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Anastomosis of endothelial sprouts forms new vessels in a tissue analogue of angiogenesis†

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Abstract

Here we describe a microfluidic device that accurately reproduces the dynamics of vascular anastomosis, the process by which vascular sprouts connect to achieve perfusion during angiogenesis. The micro-device features two parallel endothelial cell-lined vessel analogues separated by a 300 μm wide collagenous matrix into which the vessels can sprout and form perfused bridging connections. By accurately recapitulating anastomosis *in vitro*, the device will enable a new generation of studies of the mechanisms of angiogenesis and provide a novel and practical platform for drug screening.

Introduction

Blood flow is necessary for the survival of all tissue. Intense research has focused on understanding how blood vessels are recruited to avascular tissue either from pre-existing vessels (angiogenesis) or during *de novo* synthesis *via* precursor differentiation (vasculogenesis).^{1–4} Pro-angiogenic signals activate endothelial cells (ECs), which extend from the vessel wall into the surrounding matrix.^{5,6} Yet, to produce an extended, contiguous lumen for new blood flow, sprouts must connect in a process known as anastomosis.^{7–9}

Thus, to be representative of *in vivo* angiogenesis and provide interpretable data, an *in vitro* model should not only reproduce endothelial sprouts, but also their transition into newly-perfused vessel segments *via* anastomosis. Conventional *in vitro* systems for studying angiogenesis, such as the “tube formation” or microbead sprouting assays which measure network formation or endothelial sprout formation by matrix-embedded (ECs), are typically performed in static cultures where there is no pre-existing, perfused vessel from which to sprout, nor a perfused vessel toward which to sprout. Since the endothelial cells are surrounded by matrix components and not associated with a vessel lumen, the morphogenesis observed in these assays may be more related to vasculogenesis than angiogenesis.¹⁰ Furthermore, the newly formed lumens cannot be perfused,¹¹ and lacking this critical signal for vascular maintenance, they generally regress after 48 hours.¹² Consequently, results from these models are difficult to interpret in the context of *in vivo* angiogenesis.

†Electronic supplementary information (ESI) available: Endothelial sprout formation at 1.5 *versus* 3 mg ml^{-1} collagen, lateral anastomosis between adjacent HUVEC sprouts, and videos of demonstrating perfused bridging vessels *in vitro*. See DOI: 10.1039/c2ib20061a

Previously, we used a microfluidic system to describe how mechanical fluid forces and chemical factors co-direct sprouting and morphogenesis during the initiation of angiogenesis.⁶ Here we use a similar microfluidic approach to reproduce the dynamics of anastomosis. Angiogenic sprouts are induced from two parallel, pre-designated vessel analogues by treatment with vascular endothelial growth factor (VEGF), a potent pro-angiogenic growth factor.^{13–16} The sprouts migrate into the central 3-D extracellular matrix (ECM) separating the two vessels to form new bridging segments. After anastomosis, tracer particles freely pass through the new connecting segments. The bridging vessels extend between the two pre-existing vessels to span the 300 μm distance. Furthermore, by controlling the flow field within the device, we found that anastomosis is more efficient with interstitial flow than under static conditions. This device produces a fully-perfusable vessel network and has numerous potential applications for engineering vascularised tissues, for studying vessel development in solid tumours and wounds, and for screening for drugs that modulate angiogenesis.

Experimental

Microfluidic device fabrication

The microfluidic device (Fig. 1a) was comprised of two layers of poly(dimethylsiloxane) PDMS (Sylgard 184, Dow Corning). The top layer containing the monolithic channel features (50 μm in height) was fabricated using soft lithography¹⁷ and the bottom, featureless layer provided a planar substrate. The two layers were sealed irreversibly using plasma oxidation for 60 s (Harrick). At least 2 hours after plasma oxidation (to enable hydrophobic recovery of PDMS^{18,19}), a mixture of collagen gel (3 mg ml⁻¹, Type I, rat tail, pH = 7.4) and fibronectin (10 μg ml⁻¹; both from BD Biosciences) was introduced into the central channel (Fig. 1b) and aspirated through using vacuum at 4 °C to prevent rapid polymerization.

HUVEC preparation and seeding

Human umbilical vein endothelial cells (HUVECs) were acquired from the Center for Excellence in Vascular Biology, Brigham & Women's Hospital, Harvard Medical School, Boston, MA and maintained in EGM medium (2% FBS, brain bovine extract, heparin, hEGF, and hydrocortisone) (Lonza). The HUVECs were stably transfected with either green fluorescent protein (GFP) or dsRed as previously described.⁷

Following collagen gel polymerization and prior to cell seeding, the HUVEC channels in the microfluidic device were coated with fibronectin (10 μg ml⁻¹) for 3 h. Subsequently, a concentrated solution ($\sim 10^7$ cells ml⁻¹) of HUVECs at passage number 5–10, was introduced into the entire length of both HUVEC channels. The HUVECs attach to the channel walls and then spread and migrate to cover all surfaces of the channels, including the collagen exposed in the multiple perpendicular apertures that are 100 μm wide and spaced 500 μm apart center-to-center (Fig. 1b and c); it is at these interfaces that sprouting can occur. The HUVECs were grown to confluence (24–48 h after seeding) (Fig. 2a) with passive pumping²⁰ of EGM medium. The system recreates two quiescent blood vessels separated by 300 μm with multiple regions for potential endothelial sprouting.

VEGF treatment and anti-VEGF neutralization

To stimulate sprouting of HUVECs into the 3-D ECM, we treated the cells with recombinant human vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, MN). VEGF was added to EGM media at concentrations of 0, 5 or 50 ng ml⁻¹ and then introduced into the HUVEC channels by gravity flow and re-fed daily. To neutralize VEGF activity, we

used bevacizumab (Avastin[®], Genentech), an antibody that specifically neutralizes human VEGF.²¹

Immunofluorescence

HUVECs in the microchannels were initially washed with Phosphate Buffered Saline (PBS) to remove any cell media, fixed with 3% formaldehyde for 30 min, washed 3× with PBS and subsequently blocked with 5% donkey serum/0.1% Triton in PBS for 60 min. To stain for CD31 (PECAM-1), a homotypic adhesion molecule contributing to EC junction integrity,²² cells were incubated with a purified mouse anti-human CD31 monoclonal antibody (Dako) at 1 : 50 dilution for 90 min. The same procedure was used to detect type IV collagen, using a goat anti-human primary antibody (Millipore; 1 : 100 dilution). For CD31 and type IV collagen, after 3× washing with saline, the appropriate fluorescently-labelled secondary antibodies were applied for 60 min and washed three times with saline prior to confocal microscopy. Actin filaments were labelled with phalloidin conjugated with Alexa Fluor 488 (Molecular Probes). Cell nuclei were stained with DAPI nuclear stain (Invitrogen, 1 : 200 dilution).

Control of fluid flow

Flow of medium was controlled with a programmable syringe pump (Harvard Apparatus) with negative (pull) pressure flow capabilities. Before initiation of flow-based experiments, clear polypropylene barbed elbow fittings (1/16 inch, Cole-Parmer) connected to silicone tubing (Saint-Gobain) were inserted into the 1.5 mm diameter inlet/outlet ports of one of the HUVEC channels. The ports of HUVEC channels not subjected luminal flow were connected to luer adapters (Cole-Parmer) which served as fluid reservoirs. In this configuration, a pressure drop is induced across the central collagen gel, causing interstitial flow (transverse convection). The shear stress levels and interstitial flow velocity were quantified as previously described.⁶

Image acquisition and statistical methods

Phase and corresponding fluorescent images were acquired with an epi-fluorescence microscope (Olympus IX70, PRIOR automated stage, OpenLab software). Multiple-field mosaic images were stitched together with Adobe Photoshop. The number of bridging connections was measured daily for three days by counting the number of connections formed by opposing sprouts within the same aperture (Fig. S1, ESI[†]) and between adjacent apertures (Fig. S2, ESI[†]). Confocal fluorescence images were acquired with an Olympus BX61WI microscope and 20× water immersion lens (Fluoview software) with 1 µm slice thickness. Projections of confocal images were produced using Volocity software (PerkinElmer, Waltham, MA).

Statistical methods

Two or more sample populations were compared using two-way ANOVA over a course of multiple days. For two-way ANOVA, the column factor was day after initiation of experiment and the row factor was VEGF treatment. $p < 0.0001$ was the threshold for statistical significance (indicated on graphs with a “****”). Data points on the graphs represent mean values and error bars depict standard error (s.e.m.).

Results and discussion

Collagen gel localization for reproducible vessel anastomosis

The collagen concentration in the gel is an important parameter for device preparation and performance: 3 mg ml⁻¹ is sufficiently low to allow sprout extensions into the gel^{23,24} (Fig. S3, ESI†), but dense enough to inhibit sprouting under standard media conditions (Fig. 2a).

The task of filling the central channel with collagenous gel is facilitated by the rheological properties of the viscous collagen–fibronectin solution (henceforth referred to simply as “collagen”);. Capillary action and surface tension in the micron-scale channels^{25–28} help to propagate the solution within the central channel but prevent its leakage into the adjacent channels, which need to be lined with ECs post-collagen polymerization (Fig. 1c). To further enhance surface tension-mediated confinement of the collagen solution to the central channel, we angled the sidewalls of the collagen gel apertures inward by ~18° (Fig. 1d).²⁶ In addition, polymerizing the collagen gel against an air interface consistently produces a clean and slightly convex surface for HUVEC seeding (Fig. 2a).

HUVECs growing on these surfaces form monolayers with characteristic CD31 patterns at intercellular junctions²⁹ whereas CD31 outlines the lumen of sprouting HUVECs entering the 3-D collagen gel^{30,31} after ~3 days of treatment with 50 ng ml⁻¹ of VEGF (Fig. 2b). Thus, the HUVECs that migrate and sprout into the 3-D matrix adopt morphologies distinct from the cells that remain as a 2-D monolayer in the main vessel (Fig. 2b).

Previous experiments performed in PDMS microfluidic channels sealed against a glass substrate have shown that invading ECs preferentially migrate on glass instead of into the 3-D ECM space²⁸ and that this can be avoided by pre-treating the glass surface with positively-charged chemicals, supposedly by restricting collagen gel contraction.³² Our endothelial sprouts consistently invaded into the bulk of the gel rather than along the top or bottom surfaces, as verified by confocal z-scans (Fig. 2b). This may be due to the extended period of collagen polymerization (48–72 h) which potentially reduces contraction. Also, the top and bottom surfaces of our device consist of PDMS which after more than 2 h following plasma oxidation has undergone hydrophobic recovery.^{18,19} In this state, PDMS exhibits reduced cell adhesion³³ and protein adsorption³⁴ compared to untreated glass. Furthermore, previous reports of sprouting in microfabricated systems have utilized collagen gel gaps greater than 100 µm in width.^{6,28,35} We observe sprouting into the bulk of the collagen gel with apertures as small as 50 µm wide (Fig. S4, ESI†). Thus, there are no restrictions in sprouting into the collagen gel down to this width.

Anastomosis leads to perfusable vessel formation *in vitro*

VEGF stimulation (50 ng ml⁻¹, 4 days) induced the formation of actin-rich protrusions, which initiated anastomosis with the monolayer of HUVECs at the adjacent aperture (Fig. 3a). Although CD31 was localized mainly in the cytoplasm in extending tip cells, eventually, CD31-positive intercellular junctions were established at the point of anastomosis (Fig. 3b). Interestingly, these cell extensions that appeared to be mediating the connections often lacked collagen IV-positive basement membrane (Fig. 3c). This is in contrast to the trailing “stalk” of the vessel structure which developed a patent lumen, CD31-positive junctions, and a sheath of basement membrane surrounding the lumen (Fig. 3d–f). Thus, although basement membrane production and maintenance are shared by endothelial cells and pericytes *in vivo*, these observations show that endothelial cells, by themselves, can produce an evolving vascular basement membrane.

Using time-lapse imaging, we followed the dynamics of anastomosis in the device. After adding 50 ng ml⁻¹ of VEGF to both HUVEC channels and stopping flow to allow invasion

from both sides,⁶ processes extend and connect to form patent lumens at least 300 μm in length (Fig. 4). As with inflamed vessels or angiogenic tumour vessels,^{36,37} continued exposure to VEGF caused our bridging vessels to dilate (Fig. 5c). At the time of anastomosis (day 3), the bridging vessels initially formed with $\sim 20\text{ }\mu\text{m}$ diameters but then dilated to achieve nominal diameters of $\sim 50\text{ }\mu\text{m}$ by day 9 (Fig. 5). The newly formed bridging vessels had open, perfused lumens that allowed passage of $1\text{ }\mu\text{m}$ diameter fluorescent beads (Movie S1, ESI†). With bright-field microscopy, particles could be seen traversing the entire length of the newly formed bridging vessels and entering the downstream pre-existing vessel (Fig. 5d and Movie S2, ESI†).

Sensitivity of anastomosis to anti-angiogenesis treatment

To demonstrate the utility of the device in detecting anti-angiogenic agents, we applied bevacizumab (anti-VEGF antibody) to the medium. Without bevacizumab, the number of anastomosis events per collagen gel aperture increased with increasing VEGF concentration (Fig. 6 and Fig. S5, ESI†, $p < 0.0001$). Addition of 500 ng ml^{-1} of bevacizumab³⁸ with 50 ng ml^{-1} of VEGF decreased the number of anastomosis events dramatically ($p < 0.0001$), similar to levels seen when no VEGF was exogenously added ($p = 0.16$).

Inter-vascular flow facilitates formation of microvascular networks

We demonstrated in previous work that: (i) physiological levels of intraluminal fluid shear stress (3 dyn cm^{-2}) attenuates VEGF-induced endothelial sprouting and invasion into 3-D ECM and (ii) interstitial or convective flow³⁹ through the 3-D ECM enhances the rate of endothelial invasion.⁶ To test whether inter-vessel flow affects the frequency of anastomosis, we used a configuration that enables simultaneous application of physiological shear stress (3 dyn cm^{-2}) and interstitial flow ($35\text{ }\mu\text{m s}^{-1}$). To produce this condition, we administered negative pressure shear flow in the bottom channel, which resulted in transverse convection of VEGF-containing media (5 ng ml^{-1}) from fluid reservoirs connected to the top channel, through the collagen matrix and into the bottom channel. Furthermore, the flow field within the gel maintained a VEGF gradient extending from the top channel (the VEGF source).

We observed multiple, parallel anastomosis events between the two pre-existing vessels (Fig. 7), as well as connections *between* the new bridging segments, forming a more complicated vessel network (Fig. S2, ESI†). HUVEC-GFP cells lining the static top channel invaded towards the HUVEC-dsRed cells lining the bottom channel, which was subjected to shear stress. The HUVEC-GFP cells invaded away from the VEGF source and with the direction of interstitial flow (Fig. 7). In this configuration, anastomosis was much more rapid than under static and uniform VEGF conditions (Fig. 3).

To isolate the role of inter-vascular flow in the absence of a VEGF gradient, we reconfigured the device such that the HUVEC channels were exposed to interstitial flow only ($28.5\text{ }\mu\text{m s}^{-1}$) and a uniform VEGF concentration.⁶ We compared the number of VEGF-induced (50 ng ml^{-1}) anastomosis events under static conditions *versus* interstitial flow conditions. Interstitial flow significantly enhanced the number of anastomosis events (Fig. 8, $p < 0.0001$).

Conclusions

Many physiological and pathological processes such as wound healing and tumour growth rely on the formation of new vessels by connection of vessel sprouts, yet vascular biologists currently lack the appropriate technology for studying this process *in vitro*. The available

and convenient tools are only conducive for assessing sprouting and vasculogenesis^{10,40,41} but not anastomosis.

Our microfluidic-based system provides the conditions necessary for anastomosis: extension of endothelial sprouts away from an existing vessel into a 3D matrix, and connection of this sprout with another endothelial-lined structure to form a new circuit for flow. Perfusable lumens, which are not produced in previous static assays for studying angiogenesis, evolve naturally in our system in a process of coordinated migration and self-assembly. This versatile platform can provide valuable insight into the roles of various mediators of vessel maturation⁴² and anastomosis such as perivascular cells, basement membrane components, chemical signals, or fluid flow.^{6,43} Its simple design is easily adaptable, allowing the inclusion of other cell populations (*e.g.*, fibroblasts or immune cells) in the collagen gel or the addition of exogenous growth factors. Furthermore, the technology has great potential as a screening platform for agents that enhance or interfere with anastomosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Insight, innovation, integration

Using a microfluidic device that reproduces connection and perfusion of vessel sprouts, we recapitulate the dynamics of vascular anastomosis leading to a patent vessel network *in vitro*. Furthermore, we demonstrate that convective flow through the 3-D ECM enhances the rate of VEGF-induced anastomosis compared to static conditions. These findings have numerous potential applications for screening of anti-angiogenic agents, engineering vascularized tissues, and for understanding physiological situations where neovascularization is necessary for nourishing tissue such as in wound healing, tumor growth, and organ development. Furthermore, with recent emphasis on studying endothelial cells in micro-scale channels and in 3-D matrices, our micro-device represents a necessary technological evolution, improving upon previous methods that study endothelial cells either in static cultures or on the 2D substrate of flow chambers.

Fig. 1.

PDMS microfluidic device for biomimetic vessel formation. (a) Photograph of the microfluidic device. The presence of the polymerized collagen gel between the HUVEC channels prevents immediate mixing of the two coloured streams. (b) Schematic of boxed region in (a) depicting multiple apertures in the PDMS channel wall (white) that allow contact between the HUVECs and the collagen gel. The indicated dimensions are in microns (μm). (c) Collagen gel embedded with $1\ \mu\text{m}$ diameter fluorescent blue beads between channels lined with HUVEC-GFP cells. (d) Close-up view of a single aperture embedded with $1\ \mu\text{m}$ fluorescent beads. The slight inward angle of the aperture ($\theta \sim 18^\circ$) facilitates retention of collagen gel within the central channel. Dashed lines indicate interface with

PDMS channel wall. Images were recorded with epi-fluorescence microscopy (Olympus IX70, PRIOR automated stage, OpenLab software). Scale bars are 100 μm .

Fig. 2.

Vessel stability at baseline, and morphogenesis induced by VEGF. (a) HUVECs adjacent to polymerized 3-D collagen gel under standard media conditions remain quiescent for at least 4 days without supplemental VEGF. (b) CD31 expression (red) demonstrates integrity of intercellular junctions of HUVECs lining the 2-D microchannels. Stimulation with 50 ng ml⁻¹ VEGF causes the HUVECs to abandon the pre-existing vessel wall and sprout into the 3-D matrix. Dashed lines denote boundary with PDMS channel wall. Image was recorded after 1 day of VEGF treatment. Scale bars are 50 μm.

Fig. 3.

Cytoskeleton, intercellular junction, and basement membrane assembly during the initiation of anastomosis. (a–c) VEGF stimulation (50 ng ml^{-1}) induces HUVECs to form a new vessel structure whose leading edge initiates a connection with the monolayer at the adjacent aperture. The vessel structure was stained for: (a) Actin filaments and nuclei with phalloidin and DAPI respectively, (b) CD31, and (c) type IV collagen (Col IV). Insets in (a–c) are close-up views of the dashed box region. Arrows indicate cellular connections between the tip of the vessel structure and the adjacent monolayer. (d–e) Cross-sectional images demonstrating a patent lumen along the vessel structure. Numbers correspond to dashed lines in (a–c). Scale bars are $50 \text{ }\mu\text{m}$.

Fig. 4. Dynamics of anastomosis. HUVEC-dsRed cells stimulated with VEGF (50 ng ml^{-1}) sprout into the central matrix and connect to form a patent vessel. Images were recorded using epi-fluorescence microscopy. Scale bar is $100 \mu\text{m}$.

Fig. 5. Formation of a new functional microvessel by anastomosis *in vitro*. Single slice confocal images of a patent lumen in the (a) x - y and (b) x - z focal plane. The green line in (a) indicates the plane of view in (b). (c) Z-projection of the resulting formed vessel. Images (a–c) recorded 11 days after VEGF treatment. (d) Still captures from (Movie S2, ESI†) demonstrating particle advection through the lumen. Scale bars are 50 μ m.

Fig. 6. Formation of bridging segments between the HUVEC channels in the 3-D collagen gel in response to various VEGF and Bev (bevacizumab) concentrations. Numbers depict concentrations in ng ml⁻¹. Sample populations were compared using two-way ANOVA. Statistical outcome indicated for treatment condition. $n = 21-42$ per condition per day. Data points represent mean + s.e.m. *: $p = 0.044$, ***: $p < 0.0001$.

Fig. 7.

Towards functional microvascular networks *in vitro*. Negative pressure shear flow (3 dyn cm^{-2}) in the bottom channel results in interstitial flow at a rate of $35 \mu\text{m s}^{-1}$ and a VEGF gradient from the top channel to the bottom channel. The HUVEC-GFP cells lining the top channel invade towards the HUVEC-dsRed lined bottom channel resulting in the formation of multiple bridging segments. Solid arrow indicated direction of axial shear flow; dashed arrow indicates direction of interstitial flow. Blue gradient bar indicates VEGF gradient from the top channel to the bottom channel. Scale bar is $100 \mu\text{m}$.

Fig. 8. Interstitial flow enhances anastomosis. Quantification of bridging segments under uniform VEGF stimulation (50 ng ml^{-1}) and static *versus* interstitial flow conditions ($28.5 \mu\text{m s}^{-1}$). $n = 35\text{--}49$ per condition per day. Data points represent mean + s.e.m. ***: $p < 0.0001$.