransduction in ECs2, were determined by immunofluorescent imaging. In conventional culture conditions, we observed an endothelial barrier integrity associated with VECAD localization at the cell borders. After 24 hours at physiological conditions, VECAD localization at the cell borders was compromised: the cell-cell contact occurred through membrane protrusions leading to a higher number of intercellular gaps (Figure 2B (a and b-e))23–24. In some regions of the membrane, we observed VECAD-positive linear structures extending into the cell (Figure 2B (bii)). These structures were orthogonal to the cell borders and co-localized with the ends of stress fibers, suggesting that stress fibers dynamically retract and release the adherens junction proteins, thereby mediating discontinuous adherens junctions23–24. After 48 hours at physiological conditions, VECAD protein was localized to the cell borders, reestablishing the continuous cell-cell contact (Figure 2B (c-e)). Both the number and size of intercellular gaps were decreased indicating an improved endothelial barrier integrity after 48 hours in physiological conditions (Figure 2B (fi–fii)). The localization and integrity of VECAD after 48 hours in physiological conditions resembled that of an intact EC inner lining layer of mouse aorta (Figure 2B (di)) Although peripheral localization of PECAM1 did not increase after 48 hours, PECAM1 localized as thick structures at some regions of cell-cell contact (Supplementary Figure 2). This reticular organization of PECAM1 has recently been shown to contribute to endothelial barrier function25. Our gene expression analyses of the ECs exposed to

Figure 1 | Description of experimental conditions. (A) The timeline of shear stress and DO levels for each experimental condition is depicted: two control conditions (conventional and high shear), one healthy model (physiological) and two models for anti-cancer drug toxicity and ischemic vasculature. (B) DO level measurements in the culture media in the microfluidic system throughout each experimental condition demonstrating control over targeted DO levels.
physiological conditions for 48 hours showed that both KLF2 and eNOS were significantly upregulated compared to conventional culture conditions, indicating a transition to \textit{in vivo} phenotype (Figure 2C (a)). Similarly, VEGF and VEGF receptor-2 (VEGFR2) were slightly upregulated, in agreement with previous studies suggesting the anti-apoptotic role of increased autocrine levels of VEGF and VEGFR2\textsuperscript{29}. Angiopoietin 1 (ANG1), which is responsible for vascular maturation and barrier function, was not affected by the physiological conditions whereas ANG2, an antagonist of ANG1, was dramatically downregulated compared to conventional culture conditions. This finding is consistent with the analyses of intercellular gaps suggesting that physiological conditions promote improved endothelial barrier integrity similar to that found in the body (from Figure 2B (dii)). Finally, we found that the percentage of dividing ECs was significantly lower after two days in physiological conditions suggesting a transition to quiescent state (Figure 2C (b)).

Altogether, we establish an \textit{in vitro} \(\mu\)VM and demonstrate its ability to achieve \textit{in vivo} EC phenotype within 48 hours at physiological shear stress and \(O_2\) tension.

**Responses of \(\mu\)VM to vaso-effective drugs:** Administration of 5-FU impairs endothelial barrier integrity that can be alleviated by co-administration of Resveratrol. 5-Fluorouracil (5-FU) is a broadly administered anti-cancer drug targeting a wide range of solid-organ tumors including those found in gastrointestinal and breast cancer\textsuperscript{30}. Although cardiotoxicity of 5-FU is well-documented as 10% of the patients treated with this drug suffered from myocardial infarction, ischemia, congestive heart failure or sudden cardiac death, the mechanisms underlying the toxicity is not understood\textsuperscript{18}. Noncardiac vascular toxicity caused by the drug has been investigated by a limited number of studies using either animal models\textsuperscript{31} or \textit{in vitro} using conventional cultures of ECs\textsuperscript{31}. We sought to examine the response of the healthy \(\mu\)VM to 5-FU toxicity by examining the underlying mechanisms in comparison with an \textit{in vivo} mouse
model. After 48 hours in physiological conditions, 5-FU was added to the media in the μVM for 24 hours at a clinically relevant concentration of 7 mM. We could not detect cell detachment or changes in cell morphology in response to 5-FU (Figure 3A (a)). The expression levels of pro-apoptotic genes, BCL-2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) and BNIP3-like protein (BNIP3L), were downregulated compared to conventional conditions and were similar in both physiological conditions and 5-FU treated cells (Figure 3A (b)). TUNEL assay also showed that the ECs cultured in physiological conditions did not undergo apoptosis.

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Figure 3 | Administration of 5-FU impairs endothelial barrier integrity that is alleviated by co-administration of Resveratrol. (A) The apoptotic effect of 5-FU was examined on ECs cultured in physiological condition for 48 hours. (a) Inverted microscope images of ECs cultured in the μVM with and without 5-FU. Scale bar: 100 μm. (b) quantitative RT-PCR was performed to compare pro-apoptotic gene expression in ECs cultured in conventional conditions, physiological conditions or with 5-FU. (c) TUNEL assay was performed to detect apoptotic cells. The percentage of apoptotic cells after 5-FU treatment was compared to ECs treated with DNAse as the positive control. (B) (a) Fluorescent images of VECAD (red) and nucleus (blue) of ECs cultured in μVM demonstrate that (i) adherens junctions (arrows) were disrupted by treatment with 5-FU leading to the formation of intercellular gaps (arrowheads) and (ii) co-administration of 5-FU and Resveratrol alleviated the formation of discontinuous adherens junctions (arrows) and intercellular gaps (arrowheads). (b) the number and area of intercellular gaps were quantified using the immunofluorescent images. (C) In vivo experiments in mouse confirm the hyperpermeability effect of 5-FU alone and vasoprotective role of adjunct Resveratrol therapy. (a) Evans blue dye leakage in the ears of mice 20 minutes after the application of mustard oil. (b) Extravasated concentration of (i) Evans blue dye and (ii) water accumulation in the lungs of mice. (n = 5) (D) RT-PCR analyses of human ECs cultured in the μVM allowed for examining mRNA levels of the genes that are potentially responsible for the observed effects of drug treatment.
apoptosis in response to treatment with 5-FU (Figure 3A (c)). As this data demonstrated that 5-FU does not induce EC apoptosis, we continued to explore potential effects of 5-FU on EC phenotype. Performing high-resolution imaging analyses of the adherens junctions, we found that 5-FU increases both the number and area of intercellular gaps compared to physiological conditions, suggesting that 5-FU may lead to hyperpermeability of blood vessels (Figure 3B (a–b)). Resveratrol is a natural chemical that has recently been shown to enhance the efficacy of 5-FU on inhibition of tumor growth34. There is also a growing body of evidence that Resveratrol has protective effects against disruption of endothelial barrier function17,20–26. Thus, we hypothesized that co-administration of Resveratrol may overturn intercellular gap formation caused by 5-FU. We administered Resveratrol at a concentration of 100 μM in combination with 5-FU. After 24 hours of combination treatment, both the number and area of intercellular gaps were decreased, compared to treatment with 5-FU alone, suggesting a vasoprotective role of Resveratrol in 5-FU treatment (Figure 3B(aii) and (b)). In control experiments in which 5-FU treatment was applied to conventional petri-dish cultures, we observed a similar hyperpermeability effect of 5-FU (Supplementary Figure 3). However, using Resveratrol in combination with 5-FU did not overturn the formation of intercellular gaps induced by 5-FU, as opposed to Resveratrol’s suggestive protective role demonstrated in the μVM. In order to determine whether Resveratrol is vasoprotective, we performed the experiments in vivo by examining the vascular permeability of microvasculature and descending aorta in treated mice. Mice were injected with 5-FU alone (60 mg/kg) or co-administered with Resveratrol (6.5 mg/kg) for 24 hours. Intravenous tail vein injection of Evans Blue dye revealed vascular leakage in the ears of the mice treated with 5-FU alone whereas the co-administration with Resveratrol alleviated the level of the vascular leakage (Figure 3C(a)). In addition, extravascular concentration of Evans Blue dye in the lungs was significantly higher in the mice treated with 5-FU alone compared to vehicle control (Figure 3C (bi)). Similarly, wet-to-dry weight ratio of the lungs was also increased by 5-FU treatment indicating elevated water accumulation in the lung tissue as a response to 5-FU (Figure 3C (bii)). Moreover, examining the Concanaevalin A (Con A) binding throughout the layers of the descending aorta revealed that 5-FU also causes a slight increase in the permeability of the EC monolayer (Supplementary Figure 4 (a)). These data agreed with our findings using the in vitro μVM. Overall, these results suggest that the μVM better mimics the response of healthy ECs to drug treatment than conventional experiments, and therefore allows obtaining predictive cell responses. We next sought to use the in vitro μVM to begin exploring the underlying mechanism leading to the hyperpermeability response of human ECs to 5-FU. Previous reports suggested that 5-FU causes vasoconstriction which can be prevented by stimulation of eNOS content and activity35. Therefore, we first examined the mRNA levels of KL2, which mediate eNOS expression29–33. Interestingly, the expression levels of KL2, which is also a key factor found in healthy ECs in vivo34, and eNOS in human ECs were not affected by the administration of 5-FU (Figure 3D). We also validated the unchanged expression levels of eNOS in response to 5-FU in ECs of mouse aorta (Supplementary Figure 4 (b)). The gene expression levels of VEGF and VEGFR2 were also not affected in human ECs by 5-FU or adjunct Resveratrol therapy (Supplementary Figure 5). On the other hand, 3-fold increase in ANG2 expression levels accompanied with the significant downregulation of ANG1 expression in response to 5-FU. Recent hypothesis suggests that ANG1/ANG2 ratio is a more critical parameter than absolute levels of individual proteins for controlling vascular permeability35. Thus, our data highlighted the possibility that dramatically decreased ANG1/ANG2 ratio of 0.022 ± 0.003 after 5-FU treatment compared to 15.2 ± 1.3 in physiological conditions may be the mechanism responsible of discontinuous adherens junctions in response to 5-FU (Figure 3D). After the combination treatment with Resveratrol, ANG1/ANG2 ratio increased to 0.041 ± 0.01, which was significantly higher than that in 5-FU treatment, whereas it still remained lower than the levels in physiological control. Although we suggest that decreased ANG1/ANG2 ratio can be a potential mediator of the hyperpermeability effect of 5-FU, the mechanism behind the recovering effect of Resveratrol still remains unknown. One potential mediator of this may be myosin light chain phosphorylation which was shown to be both responsible for hyperpermeability of ECs36 and inhibited by Resveratrol by several studies37–40. Interestingly, Lin et al41 also showed that overexpression of KLF-2 inhibits EC hyperpermeability through MLC phosphorylation. The profound upregulation of KLF-2 after Resveratrol treatment (see Figure 3D) also agrees with this hypothesis. Additional studies, however, are required to elucidate the precise mechanism underlying the effects of these drugs on vascular permeability.

**Simulation of atherosclerosis-driven ischemia leads to human EC activation.** Atherosclerosis-associated occlusion of arteries leads to myocardial or cerebral ischemia, which may cause lethal damage to heart and brain tissues. Angiogenesis, the formation of blood vessels from pre-existing ones, occurs in ischemic tissues where the surrounding blood vessels experience a simultaneous decrease in shear stress and dissolved O2 levels42. Here, we utilized the μVM to determine the dynamics of morphological and molecular changes in human ECs by recapitulating abnormal shear stress and O2 levels found in ischemic tissues.

Following achievement of in vivo EC phenotype after 48 hours in physiological conditions, we simulated ischemia by simultaneously lowering the shear stress and O2 levels to 0.01 dyn/cm² and 1% O2, respectively. We found that after 24 hours in ischemia-mimetic conditions, VECA D distribution along the EC borders was significantly lower compared to its distribution along EC borders cultured in physiological conditions, whereas the localization of PECAM1 was not affected (Figure 4A (a)). Moreover, compared to physiological conditions (from Figure 2B (c)), we observed a decrease in the elongated morphology of ECs in response to ischemic conditions (eccentricity = 0.83 ± 0.023; Figure 4A (bi)). In fact, we observed two distinct cell populations in response to 24 hours of ischemia-mimetic conditions in terms of morphology, F-actin organization and cell-cell contact. In one population, the ECs were closely in contact with neighboring cells and occupied relatively small surface area similar to the morphology typically observed in confluent ECs in conventional cultures (from Figure 2B (a)). F-actin was randomly distributed in the cytoplasm as opposed to the peripheral F-actin distribution observed in conventional conditions (Figure 4A (bii)). In the second distinct population, the cells retained their elongated morphology; however, thick stress fibers were formed across the cytoplasm and oriented in the flow direction (Figure 4A (biii)). The cell-cell contact occurred through membrane extensions leading to a significant increase in the average number of intercellular gaps, compared to physiological conditions, indicative of a disrupted barrier integrity (Figure 4A (bii) and 4A (c)). Hypoxia (1%O2) in conventional cultures in petri-dish did not induce intercellular gap formation, and resulted in a similar morphology and cell-cell contact to the first population we observed with μVM (Supplementary Figure 6), further suggesting that the μVM is superior to conventional cultures to more accurately mimic in vivo cellular responses.

We examined the expression levels of KL2, eNOS and several angiogenic genes, which are controlled by shear stress and/or O2 tension42–44. Kruppel like factor-2 is a transcription factor, which is highly expressed in physiological phenotype of ECs and regulates the expression of a wide variety of genes including eNOS and angiogenic factors such as VEGF and VEGFR244. Indeed, KL2 and eNOS
expression were significantly downregulated in ECs after 24 hours in ischemia-mimetic conditions, while the expression of VEGF and ANG2 were found to be upregulated and the expression of ANG1 remained unchanged. The role of ANG2 in destabilization of quiescent ECs has been shown previously.45 Thus, upregulation of ANG2 in ischemia is consistent with the disrupted barrier integrity.

Interestingly, VEGFR2 expression levels were lowered by ischemia-mimetic conditions, compared to physiological conditions, reaching expression levels similar to the levels found in ECs cultured in conventional conditions (Figure 4B (a)). Twenty-four hours in ischemia-mimetic conditions also induced transition from quiescent to proliferative state of the ECs as indicated by increased percentage of Ki67-positive cells (Figure 4B (b)). Together these data demonstrate the growing ischemia-driven transition of ECs to an activated state where the endothelial barrier integrity is impaired, cell proliferation is increased, and angiogenic factors, such as VEGF and ANG2, are upregulated.

Discussion
We have established an in vitro µVM to study human vascular diseases such as atherosclerosis-driven ischemia as well as to accurately test the efficacy and toxicity of therapeutics. The µVM efficiently recapitulates physiological and pathological vascular microenvironments via simultaneous control of shear stress and O2 levels.

Although other microfluidic systems have been successfully developed to mimic physiological shear stress on ECs,46–47, the µVM in this study is the first one, to our knowledge, that permits the simultaneous manipulation of shear stress and O2 tension to produce physiological and pathophysiological EC phenotypes. In the body, ECs experience sub-atmospheric O2 levels (21%), ranging between 5–12%. Thus, in this study, we used a physiologically relevant O2 tension of 5% and achieved a healthy human µVM with a quiescent, anti-apoptotic and athero-protective endothelial phenotype. However, we did not see significant differences between ECs cultured at physiological and high shear conditions in terms of morphology and mRNA levels (data not shown). Previous studies demonstrated the varying EC responses when cultured at physiological O2 tensions11–12. A few previous studies also suggested synergistic effects of physiological shear stress and O2 levels on ECs by pre-treating ECs with low O2 prior to onset of shear stress48 or using a parallel-plate flow system with preconditioned cell culture media at desired O2 tension49. Although physiological O2 tension did not influence the transition from the in vitro to in vivo phenotype in our study, it may be specifically critical especially when studying diseased conditions that involve O2 fluctuations, and therefore influence the dynamics of O2 species generation and nitric oxide release50.

We validated the physiological proximity of the µVM to in vivo, also in terms of its responses to a broadly studied vasotoxic
chemotherapeutic drug, 5-FU, (20) and to a vasoprotective agent Resveratrol37. The anticancer drug, 5-FU, is a well-documented symptomatic cardiotoxin in patients. A very recent systematic review reported that symptoms of cardiotoxicity were detected in up to 20% of patients receiving 5-FU34. Yet, the mechanisms underlying the pathophysiology are not well understood. Although the majority of the clinical studies focuses on toxicity of 5-FU on cardiac tissue, some studies also highlighted that 5-FU may cause vascular injury by leading to a procoagulant state35, vasoconstrictions36, and even EC apoptosis44 at relatively high doses. These studies provided clinical evidence to vasotoxicity of 5-FU and proposed vascular dysfunction as a possible mechanism behind the symptomatic cardiotoxicity. We first examined immediate pro-apoptotic role of 5-FU on ECs using the \( \mu \text{VM} \). We observed no indication of apoptosis after 24 hours of treatment. While some studies detected apoptosis of cardiocytes35 and central nervous system progenitors32 at similar concentrations of 5-FU, Wada et al.36 showed that 5-FU is anti-proliferative but not pro-apoptotic on HUVECs, in agreement with our data. Another study using a rat model of intra-arterial perfusion37 showed EC apoptosis in response to intra-arterial perfusion of 5-FU. This response, however, was observed after 7 days and only near to the tip of the catheter where the ECs are exposed to much higher concentrations of 5-FU compared to complete dilution in blood.

Previous reports suggested that the vasoconstriction caused by 5-FU is endothelial- independent38 and stimulation of eNOS by exercise training can potentially reverse this adverse effect39. Thus, we examined mRNA levels of eNOS and its transcription factor KLF2 and did not detect a significant effect of 5-FU on these genes compared to physiological levels. We subsequently confirmed unaffected levels of eNOS in a mouse model suggesting that vasoconstriction is not through eNOS expression, supporting the previous evidence of endothelial-independent vasoconstriction. However, changes in the activity of eNOS and nitric oxide production and release should be further investigated.

The capability of performing high-resolution image analyses in the \( \mu \text{VM} \) system allow us to examine morphological and structural changes in response to 5-FU. We found that 5-FU induces intercellular gap formation indicating compromised endothelial barrier integrity. We also confirmed the toxic effect of 5-FU in a mouse model by showing extensive plasma leakage and accumulation in lungs. To our knowledge, this is the first study suggesting the hyperpermeability effect of 5-FU. Vascular hyperpermeability caused by 5-FU treatment may result in inflammation and contribute to complications of acute respiratory distress syndrome42 and ischemia/reperfusion injury46. In addition, induction of leaky blood vessels in tumors can limit targeted delivery of the drug and lead to cancer metastasis49. As a possible solution, we showed that 5-FU-induced hyperpermeability can be alleviated by combined treatment with Resveratrol both in the \( \mu \text{VM} \) system and in a mouse model. Resveratrol was previously shown to reduce high glucose41, tumor necrosis factor-alpha46 and staphylococcal enterotoxin B-induced vascular hyperpermeability in vitro and in vivo, supporting the vasoprotective role of Resveratrol observed in this study. Interestingly, a recent report demonstrated that combined 5-FU treatment with Resveratrol decreases tumor size to a greater extent than 5-FU only48. While the authors attributed this effect to enhanced chemosensitivity of cancer cells by Resveratrol based on their in vitro experiments, it may be that the alleviation of vascular hyperpermeability by Resveratrol is a secondary possible mechanism underlying the enhanced response to the chemotherapy. We thus propose adjunct therapy for 5-FU by the co-administration of Resveratrol as a vasoprotecting strategy.

The capability of the \( \mu \text{VM} \) to manipulate shear stress and \( O_2 \) tension independently allowed us to mimic pathological conditions found in the ischemic tissue. Several in vivo studies demonstrated that two different types of neovascularization occur at the occlusion site and post-occlusive ischemic tissue40,42. It was suggested that new capillary formation is only observed in the ischemic tissue while collateral formation is found in the site of occlusion. These findings support the idea that the perturbation of shear stress and \( O_2 \) may be the driving force for the site-specific angiogenesis. Our simulations of ischemic conditions using the \( \mu \text{VM} \) revealed the morphological, structural and genetic changes leading to EC activation. Proliferation of ECs was detected previously in animal models to occur as early as 24 hr after exposure to low shear stress and \( O_2 \) levels, reaching a maximum number of proliferative ECs on day 39. The proliferative response is usually associated with increased levels of growth factors, such as VEGF, released by the surrounding non-ECs including macrophages44. In our in vitro \( \mu \text{VM} \), we show that ECs switch from quiescent to proliferative state merely as a result of changing physical conditions. These data suggest that initial proliferative response of ECs may be controlled by the lowered shear stress and \( O_2 \) tension in the ischemic tissue while it is accelerated at later stages as a response to released growth factors by surrounding cells.

Moreover, we observed two morphologically distinct populations of cells after 24 hours of ischemia. In vivo studies previously demonstrated the temporal and spatial profile of EC activation during ischemia40,42. In one study, ANG2 overexpression was only detected in individual ECs in the first 6–24 hrs of ischemia while prolonged ischemia of 3 days resulted in overexpression of ANG2 by all ECs42. We found a similar increase in mRNA levels of ANG2 after 24 hrs of low shear stress and \( O_2 \) tension, which is in consistent with the intercellular gap formation we observed. In addition, after 24 hours, we determined a profound decrease in mRNA levels of KLF2, which was shown to inhibit VEGF-mediated42 and hypoxia-induced angiogenesis. Interestingly, eNOS and VEGFR2 levels, in our study, were also found to be downregulated by low shear stress and \( O_2 \) tension. Previous studies, however, demonstrated the predominant positive role of eNOS and VEGFR2 in angiogenesis during tissue repair and ischemia43,44. Thus, our data can be interpreted as follows; eNOS levels are independently downregulated by lowered shear stress and \( O_2 \) tension, while exogenous growth factors, such as VEGF and TGF-\( \beta \), are released in the tissue by surrounding cells in response to hypoxia to promote angiogenesis via upregulation of eNOS40 and VEGFR245 in ECs. On the other hand, individual effects of lowered shear stress and \( O_2 \) tension on angiogenesis have extensively been studied. Some previous studies, for example, reported that lowered shear stress at atmospheric conditions (21% \( O_2 \)), as occurs in lung-ischemia, causes angiogenic responses through reactive oxygen species generation and signaling47. Similarly, hypoxia has been shown to promote new vessel formation46. Although we primarily examined the synergetic effects of shear stress and \( O_2 \), individual contributions of these factors in the observed endothelial responses can further be investigated using the ischemic \( \mu \text{VM} \). In addition, the ischemic \( \mu \text{VM} \) can potentially be used to determine correct dosage and combination of potential therapeutic agents, by testing the efficacy directly on human ECs under relevant physical conditions and by examining typically encountered complications, such as hyperpermeability.

**Methods**

See on-line supplementary for complete Materials and Methods.

**Cell Maintenance in \( \mu \text{VM} \)** All components of the system were autoclaved separately and further sterilized with ethanol for 10 minutes after assembly. The glass surface of the device was coated with Fibronectin (10 \( \mu \text{g/ml} \)) prior to cell seeding. Cell seeding was achieved by injecting a cell suspension with a density of 5 million cells/ml. After 3 hrs of attachment period, growth media was supplied at a flow rate of 0.01 ml/hr overnight using a syringe pump (Chemyx, Stafford, TX). In static control experiments, the flow rate was maintained at 0.01 ml/hr, which is sufficient to provide nutrients and exerts a negligible shear stress of 0.007 dyn/cm2. In high shear and physiological condition experiments, media was circulated at a flow rate of 20 ml/hr between the microbioreactor and a media reservoir using a peristaltic pump (Ismatec, Wertheim, Germany). Physiological \( O_2 \) was generated by continuously flushing both the microbioreactor and the media reservoir with a medical grade gas.
mixture (5% CO\(_2\), 1% O\(_2\), and balance N\(_2\)). Ischemic conditions were created by lowering the shear stress and O\(_2\) tension simultaneously to 0.01 dyn/cm\(^2\) and 1% O\(_2\) (5% CO\(_2\), 5% O\(_2\) and balance N\(_2\)). Drug treatment was performed by injecting 5-FU mixture (5% CO\(_2\), 1% O\(_2\) and balance N\(_2\)). Ischemic conditions were created by lowering the shear stress and O\(_2\) tension simultaneously to 0.01 dyn/cm\(^2\) and 1% O\(_2\) overnight at 60°C.

Mouse Drug Treatment. All experimental animal protocols were approved by the Institutional Animal Care and Use Committee of Johns Hopkins Medical School. 8- to 10-week-old BALB/c mice (Charles River, Wilmington, MA) received three consecutive intravenous injections of 5-FU (60 mg/kg dissolved in 1% DMSO) or 5-FU and Resveratrol (6.5 mg/kg dissolved in 1% DMSO) every other day for mRNA extraction studies and single injection for the other analyses. Control animals were treated with equal amounts of PBS or 1% DMSO in PBS (vehicle control). The drug doses were determined to achieve the same clinically relevant drug concentrations used in the in vitro experiments. The average drug concentration in blood stream was calculated based on the first order clearance rates of 5-FU (16.4 μg ml\(^{-1}\) hr\(^{-1}\)) and Resveratrol (6.84 μg ml\(^{-1}\) hr\(^{-1}\)) from the blood. Animals were then sacrificed for the final injection. Prior to harvesting the tissue of interest for analyses, venous cava was cut and intra-cardiac injection of ice-cold PBS was performed to remove the blood.

Vascular Permeability in Mice. Permeability of mouse microvasculature was analyzed by injecting Evans blue dye (30 mg/kg in 150 μL PBS, Sigma Chemical Co.) intravenously into mice one day after injection of a single dose of 5-FU alone or 5-FU and Resveratrol. Ten minutes after the injection of the dye, mustard oil (Sigma Chemical Co.) diluted to 5% in mineral oil was applied to the surfaces of the ear. The pictures of the ears were taken after 20 minutes. Mice were sacrificed 30 minutes after the injection of the dye. The Evans blue dye was extracted from the intestines, lungs and liver by incubating specimens in 1 mL of Formamide (99.5%, Sigma-Aldrich) overnight at 60°C and measured using spectrometer at 620 nm. The amount of extravasated dye per weight of tissue was calculated for comparison between samples. Water accumulation in different organs was examined by measuring wet-to-dry weight ratio. The wet weight of lung, intestines and liver were measured immediately after removal of the organs. The dry weight of the organs was measured after drying overnight at 50°C after evaporation of the water in the organs at 60°C for 48 hours.

Concanaval A binding was used to assess the endothelial barrier function of descrent aorta in a similar manner shown by others\(^6\). The aorta segments were first fixed in 3.7% paraformaldehyde, cut longitudinally, and mounted as flat on a glass slide with the lumen side of the vessels facing up. The sample was then blocked by 5% BSA solution for 1 hour and 10 μL drop of CON A solution was added only on the luminal side. After an hour of incubation at room temperature, CON A localization was examined by taking Z-stack images using confocal microscope (LSM 510 Meta; Carl Zeiss).

**Ethics Statement.** All experimental animal protocols were conducted in accordance with National Institutes of Health Guide for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee at Johns Hopkins Medical School.

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