RAIN-Droplet: A Novel 3-D in vitro Angiogenesis Model

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Abstract

Angiogenesis is fundamentally required for the initialization, development and metastatic spread of cancer. A rapidly expanding number of new experimental, chemical modulators of endothelial cell function have been described for the therapeutic inhibition of angiogenesis in cancer. Despite this expansion there has been very limited parallel growth of *in vitro* angiogenesis models or experimental tools. Here we present the Responsive Angiogenic Implanted Network (RAIN)-Droplet model and novel angiogenesis assay using an endothelial cell culture model of microvascular endothelial cells encapsulated in a spontaneously self-assembling, toroidal hydrogel droplet uniquely yielding discrete, pre-formed, angiogenic networks that may be embedded in 3-D matrices. On embedding, radial growth of capillary-like sprouts and cell invasion was observed. The sprouts formed as both outgrowths from endothelial cells on the surface of the droplets but also, uniquely, from the pre-formed network structures within the droplet. We demonstrate proof-of-principle for the utility of the model showing significant inhibition of sprout formation ($p<0.001$) in the presence of bevacizumab, an anti-angiogenic antibody. Using the RAIN-Droplet assay we also demonstrate a novel dose dependent pro-angiogenic function for the characteristically *anti-angiogenic* multi-kinase inhibitor sorafenib. Exposure of endothelial cells in 3-D culture to low, non-lethal doses (<1 μM) of sorafenib after initiation of sprouting resulted in the formation of significantly ($p<0.05$) more endothelial sprouts compared to controls over a 48-hour period. Higher doses of sorafenib (5 μM) resulted in a significant ($p<0.05$) reduction of sprouting over the same time period. The RAIN-Droplet model is a highly versatile and simply constructed 3-D focal sprouting approach well suited for the study of vascular morphogenesis and for preclinical testing of drugs. Furthermore, the RAIN-Droplet model has facilitated the discovery of a novel pro-angiogenic capacity for sorafenib which may impact the clinical application and dosing regimen of that drug.

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Keywords

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Uncontrolled and persistent angiogenesis is the hallmark of several diseases including cancer, autoimmune diseases, age related macular degeneration and atherosclerosis. Angiogenesis is the mechanism by which new vasculature forms from a pre-existing vascular bed. It requires cell migration through extra-cellular matrix, cell proliferation, individual cell differentiation and cell-cell interaction to form multi-cellular structures generally in response to secreted mediators [1]. A broad range of fields now focus on angiogenesis as a discrete primary or controlling mechanism however functional cellular studies have been limited to a few established *in vitro* models. These may be conveniently divided into models of single cellular functions and models of multiple cellular functions able to differentiate responses of endothelial cells to varying growth conditions or exogenous mediators [2, 3]. Single function models are generally based on chemotaxis or proliferation of endothelial cells on 2 dimensional (2-D) surfaces whilst multiple function models generally combine chemotaxis with changes in cell differentiation, morphology, polarization or cell to cell interaction together combined in the formation of angiogenic capillary-like sprouting structures [3]. The latter models are based on culture of isolated endothelial cells, defined co-cultures or *ex vivo* tissue in a supportive physiological 3-D matrix such as collagen. Cells *in vivo* are surrounded by similar extracellular protein matrices so *in vitro* culture in a 3-D matrix is likely a better reflection of normal physiological growth conditions, particularly when primary cells are studied [2, 4].

In addition to workable models for study of angiogenic mechanisms, successful anti-angiogenic drug development is dependent on the ability to assay the cellular effects of current drugs and functionally screen for future efficacious compounds. By 2007 there were at least ten anti-angiogenic drugs approved for clinical use by the FDA and related international organizations [5]. Beside these specific anti-angiogenic drugs, forty other drugs in clinical trial by 2007 also possessed some anti-angiogenic capacity [5]. Due possibly to complexity of construction, lack of flexibility or requirement for specialized equipment 3-D angiogenesis models are rarely used as first line assays in the literature, instead several 2-D models predominate. Indeed despite often elegantly reflecting angiogenic cellular morphogenesis no 3-D *in vitro* model seems to have challenged the 2-D matrigel model of tube-formation, the mono-layer scratch-test assay or the modified Boyden Chamber/transwell assay for initial assessment of angiogenic events.

Here we describe a novel 3-D angiogenesis assay, the Responsive Angiogenic Implanted Network (RAIN) Droplet assay, using an original angiogenesis model combining the flexibility, utility and ease of construction of current 2-D assays, the physiological relevance of 3-D cell growth and further creating a platform for a planar, radial, focal point based assay protocol for quantification of endothelial sprouting. Uniquely, the RAIN-Droplet models angiogenic outgrowth of human dermal microvascular endothelial cells (HDMEC) from a defined structure bearing a pre-activated population of both single endothelial cells

and multi-cellular structures. We show utility of the model in solid proof–of–principle assays of angiogenesis inhibitors in current clinical use. Finally, we use the model to uncover a novel facet of pro-angiogenic activity from the normatively anti-angiogenic multi-kinase inhibitor sorafenib.

Materials and Methods

Cell Culture

Human Dermal Microvascular Endothelial Cells (HDMEC) were obtained from Lonza and were maintained in EGM2-MV (Lonza, Walkersville, USA). Lonza verify isolated endothelial cells by morphology and testing positive for acetylated Low Density Lipoprotein, positive for Factor VIII and negative for alpha smooth muscle actin. UM-SCC-22A cells (gift from T. Carey, University of Michigan, Ann Arbor, MI) were verified by genotyping and maintained in DMEM GlutaMAX supplemented with 10% fetal bovine serum, and 1 mg/ml penicillin/streptomycin, (Invitrogen Life Technologies, Carlsbad, CA, USA).

Cell Preparation in Puramatrix

Puramatrix (BD Biosciences) cell suspension was made as directed by the manufacturer. Briefly, HDMEC were trypsinized and washed once in 10% sucrose solution at 4°C to remove culture medium. All further manipulations were carried out on ice. Cells were counted by hemacytometer, placed in microcentrifuge tube and pelleted for 6 seconds at 16000 rcf. Supernatant sucrose solution was carefully removed by pipette and the cells gently resuspended in ice-cold Puramatrix.

Droplet formation and implantation for RAIN-Droplet assay

Toroid formation is illustrated in Figure 1a. Cells, 4×10^6 cells/ml, were suspended in 0.05 % w/v Puramatrix (0.2% for tumor cells). Cell suspension, 3 μl, was drawn into the tip of a 20 μl pipette. Droplet volumes of 1-15 μl were achievable with this size of pipette tip, although 3 μl was routinely used for most assays. A droplet was formed at the end of the tip by gently depressing the pipette plunger to the first stop. Holding the pipette in a vertical position the droplet was touched to the surface of complete cell culture medium causing the droplet to release and to instantly form a gel toroid. In most cases this was performed in low attachment cell culture plates to avoid loss of cells to the plastic surface during longer term cultures.

Toroid contraction occurred over 24-72 hours in floating culture. Collagen gels (Purecol, San Diego, USA) (2.3 mg/ml, 0.5 ml/well) were precast in the wells of a 12-well plate as described by the manufacturer. Droplets were transferred to the gel surface using a 200 μl pipette tip with the end 5 mm cut, or using a wide bore pipette tip. After placing the droplet on the gel any excess medium was carefully removed by pipette, and collagen (0.1 ml/well) was placed on top of the lower collagen layer to sandwich the droplet. Collagen was set at 37°C for 1hr in normal atmosphere. In some instances growth factors were added to the upper layer prior to sandwiching the droplet. Finally, culture medium was layered over the gel.

For drug treatment studies droplets were prepared as described and embedded in collagen with 50 ng/ml VEGF in the top layer (5 ng in 0.1 ml) of collagen. HDMEC were generally allowed to sprout for 24-72 hours prior to drug addition although where indicated droplets were occasionally treated immediately after embedding. Medium containing Avastin (Bevacizumab, Genentech Inc., South San Francisco, USA) (25 μg/ml) was then layered onto the collagen. In sorafenib (LC Laboratories, Woburn, USA) studies, the compound (5-5,000 nM), diluted in medium, was layered onto the collagen. Samples were incubated at 37°C, 5% CO_2 for 24-48 hours before counting sprout number.

Sprout quantification

Sprouts were quantified under phase contrast microscopy. It was used for simplicity although cells could be labeled as described below and then counted. For purposes of counting, sprouts were defined as multicellular capillary-like elongated structures radiating out from the RAIN-Droplet toroid and with the base of the sprout attached to the circumference of, or at a point within, the toroid. A start point was determined, generally at a "12 o'clock" position, then sprouts were counted clockwise around the toroid until returned to the start position.

Sprouts were observed using an Olympus IMT-2 microscope with Olympus CK20 LWD C A 20PL 20× Objective (200× final magnification). Image acquisition was performed using Evolution/QImaging microscope-mounted Digital Camera, CCD 2/3" ICX-282 with 2560×1960 resolution controlled by QCapture Pro image capture software (Media Cybernetics, Silverspring, MD, USA). Some images were converted to grayscale using greyscale conversion function on Photoshop 7.0, no further image manipulation was used for brightfield photomicrographs.

Labeling of droplets

Toluidine Blue staining of embedded droplets: Embedded droplets were washed twice in PBS (MediaTech. Inc, Manassas, USA) then fixed in 3.7% paraformaldehyde (USB, Cleveland, USA) and again washed twice in PBS. Sufficient toluidine blue (Sigma/Aldrich, St. Louis, MO, USA) in 0.1% methanolic solution was layered onto the gels to cover and incubated on the bench top for 2-5 minutes. The level of staining was monitored microscopically. To remove excess dye and destain the surrounding collagen, water was layered onto the surface of the gel, incubated for 5-10 minutes then removed. This destain process was repeated if contrast between droplet and collagen was insufficient when viewed microscopically. Samples were then imaged under the phase contrast microscope system described above using brightfield without phase ring in place.

Fluorescent labeling of embedded droplets: Embedded droplets were stained by the actin probe Alexa Fluor 488 Phalloidin (Invitrogen) or by CellTracker Green (Invitrogen). Droplets were fixed in paraformaldehyde (3.7%) then washed twice in PBS and extracted with 0.1% Triton for 40 min in PBS before washing twice with PBS. Samples were blocked for 1h with 0.1% albumin/ 0.2% fetal bovine serum at RT then Alexa Fluor 488 Phalloidin, 2 units/well, was added for 40 min at RT. Samples were washed in PBS then layered with 50% Prolong Gold plus DAPI in PBS before imaging by confocal microscope.

For CellTracker staining the embedded droplets were incubated with 10μM CellTracker Green for 30 min, then PBS for 15 minutes before fixation for 15 min with paraformaldehyde (3.7%). Samples were washed twice with PBS then layered with 50% Prolong Gold plus DAPI in PBS before imaging by confocal microscope.

For immunohistochemical staining of sorafenib or control treated cells, droplets were prepared and allowed to sprout for 48 h before adding drug or vehicle and subsequently culturing for up to a further 72 h. Then the droplets were fixed in situ as for the fluorescent phalloidin staining above. Collagen disks containing embedded droplets were then popped out of the wells using a blunt spatula and the discs processed and embedded in paraffin. Slides were prepared from thin sections cut from the paraffin blocks and sent to the University of Michigan Cancer Center Research Histology & Immunoperoxidase Lab for commercial staining using Ki-67 (mouse) antibodies and ApopTag apoptosis labeling kit.

Equipment/software specifications for confocal images

The Olympus IX71 Fluoview 500 Laser Scanning Confocal Microscope running Olympus Fluoview image acquisition software version 4.3 bearing objective lens UPLAPO 20× was used to capture the images using the following filters and dichroic mirrors: DM405/488; SDM490; BA430-460; BA505IF. 3D reconstruction of confocal stacks, without volume or surface rendering, were performed automatically by opening Fluoview multiTif files under the Surpass option using Imaris software (Bitplane Inc. St Paul, USA). To better visualize the toroidal sprouting structure, solely in actin/DAPI staining, the green and blue channels were enhanced using Photoshop Variations option, original unaltered image is available for comparison on request. Volume rendering: following 3D reconstruction the Blend option was selected and Phalloidin 488 threshold was set to 800-1400. Surface rendering was performed for DAPI stained nuclei by selecting the Surfaces option, setting Area Detail to 0.621 (smooth) and finally selecting Absolute Intensity threshold and setting to 955-3943. In all rendered images gamma was unchanged. Finally, rendered images were visually compared to original captured microscopic image for fidelity. Full image capture details available but withheld presently due to space requirements.

Transmission Electron Microscopy

Droplets were fixed in Karnovsky's fixative, a mixture of 3 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4. After several buffer rinses, they were postfixed for one hour in 1 % osmium tetroxide in the same buffer. They were then rinsed in double distilled water to remove phosphate and then *en bloc* stained with aqueous 3 % uranyl acetate for one hour. Next, they were dehydrated in ascending concentrations of ethanol, treated with propylene oxide, and embedded in Epon epoxy resin. Semi-thin sections were stained with toluidine blue for tissue orientation. Selected areas were ultra-thin sectioned 70 nm in thickness and stained with uranyl acetate and lead citrate. They were examined using a Philips CM100 electron microscope at 60 kV. Images were recorded digitally using a Hamamatsu ORCA-HR camera system operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

Deltavision movie of sprout formation

Droplets embedded in collagen within chamber coverslips (Nunc, Rochester, NY, USA) were maintained at 37°C, 5% CO_2 in a humidified atmosphere for the duration of the experiment using a Weather Station (Precision Control LLC) cell culture environment designed for the Olympus IX-71 inverted microscope. Time lapse images were captured Time lapse image capture was controlled using SoftWoRx 3.5.1. Images were captured every 600 s. Video was also constructed from captured images using SoftWoRx 3.5.1 and converted to Quicktime file using MPEG Streamclip 2.1 for Windows Vista (http://www.squared5.com) where aspect ratio was changed from 768×768 to 320×240.

Sulphorhodamine B Assay

The sulforhodamine B (SRB) cytotoxicity assay was used as described [6, 7]. Briefly, primary human dermal microvascular endothelial cells (HDMEC; Lonza, Walkersville, MD, USA) were seeded at 2.5×10^4 per well in a 96-well plate and allowed to adhere overnight. Drug or control was diluted in EGM2-MV (Lonza) and layered onto cells, which were allowed to incubate for times as indicated in the figures. Cells were fixed on the plates by addition of cold trichloroacetic acid (10% final concentration) for 1 hour at 4°C. Cellular protein was stained by addition of 0.4% SRB (Sigma/Aldrich, St. Louis, MO, USA) in 1% acetic acid and incubation at room temperature for 30 minutes. Unbound SRB was removed by washing with 1% acetic acid and the plates were air-dried. Bound SRB was resolubilized in 10 μmol/L unbuffered Tris-base and absorbance was determined on a microplate reader at 560 nm (Genius; Tecan, Graz, Austria). Test results were normalized against initial plating density and drug-free controls.

Matrigel assay

HDMEC were subject to the 2D matrigel cord formation assay. Matrigel was allowed to set in the wells and cells were added on top in the presence or absence of sorafenib (0.5 and 5 μM). After incubation for 24 - 72 h the cells were photographed and the number of endothelial cords were counted manually using Image J (NIH) point counter. A cord was defined for quantification purposes as a discrete directional cord of cells stretching between two junction nodes.

Statistics

Statistics performed were one-way ANOVA on either SigmaStat 2.0 software (SPSS, Chicago, IL, USA) or Microsoft Excel software.

Results

RAIN-droplet model

The following procedure was developed for the creation of toroidal droplet gels appropriate for 3-D embedding in collagen. Cell suspensions in PM were drawn into the tip of a 20 μl pipette and the plunger of the pipette was depressed to form a droplet at the end of the tip. The droplet, gently touched to the surface of complete cell culture media in an ultra-low attachment plate, broke the surface tension of the culture medium and was released. A

discrete, free floating hydrogel scaffold was instantly formed encapsulating the cells with little or no observable loss of cells into the surrounding media, shown pictorially in Figure 1.

Puramatrix concentration is critical to formation of toroidal gels

We hypothesized that the differential matrix resistance exerted on HDMEC by varying concentrations of Puramatrix would result in distinct droplet shapes. To test this we prepared suspensions of fixed cell number in varying concentrations of PM. Interestingly, we found that Puramatrix concentration was closely linked to polymerized droplet shape and therefore also to the utility of the droplet in the RAIN-Droplet assay (Figure 2). Using 0.05% w/v hydrogel, encapsulating 4×10^6 cells/ml, consistent flat toroidal shapes with even cell distribution were created on droplet gelation (Figure 2). Regular, toroidal gel structures are unique to this method of mold-free droplet formation and were not achievable by running the droplet down the wall of the culture well. The above preparation was used for all experimental procedures unless otherwise stated.

Contraction of the cell bearing toroidal 0.05%-0.1% hydrogel structures was observed over the first 48 hours, with little further change after this time (Figure 2, lower panel). This contraction event is in general agreement with Sieminski and colleagues[8] who observed that, in flat bed 1% and 2% PM gels, embedded endothelial cell reorganization induces gel contraction proportional to gel concentration [8].

Characterization of single and multicellular structures *within* toroidal droplet gels

Distinct cellular features were revealed within the toroidal droplets using brightfield and transmission electron microscopy (Figure 3 a-c). Common to all analyzed droplets was a continuous cell coverage around the droplet circumference (Figure 3 a, arrows). This coverage was characterized by cell-cell contacts displaying distinct electron dense (darker) regions in the electron photomicrograph (Figure 3 b, arrows). It appeared to develop over the 24-72 hour toroid contraction period, whilst newly formed droplets did not appear to display a layer of contiguous cells at the gel surface. Significantly, the HDMEC within the toroids formed organized structures of two or more intact cells, often centered around large lumenal spaces free of extracellular matrix in many cases (Figure 3 c, arrow). The exact nature of the cell-cell junctions and luminal spaces apparent in the droplets is subject to continuing investigation. However, the electron dense regions at cell-cell interfaces were common and were suggestive of tight junctions (Figure 3 b).

Characterization of radial capillary sprouts after collagen embedding of toroidal droplet gels

Sprouting was always preceded by invasion of a proportion of the endothelial cells into the surrounding matrix. An initial exploratory burst during the first 24 - 48 hour accounted for the majority of the individual cell invasion with multi-cellular sprout formation beginning over 12 -24 hour post-embed (Supplementary video 1 online). In the video one sprout is seen to form in the center of the field towards the end of the sequence. Interestingly, once initiated, the position of the sprouts generally did not change but the cells contributing to the sprout changed rapidly during the initial formation stage. Cells were frequently observed using the sprout as a migratory route from the body of the droplet into the surrounding

matrix (Supplementary video 1 online). Fluorescent labeling of HDMEC (Figure 4 a and supplementary video 2 online) show sprouts originating from both cells around the droplet circumference (Figure 4 a, arrow) and the pre-existing endothelial cell network within the droplet (Figure 4 a, arrow head). Sprouts could be maintained in culture for between 9 – 14 days with a change of media every second day although for most routine testing or assay of sprout formation described in the present study 2 – 7 days were sufficient.

Assay of angiogenic function using the RAIN-Droplet model

As proof of principle of the RAIN-Droplet assay function, we treated embedded droplets with bevacizumab (Avastin, Genentech), an anti-VEGF antibody inhibitor of angiogenesis [9, 10], which resulted in a significant (n=7-8, $p < 0.001$, representative of three independent assays) reduction in sprout number (Figure 4 b, arrows indicate control sprouts in right panel). Using the RAIN-Droplet assay we also found clear inhibition of endothelial cell invasion using sorafenib (LC Laboratories), a multi-kinase inhibitor and anti-angiogenic compound [11] (Supplementary Figure 1 online). Quantification of invasion was not the primary aim of this study and unlike the sprouting it did not occur on a narrow horizontal plane. Whilst an efficient method for quantification of 3-D invasion in this model is being investigated the radial nature of the RAIN-Droplet assay provides clear visual evidence of differences in cellular invasion patterns in both bevacizumab and sorafenib treated samples (Figure 4 b insets, Supplementary Figure 1). As proof of utility we also showed that genetically manipulated cells could be used in the RAIN-Droplet model. Droplets were created from HDMEC engineered to overexpress the anti-apoptotic protein Bcl-2 by infection with a lentiviral vector bearing the *bcl-2* gene sequence (Supplementary Figure 2). It may be seen that Bcl-2 overexpression produced highly prolific sprouting and cell invasion. Whilst clearly not optimized for quantification, this demonstrated the potential for study of cell signaling pathways using the RAIN-droplet model.

Dose-dependent stimulation of endothelial angiogenesis by Sorafenib

Interestingly, we noted a surprising event after droplets were treated with sorafenib at varying concentrations after sprouting had been allowed to occur. It was clear that sorafenib appeared to have a directly dose-dependent inhibitory effect on single cell invasion into collagen surrounding the droplet (Supplementary Figure 1). However due to limits of image resolution what may be unclear in the photographs is that the drug had a paradoxical stimulatory effect on endothelial sprout formation. To investigate this further we performed an SRB proliferation assay to determine the sensitivity of HDMEC to sorafenib. It was determined that under normal culture conditions sorafenib at less than 1 μM had no significant inhibitory effect on HDMEC proliferation (Fig 5 a). Above 1 μM sorafenib began to inhibit endothelial cell proliferation (Fig 5 a). Importantly, when non-cytotoxic, low dose sorafenib, 0.5 μM, was administered for 48 hour to droplets with pre-existing capillary sprouts the number of sprouts increased significantly in number ($p < 0.05$) and robustness (Figure 5 b). When the same low dose of sorafenib was given prior to sprouting, normal sprout formation was significantly inhibited as expected (Figure 5 c). Under both endothelial sprouting regimes higher, cytotoxic doses of sorafenib (5 μM) significantly inhibited sprout formation ($p < 0.05$) (Figs. 5 b-c). It is beyond the scope of the current study to make a detailed investigation into the molecular signaling involved in this paradoxical process but

some observations may be made at the cellular level. In figure 6 we present a time course of RAIN-droplet sprouting and sprout anastomosis induced by initial exposure to VEGF in the presence or absence of sorafenib, 0.5μM. The difference in organizational level is clear with sorafenib treatment causing a rapid and, importantly, sustained aggregation of scattered cells into clearly aligned and robust multicellular sprouting structures. The control cells are also driven to form sprouts but they are poorly organized and, without additional VEGF supplementation, do not maintain consistent structures (Figure 6). This comparison is highly representative of several separate experiments examining the effect of sorafenib on sprout structure. Immunohistochemical staining of sections cut from collagen embedded droplets did not indicate any notable difference in expression of proliferation markers (Ki-67) or apoptosis markers (ApopTag) in sprouts exposed to sorafenib compared to controls (data not shown). Furthermore when HDMEC were exposed to sorafenib in the traditional matrigel 2-D cord formation assay sorafenib treated cells formed significantly more cords than controls (p 0.01) whilst controls clearly displayed notably non-angiogenic cell growth as evidenced by the broad monolayer sheets of cells on the matrigel surface (Figure 7).

Discussion

Conceptually, angiogenesis models that allow the use of a defined cell population are attractive for further defining specific reactivities or mechanisms related to physical, chemical or biological experimental stimuli. However, traditional culture of defined cell isolates or lines as 2-D monolayers provide growth conditions relatively far removed from the normal physiological environment. Indeed, it is increasingly appreciated through gene expression analyses that the interface between the cell and the culture surface may significantly affect protein secretion and thus cell function and response to changes in external conditions [12]. Thus conditions that mimic the normal 3-D environment of the cell population, whilst still not a replacement for the *in vivo* milieu, may provide a better reflection of the normal physiological state [13]. Previous attempts at making discrete endothelial cell-bearing vehicles for 3-D endothelial sprouting and invasion have generally but not exclusively taken three directions. The simplest are variations on casting single cell suspensions of endothelial cells in matrices such as collagen [14]. In some instances cells are suspended in a gel cast within an especially designed mold but other protocols cast the gels in plates from which areas of gel are punched out with specialized equipment. Both methods produce discrete, large and fragile gel discs with usage primarily limited by the physical nature of the discs.

Foci for angiogenic sprouting have also been created by growing endothelial cells on cell culture compatible microcarrier beads, the bead effectively acting as a culture surface [15]. This may be performed by incubating endothelial cells in specialized stirring flasks for a number of days. The coated beads are then embedded in a surrounding matrix allowing cell invasion and sprouting [4, 15].

The third distinct platform for production of discrete 3-D endothelial sprouting foci is a derivative of the well-described tumor spheroid protocol. Tumor cells will aggregate if prevented from adhering to a culture surface, ultimately the aggregated cells will order into spheroid. Endothelial cells do not normally form spheroids and rapidly die by anoikis if

prevented from attaching. By suspending endothelial cells under very precise conditions in a highly viscous medium, providing limited physical support, the cells are able to survive and migrate towards each other to form endothelial spheroids [16]. The spheroids are initially loose cell aggregates which readily dissociate if cultured in 2-D or 3-D conditions. However, if allowed to mature over a period of up to 7 days the surface cells form a discrete monolayer of differentiated and polarized cells whilst spontaneous apoptosis and necrosis inside the spheroid result in a virtually cell-free lumenal void [16].

We utilized distinctive properties of a nanoscale self-assembling hydrogel, Puramatrix (PM) (BD Biosciences), to encapsulate HDMEC in free-toroidal droplets suitable for implantation in biological matrices. Uniquely within this class of *in vitro* model the RAIN-Droplets contain a heterogeneous population of angiogenically activated endothelial cells forming a branching and active network of endothelial cell structures which include capillary sprouts, multicellular spheroidal foci and active single cells. The toroidal RAIN-droplets are simple to create, require only common laboratory equipment for preparation and provide a novel platform for *in vitro* study of angiogenesis.

Puramatrix is composed of 16-mer peptide chains in 1% w/v aqueous solution [17]. This matrix instantly and spontaneously organizes into a nano-scale scaffold under physiological conditions. It promotes survival and maintains angiogenic activity of endothelial cells [8, 17]. Until now, Puramatrix has been used primarily as a replacement for extracellular matrices in cell monolayer, embedded cell suspensions or transwell invasion studies [18, 19] although microfluidic protocols have also taken advantage of the unusual polymerization properties of the hydrogel [20].

Notably, a far lower concentration of Puramatrix, ~10-fold lower, was used here than is described for any other applications of the hydrogel in the available literature [8, 17-20]. Above 0.1% hydrogel concentrations inconsistent shapes were obtained (Figure 2) and above 0.2% uneven comet shaped pellets were generally formed (Figure 2). Importantly, low concentration conditions allowing adequate toroidal gel contraction were critical for creating droplets suitable for the angiogenesis assay. Whilst culture plates bearing toroidal gels could be carefully moved immediately after droplet formation, direct manipulation or excessive shaking readily disrupted newly formed toroids of 0.05-0.1% Puramatrix. However, incubation under normal cell culture conditions allowed gel contraction, over 24-72 hours, and consistently resulted in rigid toroids that could be transferred by pipette without disruption. Thus 4×10^6 cells/ml in 0.05% w/v hydrogel produced consistent, tightly contracted, evenly shaped toroids bearing complex populations of HDMEC suitable for study or assay of angiogenic activity.

The resulting toroidal droplet gels were robust showing little obvious disruption under normal cell culture handling procedures. Droplets were easily harvested using a wide bore, 200 μl pipette tip or a regular 200 μl pipette tip with the end 4-5 mm cut. For use in the assay of angiogenesis modulation the droplets were then embedded between two layers of collagen gel (Figure 1). Embedding gels could be formed with or without added pro-angiogenic stimuli (*e.g.* VEGF, bFGF). It was most economical and sufficient to add the growth factors to the top layer of gel alone. This format maintained sprouting activity using

the smallest amount of growth factor. Implanted sprouting droplets could be consistently maintained in the presence of growth factors for up to nine days and often up to two weeks with care and bi-daily changes of VEGF supplemented medium. Despite the inherent variability and limited culture capacity of primary cells compared to immortalized cell lines for example, the RAIN-Droplet assay has been consistent enough in our hands to say that seven to nine days survival is repeatedly achievable with sprouts formed from healthy endothelial cells. Longer times are achievable and are indeed likely for low passage-number, highly viable human microvascular endothelial cells.

The initial cell invasion events of the RAIN-Droplet model after embedding droplets in collagen are currently being investigated with a view to quantitatively assaying invasion concurrently with sprout formation. This is a unique function within the existing engineered 3-D angiogenesis models. Even without numerical quantification, the RAIN-Droplet model allowed a very clear qualitative assessment of drug effects on endothelial cell invasion. The primary concern with determining invasion is that unlike the capillary sprouts, which virtually all grow out along the horizontal plane of the RAIN-Droplet like spokes on a wheel, the single invading cells have a tendency to spread the full depth of the collagen embedding gel. Quantification of unstained sprouts was easily accomplished using phase contrast microscopy. As mentioned above and distinct from other 3-D angiogenesis models the sprouts extended out from the toroid within a relatively narrow plane making quantification of sprout number or length relatively easy. By their nature bead-based or spheroid based 3-D models of angiogenesis produce multi-directional sprouts around the entire spherical structure whilst in the RAIN-Droplet model virtually all the sprouts radiated horizontally out along the plane of the toroidal droplet gel and were readily visible within virtually the same focal plane of a medium to high power microscope lens (50-200 × magnification). Alternatively, samples were fixed with paraformaldehyde at room temperature (RT), washed with water, stained with toluidine blue and viewed under a brightfield microscope. In both instances sprouts viewed at 200× magnification were clearly discerned and could be counted by eye around the full circumference of the toroid. Endothelial sprouts were defined for the purposes of this study as robust single or multicellular structures radiating out from the toroid but being attached to the toroid at their base. With particular respect to imaging sprouts, this horizontal, radial sprouting protocol is a significant improvement over most previous matrix embedded models which generally require cross sectioning of the gel in order to clearly view endothelial sprouts [14].

In evaluating the RAIN-Droplet model for its potential as an assay for angiogenesis we tested two known angiogenesis inhibitors. Both bevacizumab and sorafenib are in active clinical trials, and the mechanisms of action have been well described for both drugs[9-11]. As expected, bevacizumab, the anti-VEGF antibody, was highly effective at inhibition of sproutsover a period of 24 - 72 hours in the presence of up to 50 ng/ml VEGF contained in the embedding gel. Sorafenib was very effective at inhibiting *de novo* growth of endothelial capillary sprouts, markedly inhibiting virtually all signs of cell invasion from the droplets into the surrounding collagen. However, when the same concentrations of sorafenib were tested on droplets which had been allowed to initiate capillary sprout formation into the collagen a paradoxical increase in sprout number was observed. Indeed, microscopic

examination revealed that these new structures were highly robust, thick sprouts comprised of apparently more cells than the control sprouts. It was determined that the sorafenib concentration consistently inducing significantly more sprouts that persisted over time, 0.5 μM, was insufficient to affect cell proliferation over the same period. Thus the multikinase inhibitor sorafenib appears to display both anti-angiogenic and pro-angiogenic activity dependent on concentration of the drug and on the maturity of the capillary sprouts. It may be assumed that existing *in vitro* assays of sorafenib have failed to differentiate this important bi-phasic function of sorafenib on angiogenesis activation as there is no other discussion of it in the literature. Indeed surprisingly little literature describes pre-clinical *in vitro* anti-angiogenesis testing of sorafenib, primarily it has included *in vivo* treatment and assessment of anti-angiogenic effect on xenograft models. That *in vitro* literature which is available typically uses concentrations of sorafenib well above the 0.5 μM concentration limit we observe here for stimulatory effects on angiogenesis [21-23].

However, the observation of a paradoxical stimulatory effect of sorafenib on endothelial cells is in agreement with very recent observations by Rose and co-workers who describe a stimulatory effect of sorafenib on a variety of bladder cancer cell lines in the same dose range as the current study shows the stimulatory effects on endothelial cells [24]. Rose and co-workers also examined renal cancer cells but did not observe the same stimulation, suggesting that the stimulatory effects of sorafenib are cell specific [24]. The results of sorafenib activity on the RAIN-Droplet model suggest that endothelial cells actively undergoing angiogenic sprout formation are amongst the cell types sensitive to this biphasic drug activity. Indeed, the 2-D matrigel cord formation angiogenesis assay confirms the sprouting phenotype seen in the RAIN-Droplet model as it also demonstrates an increase in endothelial cord number by cells exposed to sorafenib. It should be noted that in the matrigel assay 5μM sorafenib significantly increased cord number whereas it was decreased in the RAIN-droplet model. Whilst the paradoxical pattern of sorafenib induced sprouting increase was confirmed, the disparity between the effects of 5μM sorafenib in the RAIN-Droplet model compared to the matrigel assay is likely due to the short term protective quality of growth factors present in the matrigel which is a non-defined extracellular matrix. It may also potentially be due to batch to batch variation inherent in primary cells such as the HDMEC. Importantly, these initial findings from the 3-D RAIN-Droplet model for angiogenesis, in conjunction with those of Rose and co-workers may have significant implications for the choice of dosing schedule used in therapeutic application of sorafenib. Furthermore they warrant continued investigation, because identification of a common mechanism would allow better classification of stimulation sensitive cell lines and potential development of compounds that could bypass this effect.

The RAIN-Droplet assay utilizes an innovative, flexible, planar, focal point based 3-D angiogenesis sprouting model. In addition to endothelial cell sprouting, this focal point based approach to the assay of migrating and invading cells has wider potential for any cells able to expand or migrate into a 3-D matrix material. The RAIN-Droplet assay combines the ease of construction of 2-D gel assays with the physiological relevance of more complex 3-D angiogenesis assays. Radial sprouting from a focal point within a narrow plane allows visual sprout quantification by the simplest of microscopical techniques but readily

translates to more complex imaging methodologies. Notably the RAIN-Droplet model allowed us to observe and quantify a new function of a known compound which had not been described to date in other angiogenesis assays. An easily performed 3-D culture protocol such as that of the RAIN-Droplet assay would provide a novel, accessible model for *in vitro* angiogenesis and expands the fine but limited repertoire of *in vitro* angiogenesis screening assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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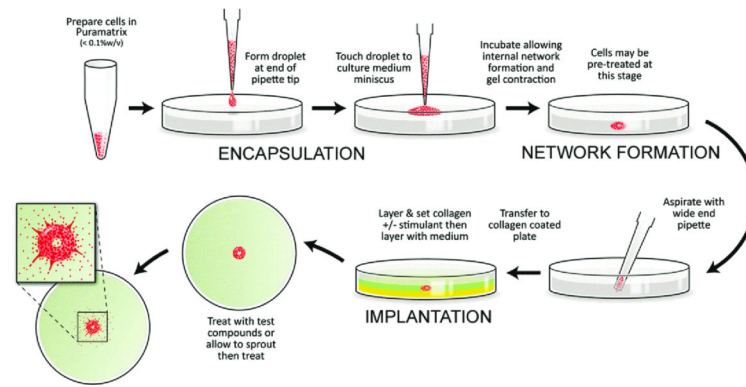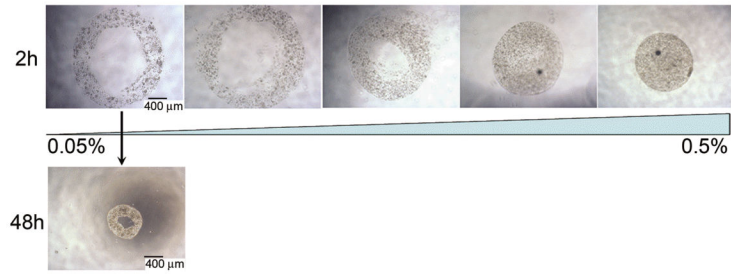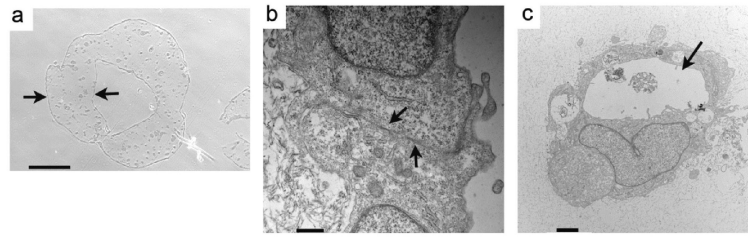
Figure 2. Formation of toroids was dependent on PM concentration
(upper panels), scale bar, 400 μm. Toroids formed from concentrations of 0.1% w/v PM and below. Toroids contracted as sprouting HDMEC networks were formed within the droplet. The same toroidal droplet is shown after 48 h incubation in culture medium (lower panel).

Figure 3. Brightfield and transmission electron micrographs of RAIN-droplets
(a) Continuous coverage of 0.05% PM toroid circumference by HDMEC (arrows), scale bar
200 μm. (b) Cell junctions at circumference, matrix is clearly visible within toroid to left of
cells. Arrows indicate electron dense regions at cell-cell interface, scale bar 500 nm. (c)
Structures in contracted toroids included a variety of cell structures, some bearing lumen-
like vacuoles empty of surrounding matrix (arrow), scale bar 2 μm.
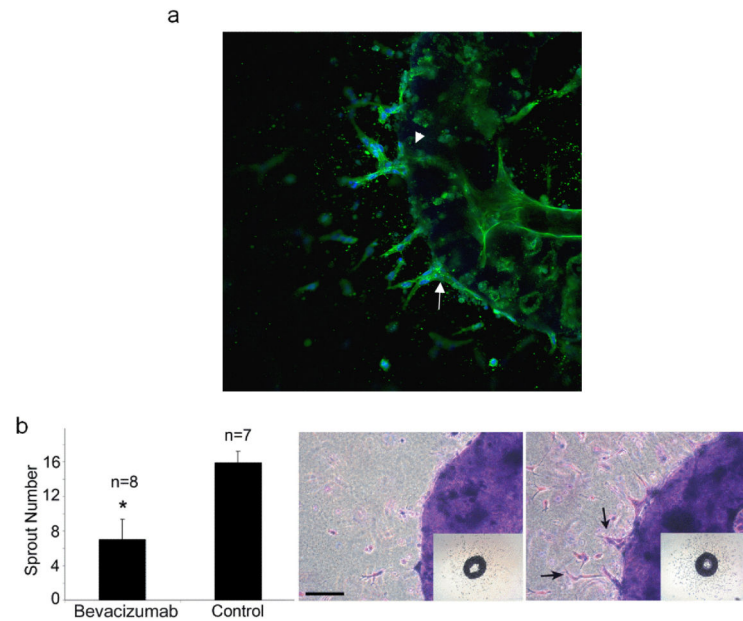
Figure 4. RAIN-Droplet model in 3-D culture and assay for effect of angiogenesis inhibitors on endothelial sprouting

(a), Actin was labelled with Alexafluor-488 Phalloidin (green) and nuclear staining with DAPI (bright blue), representative of multiple labeling studies. HDMEC sprouts originating from sprouting structures within the toroid (arrowhead) and from cells along the toroid circumference (arrow). The RAIN-Droplet toroid is visible as a diffuse, dark blue arch at the top right of the panel bordered by arches of green actin phalloidin stained cells. The droplet is potentially fluorescent due to either slight autofluorescence or differential retention of DAPI after labeling compared to the surrounding collagen. (b), Embedded RAIN-droplet toroids were exposed to VEGF (50 ng/ml) for 24 h then bevacizumab, 25 μg/ml, for up to 48 h. Scale bar is 100 μm. Bevacizumab (center panel) significantly inhibited endothelial sprouts compared to control (right panel) and caused marked attenuation of cell invasion (insets) (n=7-8, p<0.001).
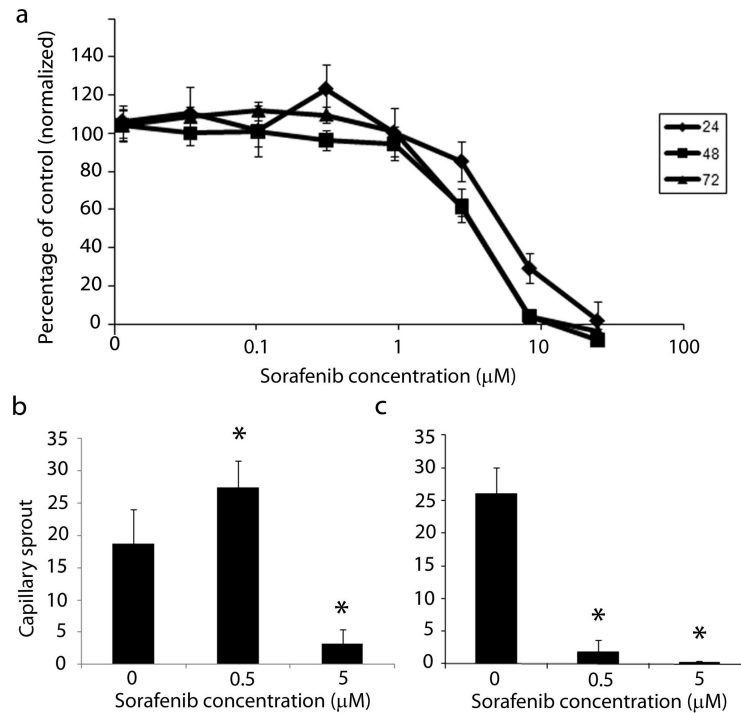
Figure 5. Concentration dependent bi-phasic stimulatory and inhibitory effects of the multi-kinase inhibitor sorafenib on endothelial capillary sprout formation

(a) Proliferation assay for HDMEC exposed to varying concentrations of sorafenib. Cells were plated in 96-well plates in the presence or absence of sorafenib at indicated concentrations. Tetrazolium dye WST-1 was added at 24, 48 and 72 hours to individual plates and absorbance was determined by microplate reader at 540 nm. Test readings were normalized against absorbance at initial plating density and also as a percentage of untreated time-point controls, (n=6 for all points). (b) HDMEC were encapsulated in RAIN-Droplet gels, embedded in collagen with VEGF (5 ng in 0.1 ml collagen). Sorafenib was added to the wells in fresh culture medium after initial formation of capillary sprouts had occurred (24-72h). Capillary sprout number was assessed by observation under high power microscope (200x). (c) HDMEC were treated as for panel (b) but sorafenib was added immediately after embedding and prior to capillary sprout formation (n=4 for all sprouting assays data points).

Control   Sorafenib(0.5μM)

Pre-treatment

24h

48h

72h
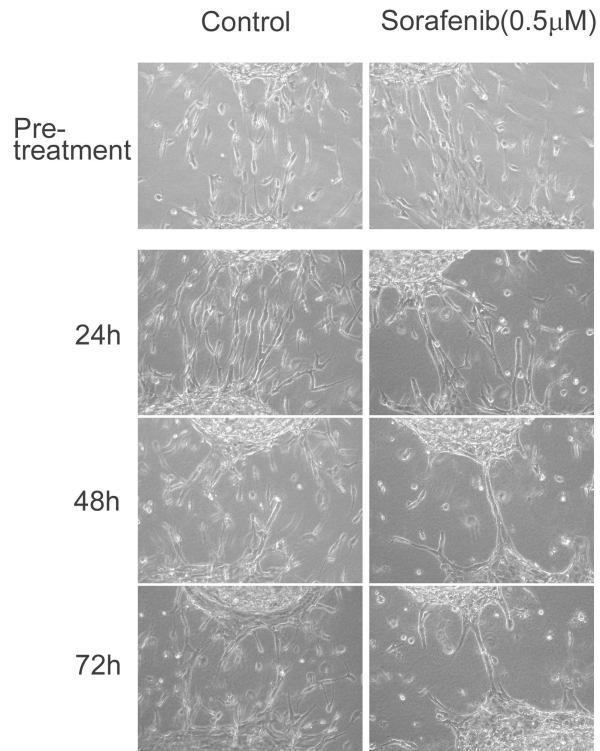
Figure 6. Sorafenib promotes formation of robust endothelial sprouts during sprouting anastomosis between two RAIN-droplets
HDMEC were prepared for the RAIN-Droplet model as previously described and embedded in collagen containing VEGF, 50 ng/ml. Droplets were incubated 72 h to establish sprouts then were treated plus or minus sorafenib (0.5 μM) for a further 72 h with images of the same field captured every 24h. Notably before treatment the fields look very similar however the sorafenib appears to drive the HDMEC to rapidly organize and aggregate in one or two robust sprouts. The control cells form thinner, less coherent sprouts which become disorganized without further stimulation.
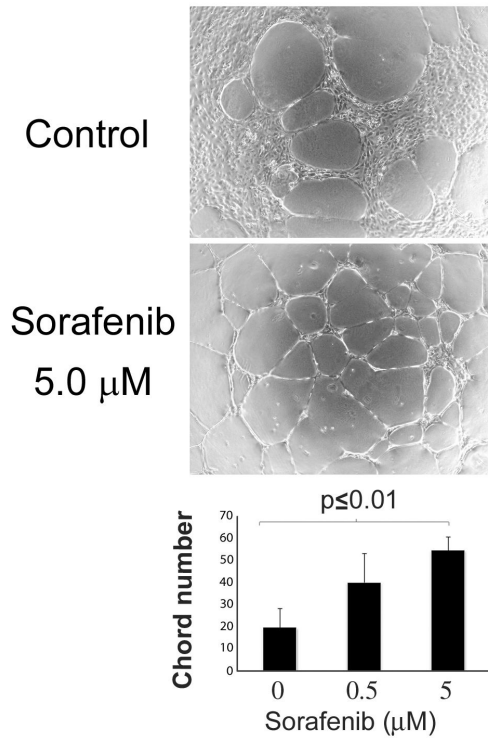
Figure 7. Effect of low concentration sorafenib on endothelial cell cord formation in the matrigel angiogenesis assay

HDMEC were plated onto matrigel in the presence or absence of sorafenib, 0.5 and 5 μM. Cells were incubated for up to 72 h with the same field being photographed per well every 24h. Cord numbers were counted manually using the Image J (NIH) point counter function, n 3 wells per concentration. Cords were defined as a discrete and linearly directional cord of cells stretching between two junction nodes. Sorafenib was found to increase cord number relative to controls with 5 μM sorafenib inducing significantly greater numbers of cords (p 0.01).