Uses of the *in vitro* endothelial–fibroblast organotypic co-culture assay in angiogenesis research

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Abstract

Angiogenesis is a complex process that involves multiple cellular events. In addition to receiving inputs from a range of stimulatory and inhibitory factors, endothelial cells undergoing angiogenesis make multiple interactions with the extracellular matrix and with other cell types in the stroma. Recreating angiogenesis *in vitro* is probably an impossible goal; however, a number of assays have been developed that recapitulate many of the key events of the process. These assays are indispensable tools for investigating the signalling pathways that control the formation of new blood vessels. In the present paper, we review the organotypic co-culture assay of angiogenesis – until recently, a comparatively underemployed assay, but one with a number of powerful advantages for angiogenesis research. We give a set of optimized protocols for its use, including protocols for siRNA (small interfering RNA)-based screens, and we discuss appropriate methods for obtaining quantitative data from the assay.

Introduction

Angiogenesis is the process by which new blood vessels form from pre-existing vasculature. Angiogenesis is most active during development, where it plays a crucial role in the elaboration of the vasculature. In adult organisms, angiogenesis is triggered by tissue hypoxia. It plays important roles in tissue healing and in recovery from ischaemic incidents, such as heart attack or stroke. In these situations, angiogenesis is beneficial; however, angiogenesis is also involved in several pathological conditions. Overactive angiogenesis is a key factor in many ocular diseases. Angiogenesis is also triggered in cancer, and solid tumours promote the formation of tumour-associated vessels that supply nutrients and oxygen to the growing mass [1].

The clinical importance of angiogenesis has driven research into the signalling pathways that control this process. Although later stages of research require *in vivo* models of angiogenesis, the initial discovery steps are reliant on *in vitro* angiogenesis assays that allow easy manipulation of signalling proteins and rapid quantifiable readouts. A large number of *in vitro* angiogenesis assays have been described, but none fully recapitulates the process of angiogenesis, and consequently each assay has its own advantages and disadvantages [2,3]. The most commonly used assay is the Matrigel chord assay, first described over 20 years ago [4]. In this assay, ECs (endothelial cells) are plated on the surface of Matrigel™, a naturally derived basement ECM (extracellular matrix) isolated from tumour cells. ECs in this assay form a network of chords within a few hours of plating and this can be stimulated by pro-angiogenic factors [5]. Interestingly, there is little or no EC migration in the Matrigel assay, making it a suitable readout for endothelial cell–cell assembly rather than EC migration and sprouting angiogenesis. Overall, the Matrigel assay is a powerful technique because of its simplicity, speed and robustness, although other non-EC types have been shown to form similar network patterns in Matrigel, raising some debate as to how specifically the assay maps to angiogenesis [3].

The co-culture assay of angiogenesis was first described by Bishop et al. [6]. Since then, it has been used in a number of studies aimed to test therapeutic agents [7]) and to delineate signalling pathways that regulate angiogenesis (e.g. [8–11]). In the original assay, HUVECs (human umbilical vein endothelial cells) are cultured with NHDFs (normal human dermal fibroblasts) for 14 days, during which time HUVECs form tubules with lumens that resemble capillaries *in vivo* [12]. In the present paper, we review the properties of the co-culture assay and discuss its advantages and limitations. We present our modifications to the original assay and provide detailed protocols for their use.

Properties of the co-culture assay

In the original co-culture assay, HUVECs are mixed with NHDFs and plated on tissue culture plastic. The interaction of these two cell types gives rise to three-dimensional tubules that resemble small capillaries *in vivo* [6,12] without addition...
The co-culture assay

The co-culture assay

Figure 1 | The co-culture assay

Left-hand panel shows an EC sprout in the assay stained for CD31 (green) and VEGF receptor-2 (red). The tip of the EC sprout displays long filopodial projections, which are involved in guidance. The nuclei of the surrounding fibroblasts can be seen stained with DAPI (blue). VEGF receptor-2 can be seen in endosomal vesicles in the body of the EC. Middle panel shows the co-culture stained with CD31 (green) and laminin (red). As tubules become stabilized, they secrete a basement lamina rich in laminin and collagen IV. Right-hand panels show two low-magnification images of the co-culture stained with NBT for quantification. The co-culture in the lower panel was supplemented with 10 ng/ml VEGF, which stimulates angiogenesis.

of exogenous ECM. Labelling of ECs before their co-culture with fibroblasts with EGFP (enhanced green fluorescent protein) by means of retroviral or lentiviral transduction, or using fluorescent markers, allows tracking of tube formation by time-lapse microscopy [11]. In the early stages of the co-culture, 3–4 days after plating, ECs appear flattened and have multiple protrusions. Over a period of 7 days, the ECs and fibroblasts proliferate, and ECs either segregate into cell clusters also referred to as ‘islands’ of ECs [11] from which sprouts emerge or remain single, adopt a bipolar morphology and migrate along cords of fibroblasts. The relative balance of these two events depends on the numbers of plated cells and the ratio of ECs and fibroblasts. Cell clustering and sprouting takes place earlier in the modified assay [8,9,13] of seeding ECs on to confluent fibroblasts (see the ‘Modification of the initial co-culture assay’ section below). As tube formation progresses, the tubules sprout, elongate and thicken, forming anastomosing structures [11].

Some VEGF (vascular endothelial growth factor) is secreted by the fibroblasts in the assay; however, tube formation can be stimulated further by the addition of exogenous VEGF (Figure 1), i.e. the assay can measure both inhibition and promotion of angiogenesis [6]. The major role of the fibroblasts in the assay is to secrete ECM components and growth factors. Importantly, the fibroblasts produce the matrix in which the tubules are embedded that is rich in collagen I and also contains fibronectin, tenascin-C, decorin and versican [6,14]. Unlike gels of Matrigel or purified collagen, the matrix produced in the co-culture assay is actively remodelled by the fibroblasts so that it contains fibrillar collagen, making its overall structure closer to in vivo ECM. This ECM is similar in composition to the provisional matrix of wound healing and tissue repair, reported to promote EC migration. Indeed, time-lapse microscopy reveals active migration of ECs and sprouting in the co-culture assay [11]. Strikingly, tubules in the co-culture assay produce numerous fine and long sensory filopodial projections (Figure 1) that protrude either at the tip of the tubules, resembling tip cell filopodia in vivo [15], or at lateral sites where they are associated with the generation of sprouts (G. Mavria, unpublished work).

Optical sectioning of the co-culture assay shows that it is three-dimensional, with a depth of three to five cells in the mature assay with tubules forming within layers of fibroblasts and ECM [14,16]. As tubules mature, they secrete a basement lamina that is rich in laminin and collagen IV [6,14] (Figure 1). At 14 days following co-culture of ECs and fibroblasts, the tubules become quiescent, and there is strong accumulation of VE-cadherin (vascular endothelial cadherin) at cell–cell junctions [8]. Accumulation of VE-cadherin at cell–cell junctions leads to an increase in actomyosin contractility that suppresses sprouting and promotes tubule quiescence [8].

Electron microscopy of mature tubules shows the presence of a lumen [6], which is taken as the true marker of the formation of capillary-like structures.

Modifications of the original co-culture assay

In the original protocol, HUVECs are mixed with NHDFs at a ratio that depends on the batch of ECs and fibroblasts and the desired basal level of tube formation. A major modification of the assay involves plating ECs directly on to fibroblasts that have been allowed to reach confluence over a period of 5–7 days following plating [11]. Seeding of ECs on confluent fibroblasts reduces the time necessary for basal tube formation to 5 days [11], while tubules establish and form lumens within 11 days of co-culture, thus significantly shortening the length of the assay. Although the ECs are plated on top of the fibroblast layer, the resultant tubes are embedded in three-dimensional matrix in a manner similar to the original 14-day co-culture assay [14].

The culture conditions of ECs and fibroblasts, detailed protocols for the original and modified assays, together with associated methods detailed below, can be found in the Supplementary Online Data at http://www.biochemsoctrans.org/bst/039/bst0391597add.htm.

Gene silencing using siRNAs (small interfering RNAs)

The 14 days of the original assay is too long to allow the use of siRNA oligonucleotide-mediated gene silencing. The modified assay of plating ECs on confluent fibroblasts allows a readout of basal tube formation to be taken at 5 days following plating of ECs, and we find that siRNA-mediated gene silencing is effective over this period [7–9,11]. ECs
are transfected with siRNA oligonucleotides using lipid-mediated transfection before plating in the assay [7–9]. We find that lamin is a useful control gene, as its silencing does not inhibit tube formation in this assay. The use of siRNA oligonucleotides provides a relatively low-cost and scalable basis for the design of screens for regulators of angiogenesis.

**Gene silencing using viral shRNAs (short hairpin RNAs)**

In order to determine the roles of proteins of interest in different stages of tube formation, it is necessary to achieve long-term stable silencing of genes. This can be achieved by stable transduction of ECs using recombinant retroviruses or lentiviruses harbouring shRNAs. Retroviral and lentiviral infection of ECs typically achieve transduction efficiencies of 30% and >80% respectively. Retroviral and lentiviral vectors harbouring shRNAs are commercially available, whereas shRNA lentiviruses from several commercial sources also harbour the EGFP gene, allowing modified ECs in the coculture assay to be followed in real time by time-lapse microscopy. Infection with lentiviruses harbouring EGFP should be at low multiplicity in order to avoid expression of high levels of EGFP that compromise the tube-forming ability of ECs in the assay. Following viral infection, EGFP-expressing cells can be FACS-sorted before their co-culture with fibroblasts. Recombinant retroviruses and lentiviruses can also be used to stably overexpress genes of interest, or their dominant-negative or constitutively active counterparts [11]. In addition to lentiviruses harbouring EGFP, we also use lentiviruses with the puromycin selection marker, which allows rapid selection of stably transduced ECs before their co-culture with fibroblasts and visualization of tube formation by CD31 staining.

**Quantitative analysis**

For quantification, we take low-magnification images of co-cultures stained for CD31 and using the high-contrast immunohistochemical detection reagent NBT (Figure 1). This allows for large areas of tubules to be quantified (cm²). There are several kinds of quantitative data that can be extracted from the assay. The most valuable parameter is the total tube length, which can be best calculated as total length of tubules per unit area. Tube number and average tube length can be misleading, since the tube number decreases as tubules become a highly connected network. Branching is another valuable parameter indicative of sprouting that can be calculated as the number of branch points per unit length. However, there is a caveat when counting branches as a measure of sprouting. Branches form in two ways: by sprouting from existing tubules (bifurcation), and from a lateral join between separate tubules (anastomosis). The second mechanism is influenced by the total tube length: if there are more longer tubules in the assay, then they are likely to form more connections. Consequently, in a dense culture, treatments that reduce tube length will reduce the average number of branches. Meaningful quantification of sprouting by counting the number of branches may be best carried out using co-cultures with fewer ECs, where tubules have a lower chance of forming connections by anastomosis. Alternatively, time-lapse microscopy may be employed to determine the effects of gene expression, silencing or pharmacological inhibition on sprouting angiogenesis in the co-culture assay. Another useful parameter is tubule thickness, which can be expressed as the total area of EC staining divided by the total tubule length. This readout gives a measure of site-to-site cell–cell adhesion that may give rise to lumens with extracellular hollowing [17]. Manual quantification of the above parameters is time-consuming, but accurate. Software is also available that semi-automates this process to give reliable results with careful monitoring (Angioquant [18]; Angiosys; TCS Cellworks).

**Visualization of tube formation by immunohistochemistry and immunofluorescence**

Tube formation in the co-culture assay is typically visualized by immunohistochemistry using high-contrast colorimetric stains such as NBT (Nitro Blue Tetrazolium) (Figure 1) or immunofluorescence using antibodies against PECAM-1 (platelet/endothelial cell adhesion molecule 1)/CD31. An advantage of the co-culture assay over other three-dimensional angiogenesis assays is the relative ease of high-resolution confocal microscopy. The matrix deposited by the fibroblasts is relatively thin, allowing rapid fixation and easy penetration of antibodies. The ECs are close enough to the coverslip to be within the focal range of high numerical aperture lenses, allowing performance of high-resolution confocal microscopy. In addition to imaging of fixed cells, the assay can be used to image angiogenesis in real time by time-lapse microscopy, as ECs can be transduced using viral vectors harbouring EGFP (see the previous section).

**Limitations of the assay**

We find two main limitations with the co-culture assay. The first comes from the presence of the fibroblasts. In the Matrigel assay and other matrix-based assays, ECs can be harvested and analysed by Western blotting and RNA samples can be extracted to measure gene expression by quantitative PCR. The presence of large numbers of fibroblasts means that this is not an easy task with the co-culture assay. Although it is theoretically possible to isolate the ECs from the assay by FACS, this is cumbersome and gives low yields of material (ECs represent <10% of the total cell number after 14 days of co-culture). This problem can be overcome by generation of a cell-free fibroblast matrix before the plating of ECs; however, we have found that ECs sprout, but do not form luminized tubules in a cell-free matrix, suggesting that fibroblasts are necessary for lumen formation potentially by remodelling the ECM.
The presence of fibroblasts can also complicate analysis by immunofluorescence, since it can be difficult to discriminate cellular structures belonging to the two different cell types. Even with large structures such as nuclei, it can be hard to be sure to which cell they belong. This problem can be overcome by using lentiviral transduction of GFP (green fluorescent protein)-tagged proteins before plating ECs on to the fibroblasts. The second issue concerns gradients of angiogenic growth factors. During developmental angiogenesis in the retina in vivo, ECs see a gradient of VEGF that polarizes ECs and controls the direction of migration of the growing vessels. We have not succeeded in incorporating a gradient into the co-culture assay because creating and maintaining a chemotactic gradient in vitro is technically difficult, although this may be possible by the use of small flow chambers. On the other hand, since pathological angiogenesis such as cancer angiogenesis has not been reported to involve chemotactic gradients, the co-culture assay may recapitulate better pathological rather than physiological angiogenesis. While the incorporation of chemotactic gradients in the co-culture assay is under development, angiogenesis assays in three-dimensional collagen gels offer the easiest way of studying gradient effects in vitro [19].

Conclusions
The organotypic co-culture assay is a powerful tool in angiogenesis research that recapitulates a remarkable number of the cellular processes undergone by ECs during in vivo tube formation including EC migration, assembly, sprouting, anastomosis and lumen formation. Consequently, it allows elucidation of the precise roles of proteins of interest in these different stages of angiogenesis in a visual and quantitative manner. In addition to this macroscopic analysis of tube formation, the unique strength of the co-culture assay in its amenity to high-resolution microscopy allows relatively easy analysis of subcellular processes important for angiogenesis.

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Co-culture assay protocols

Materials
Mouse monoclonal antibody against PECAM-1 (platelet/endothelial cell adhesion molecule 1)/CD31 is from R&D Systems (clone 9G11). Alexa Fluor-conjugated fluorescent secondary antibodies are purchased from Invitrogen. Alkaline phosphatase-conjugated secondary antibody (NB720-AP) is from Novus Biochemicals. Recombinant human EGF (epidermal growth factor) and bFGF (basic fibroblast growth factor) are from R&D Systems.

Cell culture
HUVECs are collected from umbilical cords as described previously [1] or purchased from Promocell or TCS CellWorks. ECs are maintained in commercial ECGM (endothelial cell growth medium) (Promocell or Large Vessel Endothelial Cell Medium; TCS Cellworks), or in DMEM (Dulbecco’s modified Eagle’s medium)/Ham’s F12 nutrient mixture (Sigma) containing 2% (v/v) heat-inactivated FBS (fetal bovine serum), 1 μg/ml hydrocortisone, 10 ng/ml EGF, 20 μg/ml heparin sulfate, 250 μg/ml insulin, 100 units/ml penicillin and 100 μg/ml streptomycin. Growth of ECs will be better in the commercial cell media. ECs may be cultured on plates coated with human fibronectin (Sigma #F0895) (10 μg/ml for 30 min) or directly on tissue culture plastic. It is important that cells are always maintained subconfluent as EC differentiation influences negatively the co-culture assay. NHDFs (PromoCell or TCS CellWorks) are maintained in DMEM with 10% (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml l-glutamine. ECs are used up to passage 6 for commercial HUVECs and fibroblasts are used up to passage 12. Use of cells beyond these passage numbers is not recommended, as the cells do not perform in the co-culture assay despite their normal appearance in monocultures.

Co-culture assay of ECs and fibroblasts
In the original assay, HUVECs and NHDFs are co-cultured at a ratio that depends on the desired basal levels of tube formation. We typically start with 10⁴ HUVECs that are co-cultured with 3×10⁴ NHDFs in a 24-well. The cells are initially co-cultured in a 1:1 mixture of DMEM with 10% (v/v) FBS and Large Vessel Endothelial Cell Medium and changed to Optimized Medium (TCS CellWorks) 4 days after initial co-culture. The assay takes 14 days and the medium is refreshed every 2 days.

Modified co-culture assay (plating of ECs on to confluent fibroblasts)
NHDFs are harvested with trypsin and collected in DMEM with 10% (v/v) FBS. NHDFs are diluted in ECGM to 3×10⁴ cells/ml and seeded directly on tissue culture plastic or on glass coverslips. The medium (ECGM) is refreshed 4 days after plating and HUVECs are plated on to the fibroblasts the next day. The NHDF should reach confluence 5 days after plating, depending on the batch and passage number. ECs are trypsinized and collected in DMEM/Ham’s F12 (or basal ECGM) containing 20% (v/v) FBS by centrifugation at 700 g for 5 min. The cell pellet is resuspended at 3×10⁴ cells/ml in complete ECGM and seeded on to the NHDF monolayer (3×10⁴ cells per six-well plate). The medium may be refreshed at 2 and 4 days following plating of ECs on to the fibroblasts and the co-cultures are fixed 5–6 days after plating. If Large Vessel Endothelial Cell Medium is used for the growth of ECs, NHDFs are grown to confluence in DMEM with 10% (v/v) FBS, and ECs are plated on to the fibroblasts in a 1:1 mixture of Large Vessel Endothelial Cell Medium and DMEM with 10% (v/v) FBS.

Preparation of co-cultures for quantification
Cells are fixed for immunohistochemistry 5–6 days after plating of ECs on to confluent fibroblasts. Fixation is carried out using 70% ethanol at −20°C for 30 min. Cells are washed with PBS between all fixation and antibody treatments. Cells are treated with 0.3% hydrogen peroxide in methanol for 15 min to remove endogenous alkaline...
phosphatase activity. ECs are then labelled with mouse anti-CD31 antibody (0.25 μg/ml) in 1% (w/v) BSA by incubation for 1 h at 37°C. Cells are washed and incubated with 0.6 μg/ml alkaline phosphatase-conjugated secondary antibody in 1% BSA for 1 h at 37°C. Cells are washed in water before adding the BCIP (5-bromo-4-chloroindol-3-yl phosphate)/NBT substrate (Sigma), and the stain is allowed to develop for 15–30 min at 37°C. Cells are then washed with water and air-dried before imaging the co-cultures by brightfield microscopy. Imaging without phase contrast gives best results.

**Preparation of co-cultures for immunofluorescence microscopy**

The co-cultures are routinely prepared for confocal microscopy by fixation in PFA (paraformaldehyde). The cells are washed three times in PBS and fixed in 4% (w/v) PFA in PBS for 15 min. The cultures are washed again and permeabilized in 0.2% Triton X-100 in PBS for 5 min. An optional last wash with 0.5% fresh sodium borohydride in PBS reduces autofluorescence. After three final washes in PBS, the cells are ready for incubation with antibodies. Alternatively, the cultures may be fixed in methanol. This method of fixation gives better preservation of microtubules and the endoplasmic reticulum; however, preservation of the actin cytoskeleton is suboptimal and a lot of soluble cytosolic material is lost. For methanol fixation, incubate the cells with 100% methanol pre-cooled to −20°C for 5 min at −20°C. Methanol also permeabilizes the cells, so the Triton permeabilization is not required. Following fixation, cells are incubated with primary antibodies for 1 h in 1% (w/v) BSA in PBS. The cells are then washed three times in PBS and incubated with secondary antibodies for 1 h in PBS. Following incubation with the secondary antibody, the cultures are washed three times with PBS before mounting.

**Using siRNA oligonucleotides in the co-culture assay**

ECs are seeded at 4 × 10^4 cells/ml on to fibronectin-coated six-well plates and incubated overnight. The following day, ECs are transfected with siRNA oligonucleotide duplexes using GeneFECTOR™ (Venn Nova). For transfection in a six-well plate, 4 μl of siRNA oligonucleotide (20 μM) and 6 μl of GeneFECTOR™ are each diluted in 250 μl of OptiMEM™ (Invitrogen) in two separate microcentrifuge tubes. The siRNA solution is then added to the GeneFECTOR™ solution and incubated for 5 min at room temperature. ECs are washed twice in OptiMEM™ and then left in 1 ml of OptiMEM™. The siRNA and GeneFECTOR™ solution is then added dropwise while swirling. After incubation for 3 h at 37°C, the ECs are harvested by trypsinization and seeded on to the confluent NHDFs at 3 × 10^4 cells/ml in ECGM. The co-culture is continued as described above.

**Using lentiviral and retroviral shRNAs in the co-culture assay**

For viral infection, ECs are seeded on to six-well plates as described above, and supernatants of retroviruses or lentiviruses harbouring shRNAs that have been harvested from virus-producing cells using standard methods are applied on to the ECs for 5 h in the presence of polybrene (8 μg/ml). The viral supernatants are then removed, the ECs are washed three times with PBS and the EC media are replenished. At 2 days after infection ECs stably expressing shRNAs together with EGFP or a selectable marker, are either FACS-sorted or selected using appropriate drug selection. Following FACS or drug selection, the ECs may be expanded further before co-culturing with the fibroblasts in the assay as described above. For lentivirus-mediated delivery of shRNAs, we routinely use the pGIPZ lentiviral backbone that also harbours EGFP (Open Biosystems) or the pLVX puro lentiviral backbone (Clontech).

**Reference**