Angiogenesis (2006) 9:111–125
DOI 10.1007/s10456-006-9037-x

ORIGINAL PAPER

Fine-tuning of a three-dimensional microcarrier-based angiogenesis assay for the analysis of endothelial-mesenchymal cell co-cultures in fibrin and collagen gels

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Received: 19 March 2006 / Accepted: 14 June 2006 / Published online: 19 October 2006

Abstract A prerequisite for successful tissue engineering is the existence of a functional microvascular network. We hypothesized that such networks can be created and quantified in an in vitro setting by co-culturing endothelial cells (ECs) with tissue-specific 'bystander cells' in 3-D gel matrices. To test this hypothesis we adapted a previously described in vitro microcarrier-based angiogenesis assay (V. Nehls and D. Drenckhahn, 1995, Microvasc Res 50: 311–322). On optimizing this assay, we noted that the initial EC-microcarrier coverage depended on EC type and seeding technique employed to coat the microcarrier beads with the ECs. A confluent EC monolayer on the microcarrier surfaces formed only when bovine aortic endothelial cells (BAECs) were admixed to the beads under gentle agitation on an orbital shaker. After embedding BAEC-covered microcarrier beads into a sandwich-like arrangement of collagen or fibrin gels, we assessed cellular outgrowth at different serum concentrations in terms of migration distance and sprout formation. Quantifiable sprout formation was highest at 1% fetal bovine serum (FBS) in collagen matrices and at 0.1% FBS in fibrin matrices. At higher serum concentration, excess cell migration and formation of clusters prevented quantitative analysis of sprouting. Following the fine-tuning of this angiogenesis assay, we co-cultured BAECs with adipose tissue-derived fibroblasts (FBs) and vascular smooth muscle cells (SMCs). While FBs were able to increase the average migration distance of BAECs in both matrices, SMCs enhanced BAEC migration in fibrin, but not in collagen gels. By contrast, the number of newly formed sprouts in fibrin gels was increased by both cell types. We conclude that in this model bystander cells enhance EC network formation in a matrix-dependent manner. Additionally, these results stress the importance of carefully selecting the experimental parameters of a given in vitro angiogenesis model.

Keywords Angiogenesis · Co-culture · Collagen gel · Endothelial cells · Fibrin gel · Fibroblast cells · Microcarrier · Serum concentration · Smooth muscle cells

Abbreviations

3-D	three-dimensional
AMD	average migration distance
BAEC	bovine aortic endothelial cell
bFGF	basic fibroblast growth factor (also FGF2)
DMEM	Dulbecco's modified Eagle's medium
EC	endothelial cell
ECM	extracellular matrix
ECGS	endothelial cell growth supplement
FB	bovine adipose fibroblast cell
FBS	fetal bovine serum
HAEC	human aortic endothelial cell
HMEC-1	transformed human microvascular endothelial cell
HMVEC	primary human microvascular endothelial cell

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PBS	Dulbecco's phosphate buffered salt solution
SMC	porcine pulmonary artery smooth muscle cell
rhEGF	recombinant human epidermal growth factor
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor

Introduction

In spite of great scientific and commercial efforts over the past decade, only few engineered tissue constructs are in clinical use to date. These products, such as artificial skin, are necessarily of limited thickness due to their reliance on passive diffusion to supply the grafted cells with oxygen until the neo-tissue becomes vascularized. In most other cases, the establishment of a functional vasculature is a pre-requisite for the successful engineering of larger tissue constructs. Thus, the formation of a vascular network within engineered constructs, is one of the greatest challenges in tissue engineering [1–3].

One approach to engineering vascular networks in vitro is to mimic the natural process of angiogenesis. Numerous in vitro angiogenesis models have been developed, including two-dimensional cell migration and proliferation assays and three-dimensional (3-D) organ culture assays. Two-dimensional assays, are overly simplistic and model only a few aspects of the angiogenic process, such as endothelial cell (EC) migration or proliferation, while organ culture assays are usually far too complex for mechanistic studies [4, 5]. In contrast, 3-D hydrogel-based assays combine advantages of both types of these assays. Gel-based assays are generally comprised of ECs cultured in natural extracellular matrix (ECM) protein-derived 3-D gel matrices and can mimic several key aspects of the angiogenic process in vitro, such as EC sprouting, migration, and tube formation. Collagen and fibrin are the two most commonly used ECM proteins for these assays. Both materials are well characterized as scaffolds for tissue engineering purposes and have been shown to support EC migration, proliferation, and differentiation [6–9]. In the first 3-D gel assay, Montesano and Orci [10] grew confluent monolayers of capillary endothelial cells atop a collagen gel. Upon stimulation with 4β-phorbol 12-myristate 13-acetate, the ECs invaded the underlying matrix and formed capillary-like structures containing a lumen.

Since their introduction, gel-based in vitro angiogenesis assays have been frequently modified to study the effects of co-cultured mesenchymal and tumor cells on EC activities. Fibroblasts secrete pro-angiogenic factors that enhance EC migration and/or induce tubular morphogenesis [11, 12], while EC migration and formation of capillary-like structures is inhibited by smooth muscle cells. However, these data are difficult to compare, since the observed effects depended on a variety of divergent assay parameters, such as cell–cell contacts, assay setup or the type of endothelial cells used [11, 13].

In 1995, Nehls and Drenckhahn developed a microcarrier-based angiogenesis assay, in which EC-covered Cytodex3^{TM} microcarrier beads were embedded into a 3-D fibrin gel and capillary-like structures formed in response to different angiogenic factors or signals emanating from the type of co-cultured cells [14, 15]. This assay has been well characterized [16–19] and is advantageous over other assays in that the radial outgrowth of the ECs offers a fast and simple means to quantify EC sprout formation. In this study, we modified the originally described technique of Nehls and Drenckhahn, and combined it with a more recent image analysis-based approach to quantify additional aspects of the angiogenic process. Vernon and Sage [20] described a method for improved quantification of migration in 3-D gels by employing a radial grid to measure the distances that cells had traveled from a source of aggregated ECs embedded in a gel. We surmised that a combination of these two techniques would enable us to simultaneously analyze sprout formation and cell migration.

In testing our working hypothesis that co-culture of tissue-specific 'bystander cells' with ECs in a 3-D matrix modulates EC migration and sprout formation, we compared the effects of two mesenchymal cell types, fibroblast and smooth muscle cells, on EC migration and sprout formation in the two most commonly used gel matrices, collagen type I and fibrin gel.

Materials and Methods

Materials

Bovine fibrinogen, bovine thrombin, Cytodex3^{TM} microcarrier beads, MCDB131 basal medium, hydrocortisone, recombinant human epidermal growth factor (rhEGF), endothelial cell growth supplement (ECGS), heparin, mitomycin C, and bisbenzimide (Hoechst 33258) were purchased from Sigma (St. Louis, MO). 10 × Dulbecco's phosphate buffered salt solution (PBS), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, and antibiotic-

antimycotic-solution were from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was from Hyclone (Logan, Utah). Anti VE-cadherin (sc-6458, goat polyclonal IgG) was from Santa Cruz (Santa Cruz, CA) and Alexa Fluoro 488-conjugated 488, donkey anti-goat IgG (A11055) from Molecular Probes (Carlsbad, CA). Rat tail collagen type I solution was purchased from BD Bioscience (San Jose, CA), as was basic fibroblast growth factor (bFGF). For some studies, highly purified and well-characterized bFGF (a kind gift from Dr. Philip Lazarovici; Hebrew University of Jerusalem, Israel) was used.

Cell culture

The isolation of bovine aortic endothelial cells (BAECs), human aortic endothelial cells (HAECs), porcine pulmonary artery smooth muscle cells (SMCs) and bovine adipose fibroblast cells (FBs) has been described previously [21]. Both the HAECs and BAECs used for the experiments described here were pooled from several independent isolations following purification of the primary isolate (to > 99%) by manual weeding and/or flow cytometric sorting [21, 22]. Subsequently, in order to minimize the differences between the various isolates, several batches of 2nd-passage cells from three individual preparations each were pooled. For the studies describe here, several vials of two different pools were used through passage 6–9 each. No differences were observed between the different pools and for the passages indicated.

Primary human dermal microvascular endothelial cells (HMVECs) were purchased from Clonetics (San Diego, CA). Immortalized human dermal microvascular cells (HMEC-1) were obtained from the Center for Disease Control (CDC, Atlanta, GA) [23]. HAECs and HMVECs were maintained in MCDB 131 basal medium, supplemented with 5% FBS, 10 ng/ml rhEGF, 1 μg/ml hydrocortisone, 1 U/ml heparin, 30 μg/ml ECGS, 0.3 mg/ml L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin and 125 ng/ml amphotericin B (modification of Clonetics' MGM-2 medium). HMEC-1 cells were cultured in MCDB131 basal medium supplemented with 10% FBS, 10 ng/ml rhEGF, 1 μg/ml hydrocortisone, 0.3 mg/ml L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin and 125 ng/ml amphotericin B (modified from [23]). Except where noted otherwise, BAECs were routinely grown in DMEM (1 g/l glucose) supplemented with 10% FBS, 0.3 mg/ml L-glutamine, 10 U/ml penicillin G, 10 μg/ml streptomycin and 25 ng/ml amphotericin B. In comparing adhesion of different endothelial cell types to microcarrier beads, BAEC were initially also adapted to and cultured in the same MCDB131-based medium as the other endothelial cells. However, since in our hands BAEC coverage of the beads was not affected by the different culture media (not shown) all subsequent experiments with BAECs, especially the serum concentration and co-culture studies, were carried out in the original DMEM-based medium described above.

Three of the four endothelial cell types used in this study were cultured in gelatin-coated flasks, with the exception of HMVECs, which were cultured in fibronectin-coated flasks only. FBs and SMCs were cultured in DMEM (4.5 g/l glucose) supplemented with 10% FBS, 0.3 mg/ml L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin and 125 ng/ml amphotericin B. Basal medium for the angiogenesis assays consisted of DMEM (1 g/l glucose), 10% FBS, 0.3 mg/ml L-glutamine, 10 U/ml penicillin G, 10 μg/ml streptomycin and 25 ng/ml amphotericin B. For co-culture studies, this medium was additionally supplemented with 1 U/ml heparin. All angiogenesis assays were conducted with BAECs between passages 6–9. For co-culture studies, FBs and SMCs between P6 and P9 were growth arrested by treating the cells for 1 h with 25 μg/ml mitomycin C. All cells were cultured in a humidified incubator at 37°C and a CO_2 level of 5%.

Microcarrier preparation

Gelatin-coated Cytodex3^{TM} microcarrier beads were hydrated, according to the manufacturer's protocol. Briefly, 500 mg of Cytodex3^{TM} microcarrier beads were swollen for three hours in 100 ml 1 × PBS, the hydrated beads were washed, resuspended in 25 ml 1 × PBS and then autoclaved at 121°C for 15 min. The bead suspension was stored at 4°C.

Microcarrier seeding

Four different methods for seeding ECs on microcarrier beads were tested. For the 1st method (*original method)*, described by Nehls and Drenckhahn [14], 1.8×10^5 ECs and 6×10^3 beads were suspended in 2 ml medium and incubated for four hours under static conditions. After the addition of 28 ml fresh medium, the suspension was gently agitated. For the 2nd method (*monolayer method)*, 6×10^3 beads were added to a monolayer of ECs grown in a 25 cm^2 tissue culture flask. The ECs were allowed to migrate onto the beads, before the cell-covered microcarriers were mechanically dislodged from the flask [1]. For the two other methods, 1.2×10^6 ECs were mixed with 6×10^4 Cytodex3^{TM} microcarrier beads in 30 ml of basal medium. Beads and cells were either incubated under

static conditions (3rd method: *static method*) or cultured under gentle agitation on an orbital shaker (Stovall Life Science, Greensboro, NC; 4th method: *dynamic method*) in the incubator. For visualization of the degree of monolayer formation, EC-covered microcarriers were washed with 1 × PBS and fixed with 5% formaldehyde. The nuclei of attached ECs were then stained with 10 μg/ml bisbenzimide (Hoechst 33258) for microscopic evaluation (Leica DMRX microscope, 360/460 UV filter settings, 10 × objective, Leica DC 300F camera). The number of ECs per microcarrier bead was determined, as was the radius of the microcarrier beads.

Immunostaining of EC–EC contacts

BAEC-covered microcarrier beads were washed thrice with 1 × PBS and fixed with 5% formaldehyde. Immunostaining was performed as previously described [24]. Cells were stained with a polyclonal anti-VE-cadherin antibody at a concentration of 1:250 and visualized with Alexa Fluoro 488 conjugated secondary antibody (anti-goat IgG) at a 1:1500 dilution. Digital images were acquired, as above, using the FITC filter settings.

Gel preparation

For collagen gels, a collagen type I solution with a final concentration of 3 mg/ml was prepared according to the recommendations of the manufacturer. Briefly, depending on the final volume required for each experiment, calculated amounts of 10 × PBS, 1 N NaOH, cell culture-grade H_2O, and collagen were mixed and the pH was adjusted under sterile conditions to a value of 7.4 ± 0.05 using a pH electrode (Fisher Scientific, Pittsburgh, PA). For fibrin gels, a fibrinogen solution with a final concentration of 10 mg/ml was prepared by layering the fibrinogen powder onto prewarmed 1 × PBS. The solution was gently agitated for 2 h at 37°C (until the fibrinogen was dissolved), passed through a 0.2 μm filter and the pH was adjusted to 7.6 ± 0.05. Both solutions were used immediately.

Microcarrier-based angiogenesis assay setup

All our assays were 'sandwich assays', i.e., they were comprised of at least two layers of gels, which were poured sequentially. The medium used for gel formation, DMEM w/o sodium bicarbonate, enabled us to maintain a constant pH of the protein solutions during all manipulations outside the incubator; a vital criterion for EC sprouting in fibrin gels [25, 26]. All gel layers were comprised of a 1:1 (v:v) mixture of the protein solutions and serum-free bicarbonate-free DMEM basal medium to create collagen and fibrin gels at final concentrations of 1.5 mg/ml and 5 mg/ml, respectively. In preliminary experiments, we determined that this higher concentration of fibrinogen was necessary to prevent excess lysis of the fibrin gels by the bystander cells, thus avoiding the use of the serine protease inhibitor aprotinin, which might interfere with the ability of the ECs to migrate and proliferate.

To generate the first layer, 400 μl of the protein solutions were added to each well of a 24-well plate (800 μl for 12-well plates) and allowed to gel for 15 min at 37°C. For the second gel layer, the EC-covered microcarrier beads were transferred into one volume of serum-free medium (100–150 beads/ml), which was then mixed with the respective protein solutions. Five-hundred microliter (1 ml for 12-well plates) of this suspension was added on top of the first layer. The microcarriers were evenly distributed by a brief agitation of the culture plates. The culture plates were then incubated for 15 min in a hybridization oven (37°C).

For co-culture studies, the volume of the second gel layer was halved and overlaid with an additional 250 μl (500 μl for 12-well plates) of a third layer containing the growth-arrested bystander cells at a final concentration of 2×10^5 cells/ml (Fig. 1). The control groups consisted of a cell-free third gel layer. For the fibrin gels, cleavage of fibrinogen and gel polymerization were induced by addition of thrombin during each step at a concentration of 1 U/ml. After complete polymerization of the various gel assemblies, 1 ml (2 ml for 12-well plates) of complete bicarbonate-buffered basal medium, supplemented as above, was added to the sandwich-like gel assemblies. The cells were then cultured for up to eight days in a conventional CO_2

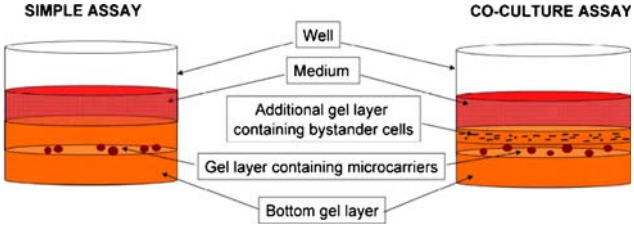

Fig. 1 Overview of the microcarrier-based angiogenesis assay: The assay was comprised of several gel layers. The bottom layer served as a "coating" to ensure a strictly three-dimensional environment for various experiments. The cell-covered microcarrier beads were contained in the second layer. The assay for the co-culture experiments (right side) was comprised of an additional third layer, in which 2×10^5 growth-arrested mesenchymal cells were uniformly dispersed. For details see Materials and Methods section

incubator (5% CO_2, 37°C). The medium was changed every other day.

Analysis of EC sprouting and migration

Within the first hours after assay assembly, the well plates were placed onto the stage of an inverted Nikon TE-2000U microscope equipped with a computer-controlled *x*–*y*–*z* positioner. In order to monitor the process of EC migration/sprouting in each well, individual microcarrier beads were selected randomly; each of these beads was at least 300 μm away from the nearest neighboring bead to avoid EC–EC interference. The positions of the microcarrier beads were noted. Microphotographs of the same microcarrier beads were taken sequentially at different time points (day 0, day 4, day 8), using 4 × and 10 × objectives. The micrographs were evaluated by counting sprouts and analyzing the migration distances from the microcarrier surfaces (Fig. 2). A sprout was defined as a structure originating from the microcarrier bead surface that was > 50 μm in length. Migration distance measurements were performed as described by Vernon and Sage [20] with some modifications. In brief, a radial grid, consisting of 36 radii, originating from the center of the bead, was overlaid over each micrograph. On each radial line, the distance of the EC that had migrated furthest into the surrounding gel matrix was recorded, all 36 distances were averaged and the radius of the bead was subtracted to yield the migration distance for that particular bead.

Statistical analysis

All assays were repeated independently at least three times. For each experiment, migration/sprouting data from approximately 20 beads per condition were assessed. All values were averaged and expressed as mean ± SEM (standard error of the mean). Statistical analysis (one-way ANOVA, Student's *t*-test) to compare the differences between the test groups was performed using Microsoft Excel. Measurements were deemed statistically significant if $P < 0.05$.

Results

Endothelial cell attachment to microcarrier beads

Complete cell-coverage of the microcarrier bead surfaces is an essential requirement for the validity of any microcarrier-based in vitro angiogenesis assay. Only under these conditions can the cell layer covering the entire bead surface mimic the confluent EC monolayer that is present at the beginning of angiogenic sprouting in vivo. In selecting a suitable cell type for our assay, which yielded confluent monolayers on the microcarrier bead surfaces, we studied four different seeding techniques and four distinct EC types. The methods for seeding the microcarriers were (i) the original method described by Nehls and Drenckhahn [14], (ii) a dynamic method, (iii) a static admixing method, with

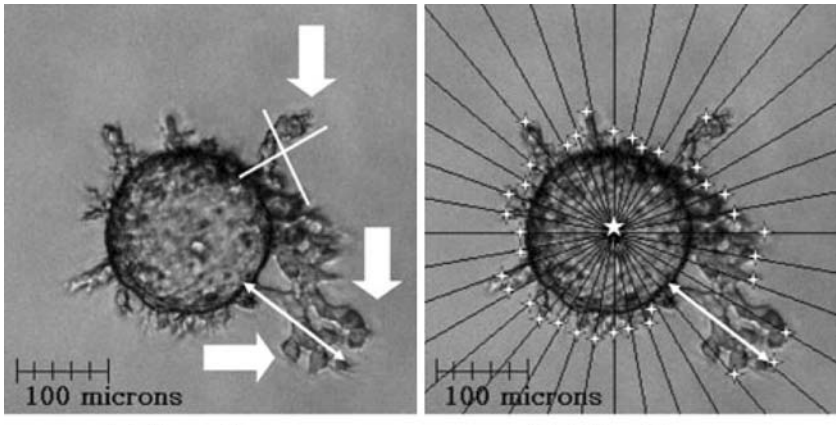

Fig. 2 Analysis of radial outgrowth: Endothelial cell invasion was characterized by two parameters: (**a**) Number of sprouts per microcarrier bead, and (**b**) Average migration distance (AMD) for different experimental conditions. The number of sprouts was determined by counting all clearly distinguishable multicellular sprouts > 50 μm for each microcarrier bead. EC migration was measured utilizing a radial grid of 36 radii centered over the microphotograph of the microcarrier. On each radius the position of the leading migratory EC was marked and the distance to the midpoint mark was measured. The values were averaged and the radius of the bead subtracted yielding the migration distance of the investigated microcarrier. The mean for all microcarriers cultured under the same conditions corresponded to the AMD

(ii) and (iii) being variations of the original method, and (iv) an alternative seeding technique, in which the microcarrier beads were placed on top of a confluent monolayer of ECs grown in a tissue culture flask. The four endothelial cell types tested were primary human aortic ECs (HAECs), bovine aortic ECs (BAECs), human microvascular ECs (HMVECs), and an immortalized primary human microvascular EC line (HMEC-1s).

The average number of ECs attached to the surfaces of individual microcarrier beads for each of these conditions was assessed four days after microcarrier seeding. In these experiments, we found that the optimal seeding method was dependent on cell type and that the tested cell types exhibited differential affinities for the microcarrier bead surfaces (Fig. 3). In general, BAECs attached to the microcarrier beads much more readily than the other endothelial cell types that were all of human origin. After four days of culture, we counted, on average (across all seeding methods), 14 ± 1 HMVECs, 22 ± 1 HAECs and 65 ± 5 HMEC-1s per microcarrier, and 208 ± 9 cells per bead for BAECs. For the human cells, the most efficient seeding was achieved by allowing the immortalized HMEC-1s to crawl onto the beads from a confluent monolayer (Fig. 3b). Optimal seeding of HAECs was also achieved using this method, but HMVECs attached to and grew best on the beads when the static seeding method was used (Fig. 3c). It is noteworthy that the attachment of BAECs to the microcarriers in comparison to the three other cell types was only minimally dependent on the seeding method used.

Of all EC types tested, BAECs consistently yielded the highest attachment to the beads and were the only cell type to form a confluent monolayer on the microcarrier surface within 2–4 days of culture. Fig. 4 shows a typical BAEC-covered microcarrier bead. The confluence of the BAEC monolayer on the bead surface is clearly visible both under phase contrast visualization (Fig. 4a) and, especially, after staining the EC boundaries with an antibody to VE-cadherin (Fig. 4b). Since three (original, static and dynamic) of the four seeding techniques resulted in comparable attachment of BAECs ($P > 0.05$) to the microcarrier beads, the appropriate seeding method was chosen by also taking into account other parameters. We note that both the initial attachment of BAECs to and the rapid formation of a confluent monolayer on the microcarrier beads were independent of the medium in which they were cultured. We did not observe significant differences in bead coverage irrespective of whether the cells were cultured in DMEM (their regular growth medium) or in a MCDB 131-based medium, as used for the other EC types (data not shown).

An essential prerequisite for the analysis of EC migration and sprouting is to start with individual EC-

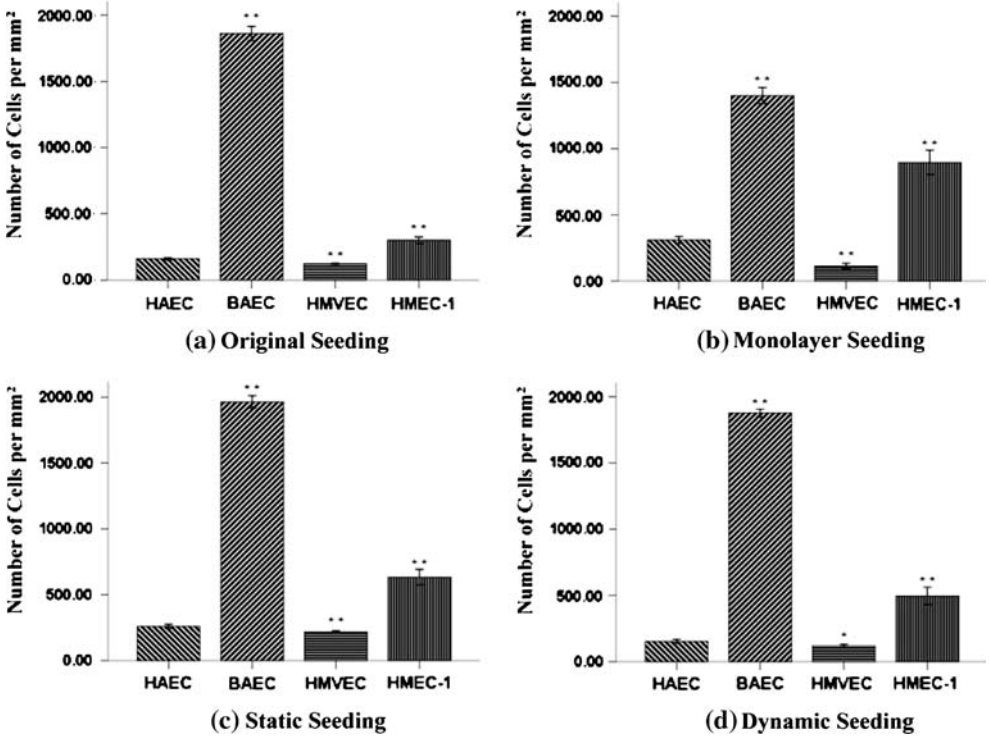

Fig. 3 Cell type- and seeding technique-dependent variation of cell adhesion to microcarrier beads: Microcarrier beads were seeded with four different endothelial cell types using four different seeding techniques (**a**–**d**), for details see Materials and Methods. Four days after initial seeding, the EC-covered microcarriers were fixed and fluorescently stained. The bisbenzimide (Hoechst 33258) stained nuclei were counted for 25 microcarriers, and the bead radius was determined to calculate the number of cells/mm^2 microcarrier surface. Bars represent means ± SEM. *$P < 0.05$, **$P < 0.01$ (compared to HAECs)

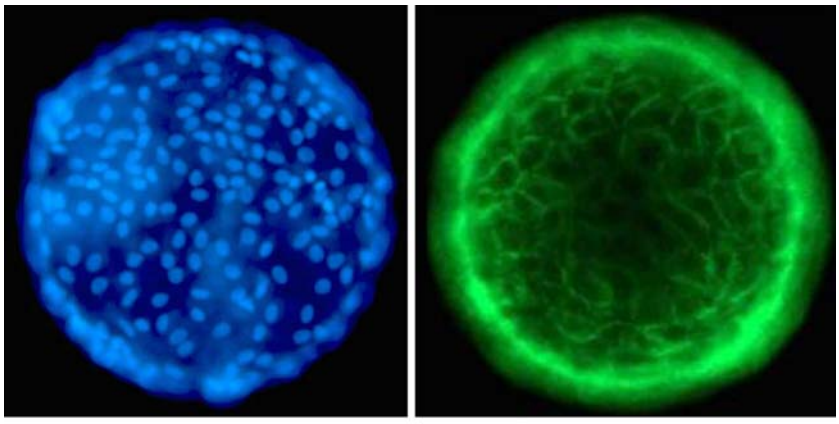

Fig. 4 Monolayer formation of BAECs on Cytodex3™ Microcarrier beads: BAECs and microcarrier beads were admixed and cultured under constant agitation for four days, before BAEC-covered microcarriers were fixed and cells attached to microcarrier surfaces were visualized by staining (**a**) nuclei with 10 μg/ml Hoechst 33258, (Bisbenzimide, BBZ) and (**b**) EC–EC junctions with a VE-cadherin specific antibody followed by a secondary antibody coupled to Alexa Fluoro 488. Large numbers of BAECs attached to the microcarrier bead surface. The presence of VE-cadherin at the cell borders verified the existence of close cell–cell contacts and suggested the establishment of a confluent EC monolayer. Original magnification: 100×

covered microcarrier beads. Using the original and the static seeding techniques, BAEC-lined microcarrier beads frequently aggregated during the initial culture phase, forming large aggregates (> 5–10 beads), resulting in inhomogeneous cell coverage. By contrast, during dynamic seeding, the majority of the microcarrier beads remained single, with only occasional formation of small aggregates of 2–3 microcarriers. Therefore, BAEC-covered microcarrier beads seeded under constant agitation were selected as the model for all subsequent studies.

Effects of serum concentration on EC migration and sprouting in 3-D matrices

To avoid masking the EC responses to exogenous growth factors and co-culture conditions by serum-contained factors, experiments are usually carried out at low serum concentrations [15]. In order to assess the effects of serum per se on 3-D EC migration and sprout formation in collagen and fibrin gels, we tested four different serum concentrations (0%, 0.1%, 1% and 10% FBS). As seen in Fig. 5a, in the collagen gel system, BAEC migration was proportional to the serum concentration. In serum-free medium, the average migration distance (AMD) was 35 ± 2 μm and increased only slightly, though significantly ($P < 0.05$), in the presence of 0.1% FBS. In 1% serum the AMD increased to 144 ± 5 μm, further increasing in 10% serum to 197 ± 9 μm ($P < 0.01$). Most BAECs migrated primarily as individual cells. We note that in the collagen gels (at all tested serum concentrations), the cells migrated preferentially individually rather than collectively (sprouting) (Fig. 6a–d). Due to the dense cellular outgrowth at 10% FBS (Fig. 6d), sprout formation could only be evaluated at lower serum concentrations. The number of sprouts per microcarrier and the sprout length at 0% and 0.1% FBS were comparable (approx 2 sprouts/bead, $P > 0.05$). For a serum concentration of 1%, the number of sprouts increased more than two-fold ($P < 0.01$) (Table 1). The sprout length increased only slightly (by approximately 14%) from 0% to 1% FBS ($P < 0.05$).

The effects of serum on the AMD of BAECs in fibrin gels were quite distinct from those in collagen gel (Fig. 5b). In the absence of serum, there was only minimal EC outgrowth from the microcarrier beads. The average migration distance in fibrin gels increased 3- and 20-fold when raising the serum concentration from 0% to 0.1% and 1%, respectively, with no further increase at 10% FBS. In serum-free medium, or at 0.1% serum, cell migration was largely collective, a prerequisite for sprout formation (Fig. 6e–h), while at serum concentrations of ≥ 1%, BAECs migrated both individually and collectively. Similar to the case for collagen, at elevated serum levels the observation and quantitative evaluation of distinctive outgrowth features in the fibrin gels was often thwarted due to the high numbers of cells surrounding each microcarrier bead (Fig. 6g–h). Therefore, sprout formation in fibrin gel could not be evaluated at 1% and 10% FBS. At a serum level augmentation from 0% to 0.1% FBS the

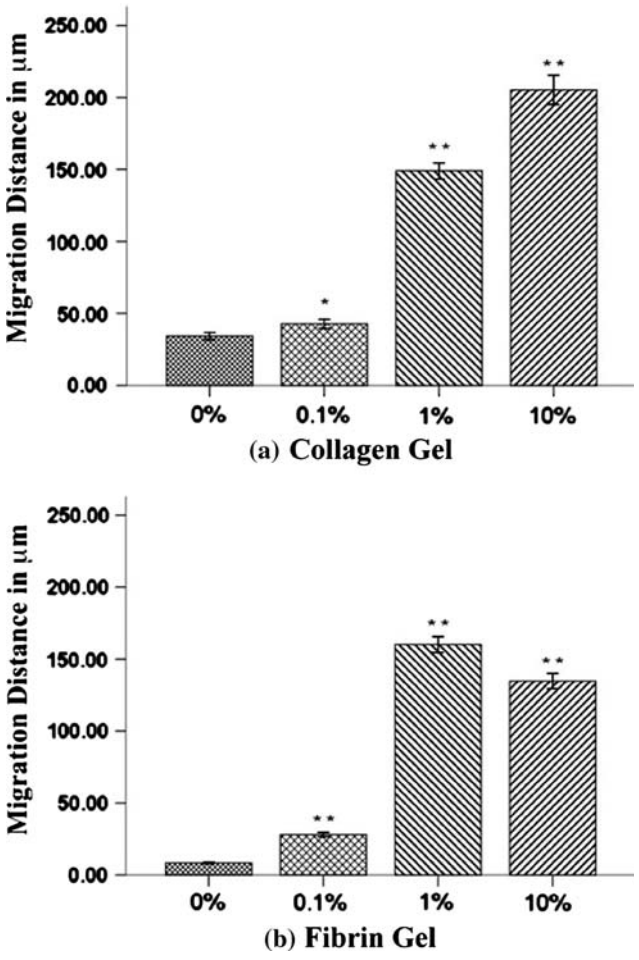

Fig. 5 Serum concentration effects on the AMD of BAECs in collagen and fibrin hydrogels: The AMD was measured after four days of culture of hydrogels containing BAEC-covered microcarrier beads in basal medium supplemented with different concentrations of FBS. The serum-induced changes were different for collagen and fibrin hydrogels. While the AMD increased steadily with an increase in serum levels for collagen, in fibrin gels the AMD increased only up to a FBS concentration of 1%. Thereafter, the AMD decreased with an increase in FBS concentration. Bars represent mean ± 1 SEM. *N* (from five replicates) ranged from 95 to 110 in collagen gels; in fibrin gels *N* (from five replicates) ranged from 95 to 105. $*P < 0.05$, $**P < 0.01$ (as compared to baseline values at 0% FBS)

number of sprouts increased from 1.0 ± 0.2 to 3.4 ± 0.3 sprouts per bead (Table 1), while the average sprout length did not change significantly.

In order not to confound the effects of bystander-derived angiogenic agonists/antagonists by the complex responses of the cells to serum, the subsequent co-culture studies in collagen and fibrin gels were conducted at reduced serum concentrations of 1% and 0.1%, respectively; these serum concentrations supported similar extent of sprout formation in the two gel systems.

Effects of mesenchymal cells on EC migration and sprouting in different matrices

Mesenchymal cells, such as smooth muscle cells and fibroblasts, are integral to the biological process of angiogenesis in vivo. Therefore, we hypothesized that the modulatory effects of these cells can also be observed in a 3-D in vitro angiogenesis model, which in addition to ECs also comprises some of these tissue-specific bystander cells. The influence of two mesenchymal cell types (bovine adipose fibroblast cells (FBs) and porcine pulmonary artery smooth muscle cells SMCs) on endothelial cell migration and sprouting was studied in both collagen type I and fibrin hydrogels. As seen in Fig. 7a, in collagen gels only bFGF (positive control) and FBs were able to significantly increase the AMD of BAECs from 150 ± 9 µm (negative control) to 286 ± 8 µm and to 253 ± 10 µm, respectively ($P < 0.01$), while SMCs failed to induce any observable increase in the AMD ($P > 0.05$). In the absence of bystander cells or in the presence of SMCs, the migrating ECs exhibited a mainly rounded morphology (Fig. 8a, d). By contrast, when cultured in the presence of bFGF or upon co-culture with FBs, EC outgrowth was denser and the cells were more elongated, precluding a more quantitative analysis of sprout formation, even though sprout formation could be observed (Fig. 8b, c).

In fibrin gels, FGF caused only a small (approx. 20%), albeit significant ($P < 0.05$), increase in EC migration (Fig. 7b). By contrast, the AMD of BAECs in the presence of either FBs or SMCs was increased by 40% from 30 ± 2 µm to 42 ± 2 µm ($P < 0.01$). This difference was also visible in the migration mechanisms. In the control groups (w/o bystander cells), BAECs migrated both individually and collectively (Fig. 9a, b). By contrast, co-culture with the bystander cells resulted in collective cell migration and enhanced sprout formation (Fig. 9c, d). In comparison to the control group, the average number of sprouts per bead increased 2.5-fold in co-cultures with FBs ($P < 0.01$), and about two-fold ($P < 0.01$) in the presence of smooth muscle cells. Basic FGF did not significantly change either the number of sprouts per bead or the average length of sprouts ($P > 0.05$) (Table 2).

In our model, the virtual lack of an effect of bFGF in fibrin gels on EC migration distance and sprouting might have been due to insufficient sprout stabilization. When cultured in the presence of bFGF, initial BAEC sprout formation occurred more rapidly than in ECs co-cultured with either FBs or SMCs. However, bFGF-induced sprouting was transient in nature; the sprouts

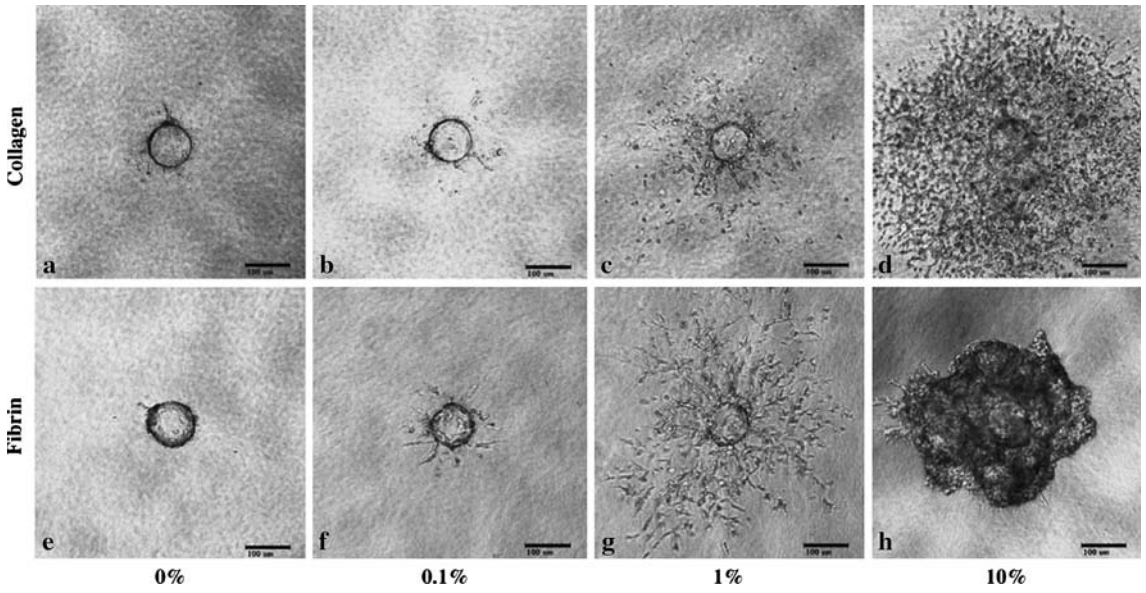

Fig. 6 Outgrowth pattern of BAECs in hydrogels under different serum concentrations: After four days of culture, ECs readily invaded the surrounding collagen matrix (1.5 mg/ml) using individual and collective cell migration strategies. Remarkably, many BAECs invading the surrounding collagen matrix exhibited a rounded shape. BAECs invading fibrin gels (5 mg/ml) showed a more elongated morphology. In fibrin gels, EC migration distance was usually smaller than in collagen gels (exception 1% FBS). Elevated proteolytic activities of ECs might also explain the very condensed cellular outgrowth of BAECs cultured in fibrin matrices at 10% FBS. Bars represent 100 μm

Table 1 Average number of sprouts per bead and their average length depended on the serum concentration of the basal medium

Condition	Sprouts per bead in collagen gel	Sprouts per bead in fibrin gel	Average sprout length in μm in collagen gel	Average sprout length in μm in fibrin gel
0% FBS	1.7 ± 0.2	1.0 ± 0.2	94.2 ± 4.3	79.6 ± 4.3
0.1% FBS	1.7 ± 0.2	3.4 ± 0.3**	102.9 ± 5.5	84 ± 2.8
1% FBS	4.2 ± 0.2**	NA	107 ± 4.0*	NA
10% FBS	NA	NA	NA	NA

BAEC-covered microcarrier beads were cultured in either collagen or fibrin gel matrices for four days under different serum supplementations, before sprout formation was analyzed. The average number of sprouts per bead and the average sprout length increased with an increase in FBS concentration. Radial EC invasion under high serum levels (at least above 1%) led to accumulation of cells nearby the microcarrier, impairing analysis of sprouts numbers. *N* (from three replicates) ranged in collagen matrices from 72 to 79 and in fibrin hydrogels from 48 to 51. $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to 0% FBS

had largely disintegrated by day 4. Hence, the effects of bFGF on sprouting could not be adequately assessed under our experimental conditions. The destabilization of EC sprouts could also be observed in co-cultures at day 8 (Fig. 10a, b). The average number of sprouts at day 8 was reduced drastically compared to the values at day 4 for all groups (unpublished observation).

Taken together, these results support the hypothesis that EC sprouting, as a prerequisite for vascular network formation, can be enhanced by co-culturing ECs with mesenchymal bystander cells. In addition, our data strengthen the notion that EC sprouting does not depend solely on the mesenchymal cell types, but also on the 3-D matrix utilized in the study.

Discussion

The in vitro microcarrier-based angiogenesis assay was originally introduced as a system for fast quantification of angiogenic sprouting [14]. We modified the original model, in order to apply this assay for assessing in vitro angiogenesis in co-cultures with mesenchymal cells. With the optimized system, we investigated the effects of fibroblasts and smooth muscle cells on EC migration and sprout formation in two different 3-D matrices. Our data suggest a critical role for both the mesenchymal cell type and the matrix composition for EC migration and sprouting. We stress that the main purpose of the methodological studies reported here was

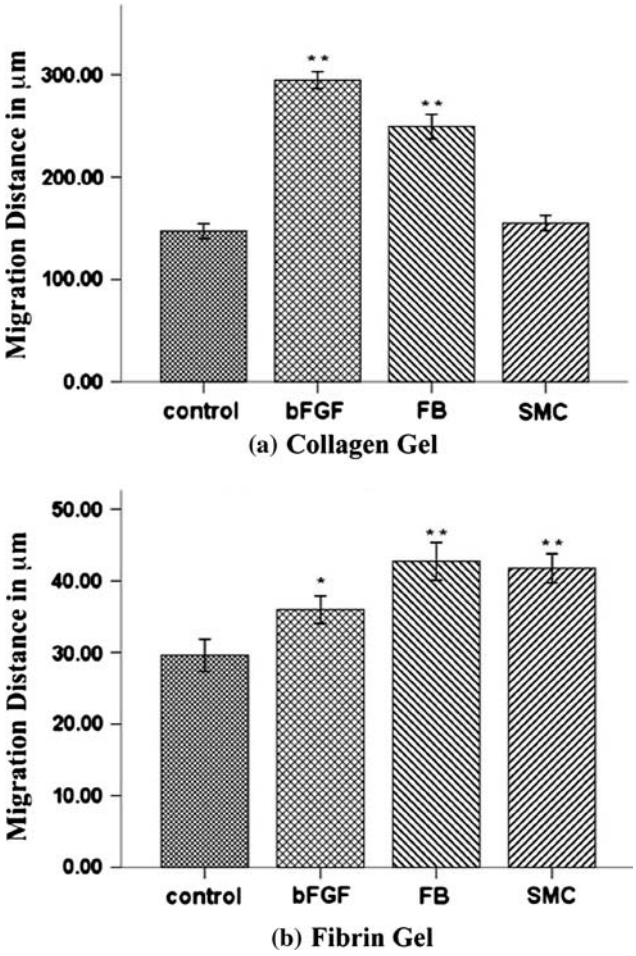

Fig. 7 Co-culture effects on the AMD of BAECs in collagen and fibrin hydrogels: The AMD depended on bystander cell type and 3-D hydrogel composition. The AMD was measured four days after co-culture assay setup for both gel matrices. The effects of FBs and SMCs were different for collagen and fibrin gel matrices. While an increase in AMD was induced by FBs for collagen gels, in fibrin gels the AMD increased also in the presence of SMCs. Interestingly, bFGF (positive control) affected the AMD of BAECs in collagen more profoundly than in fibrin gels. Bars represent mean ± 1 SEM. *N* (from four replicates) ranged from 70 to 84 in collagen gels; in fibrin gels *N* (from four replicates) ranged from 66 to 84. Label $*P < 0.05$, $**P < 0.01$ (compared to baseline values of negative control)

to adapt a previously described in vitro angiogenesis assay [14] for co-culture studies, rather than to address mechanistic issues. Having optimized and validated our model system, the mechanism of EC-parenchymal cell interactions is the focus of ongoing studies in our laboratory.

Endothelial cell attachment to microcarrier beads

A confluent monolayer is a critical requirement for the initiation of angiogenesis in any realistic in vitro model, since in situ ECs are in close contact prior to the initiation of neovascularization [27]. In our hands, establishment of the confluent monolayer on the microcarrier bead surface depended on both the EC type and the microcarrier seeding method. For the initial comparison of EC adhesion to the culture beads, all of the primary cultures were used at similar low passage numbers, based on prior observations (by others and us) that low passage ECs maintain many of their original heterotypic traits found in situ and hence, are useful for angiogenesis assays [9, 21]. Specifically, in the case of BAECs, we also established in preliminary studies that different culture media (MCDB131 vs. DMEM) did not significantly affect the ability of the cells to adhere to the microcarrier beads and form confluent monolayers (data not shown).

Different degrees of EC coverage on the microcarrier bead surface could be caused either by differences in the initial EC attachment to, or by differential cell growth on, the microcarrier surfaces. Differences in the ability of the various cells to establish monolayers on microcarrier beads could be due to the cell type, viz. macrovascular vs. microvascular, or primary vs. transformed. Such differences could manifest themselves in the degree by which these cells express cell–cell and/or cell–substrate adhesion molecules, and hence affect the initial adhesion to the bead surfaces. Indeed, in preliminary studies we found significant differences in the integrin profiles of endothelial cells derived from various anatomic locations and divergent species (S. Sarkar, C. Mancinkiewicz, and P.I. Lelkes, unpublished observations). At this point, we cannot clearly distinguish between the relative importance of initial adhesion versus the subsequent cell growth, since we only determined the number of cells covering the beads once after four days. We note that another group that used this bead-based angiogenesis assay also changed the initial seeding method, employing a complex mixture of static and dynamic seeding [17]. Taken together, we conclude that the method by which ECs are seeded onto microcarrier beads profoundly affects cell attachment and/or growth, and hence has to be selected carefully.

In our hands, BAECs were the only EC type that consistently formed a confluent monolayer on the microcarrier surface. These cells are derived from the aorta, which is usually not involved in angiogenesis. The usefulness of large vessel-derived ECs as models for angiogenesis is controversial [18, 28, 29]. For example, Bastaki et al. [28] reported capillary-like structure formation by murine brain and heart microvascular ECs in fibrin matrices, while aortic ECs did not form these structures. Similarly, Cavallaro et al. [29] reported impaired lumen formation by bovine

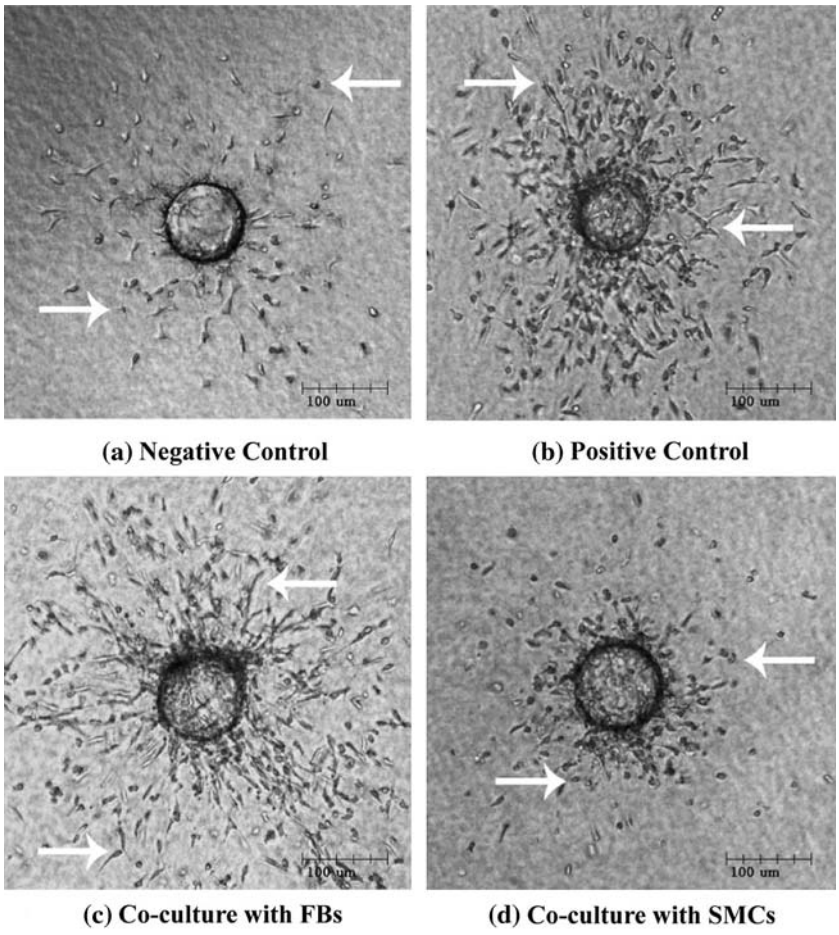

Fig. 8 Outgrowth patterns of BAECs co-cultivated with FBs and SMCs in collagen hydrogels: ECs readily invaded the surrounding collagen matrix (1.5 mg/ml) using individual and collective cell migration strategies. Sprouts formed by BAECs were multicellular (confirmed by bisbenzimide staining). On day 4, 20 ng/ml bFGF (positive control) and FBs induced enhancement of cell migration and proliferation. SMCs had no significant effect. High density of EC numbers around the microcarrier prevented sprout formation analysis. Arrows in subfigure (**a**) and (**d**) mark characteristic rounded EC phenotypes. In subfigure (**b**) and (**c**) arrows point to elongated ECs. Bar represents 100 μm

aortic endothelial cells in a collagen sandwich assay. By contrast, several other groups have successfully used large vessel-derived ECs, specifically BAECs, for in vitro angiogenesis studies, describing both invasion and capillary-like structure formation [14, 30]. These experiments, while attesting to the usefulness of large vessel endothelial cells for these kinds of angiogenesis models, also stress the variability of cell activities depending on cell origin and experimental setup.

The effects of serum concentration on ECs in 3-D matrices

The presence of serum influences many aspects of cell physiology, such as growth, proliferation and migration. For co-culture studies, a decrease in serum concentration is desirable, since serum contains many ill-defined factors in variable amounts, which in some instances may either enhance or antagonize endothelial cell differentiation induced by factors derived from bystander cells [31]. To the best of our knowledge, the effects of the serum concentration on angiogenesis assays have rarely been studied in great detail. For example, Go et al. [31] investigated angiogenic activity at very low serum concentrations in fibrin gels using the aortic ring assay and observed spontaneous angiogenesis in serum free minimal medium after 12 days of culture. Serum supplementations (1–3% FBS) resulted in a dose-dependent stimulation of angiogenesis. Our results confirm in part the data by Go et al. *viz*. the occurrence of spontaneous migration and sprouting in the absence of serum in collagen, but not in fibrin gels. However, an important difference between the two systems is the inevitable presence of fibroblast and smooth muscle cells in the aortic ring assay, which might have influenced EC behavior. Taken together, our data suggest that the serum concentration affects EC migration and sprout formation; these effects, however, are matrix-dependent. While in collagen the migration distances increased steadily with increasing FBS levels, the effects of serum in fibrin gels plateaued at ~1% FBS (Figures 5 and 6). This observation might be explained by differences in the major proteolytic/matrix degrading systems affecting the gel systems [32]. For example, Collen et al. [33] studied fibrin matrices containing 10% collagen type I

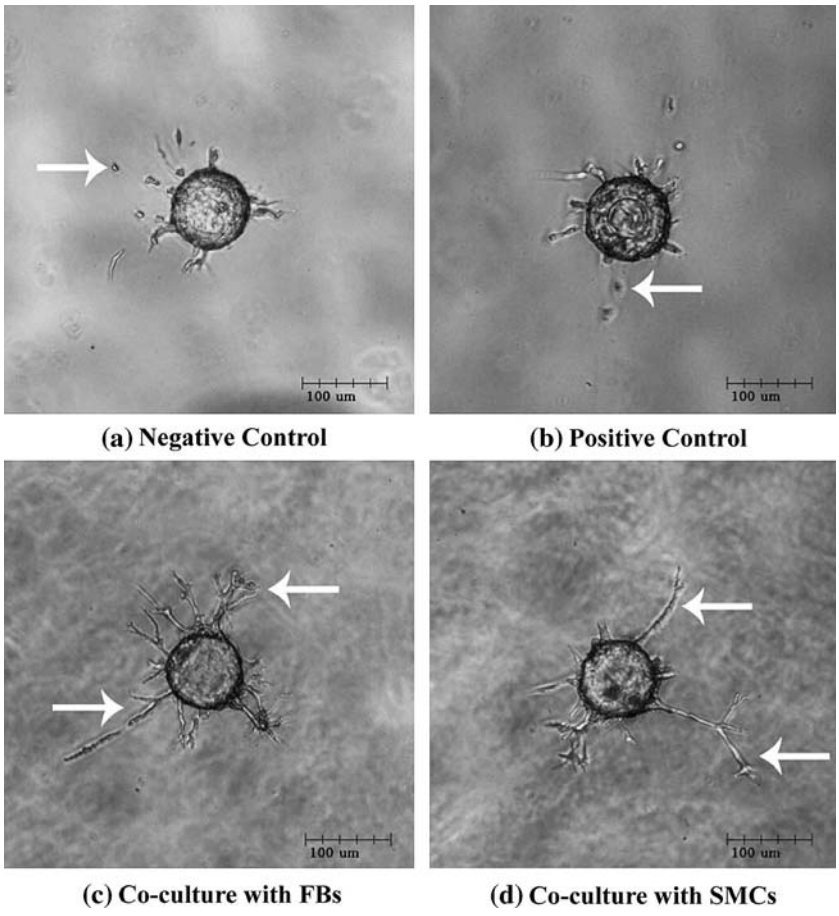

Fig. 9 Outgrowth patterns of BAECs co-cultivated with FBs and SMCs in fibrin hydrogels: ECs invaded the fibrin matrix (5 mg/ml) using mainly collective cell migration strategies. Sprouts formed by BAECs were multicellular (confirmed by bisbenzimide staining). On day 4, 20 ng/ml bFGF (positive control) did not induce significant changes. As a result of co-cultivation with tissue-specific bystander cells, the average sprout number and length was increased. Sprouts of co-cultivated BAECs were thick multicellular structures. Only few individual cells were observed. Arrows mark individual, rounded ECs (**a**, **b**) or elongated, aligned EC structures (**c**, **d**). Bar represents 100 μm

Table 2 Average number of sprouts per bead and their average length depended strongly on the type of tissue-specific bystander cells

Condition	Sprouts per bead in fibrin gel	Average sprout length in μm in fibrin gel
Control	2.3 ± 0.2	81.3 ± 2.6
bFGF	2.6 ± 0.3	91.2 ± 6.1
FB	6.2 ± 0.4**	100.8 ± 2.9**
SMC	5.2 ± 0.4**	96.9 ± 2.8**

BAEC-covered microcarrier beads embedded in fibrin hydrogels were cultured for four days, before sprout formation was analyzed. Co-cultivation of BAECs with FBs or SMCs increased the average number of sprouts per bead as well as the average sprout length. Addition of 20 ng/ml bFGF did not induce significant changes. N (from four replicates) ranged from 58 to 73. $^{**}P < 0.01$, as compared to controls

and observed that capillary-like structure formation was largely driven by matrix metalloproteinases. By contrast, pure fibrin matrices were primarily degraded via the plasminogen activator/plasmin system. As a caveat, the experiments by Collen et al. were all carried out in the presence of 20% FBS. In our experiments, the observed decrease in migration distance at 10% vs. 1% FBS in fibrin gels (Fig. 5) may have been caused by excessive matrix dissolution in the 10% FBS-supplemented medium, thus removing important guidance cues and inhibiting cell migration [27, 34].

Some of the differences in EC migration in the two types of hydrogels may also be explained by differences in the adhesion to the different matrix components. During angiogenesis, EC interaction with and adhesion to ECM components is regulated via different sets of integrin receptors [35, 36]. In our study, indirect evidence supporting this notion is found in the differential EC morphology, which was rounded in collagen gel and more elongated in fibrin gel. Interestingly, at 1% serum BAEC migration in both gel systems is nearly identical (~150 μm, Fig. 5), in spite of differences in concentration of the hydrogel-forming proteins (1.5 mg/ml for collagen and 5 mg/ml for fibrin), which might be a result of differences in the cytoarchitecture of the gels and/or in their mechanical properties. Both of these parameters are of importance in generating engineered tissue constructs; hence, current experiments in our laboratory focus on elucidating their role in endothelial cell migration and sprout formation.

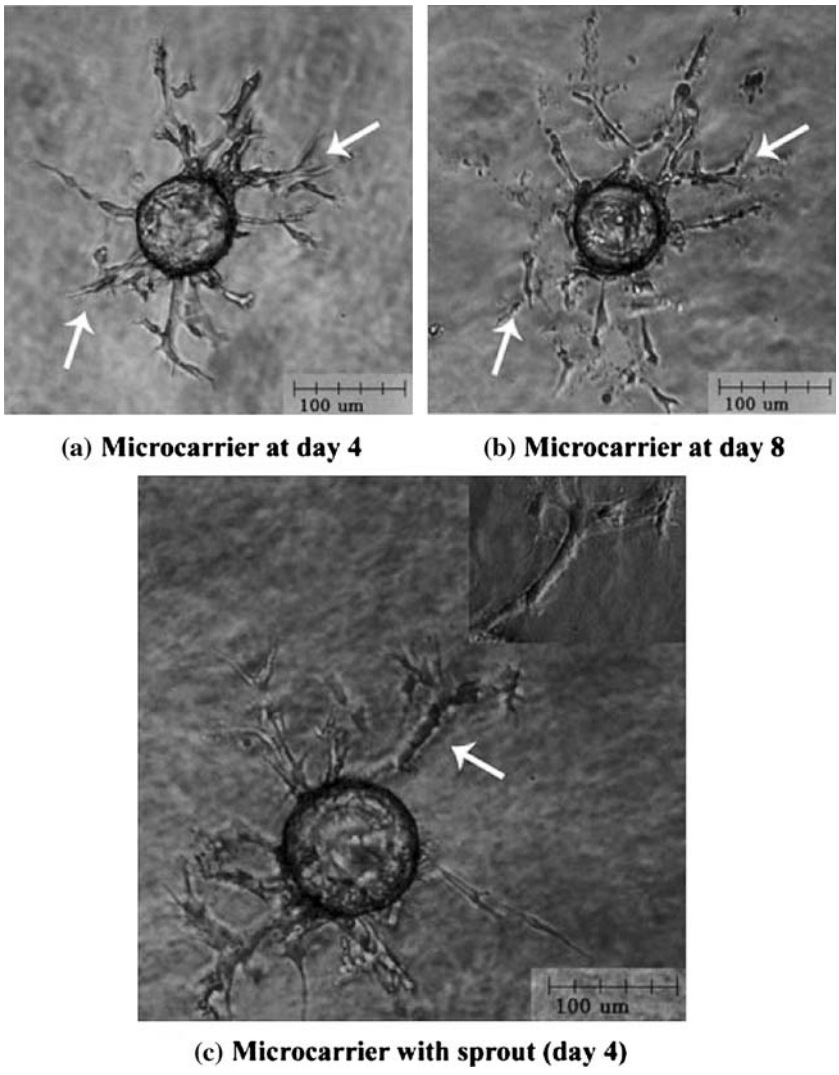

Fig. 10 Sprout decomposition in fibrin hydrogels: panel (**a**) shows a typical BAEC covered microcarrier bead after four days of co-culture with FBs. ECs readily invaded the fibrin matrix (5 mg/ml) and formed multiple sprouts (arrows mark two selected sprouts). Sprouts were thick interconnected structures, shown in panel (**c**). At day 8 most of these sprouts had disintegrated substantially (**b**) confirmed by marked cellular debris (arrow). The disintegration was accompanied by a decrease in sprout number and length

Effects of mesenchymal cells on EC angiogenic activity in different matrices

Based on the data shown in Fig. 6 and Table 1, we selected two different serum concentrations for our co-culture studies (1% for collagen and 0.1% for fibrin gels), which yielded equal numbers of basal EC sprouts in collagen and fibrin gels. Our results (Figures 8 and 9) demonstrate that FBs and SMCs differentially affect BAEC migration and sprouting and that these effects are matrix-dependent. The importance of bystander cells for the promotion of angiogenesis has been previously demonstrated [11, 37]. Montesano et al. [11] reported that Swiss 3T3 fibroblasts induced capillary-like structure formation in a three-dimensional collagen gel by releasing unknown factor(s) that resulted in increased production of plasminogen activators by the ECs. However, in the study by Montesano et al., only the fibroblastic Swiss 3T3 cells produced these factors, while other types of fibroblasts failed to induce significant capillary-like structure formation in the same model. Our data extend the above report, since FBs derived from bovine adipose tissue also induced endothelial cell migration in collagen gels.

Smooth muscle cells are known to regulate endothelial cell quiescence and also to control endothelial responsiveness to angiogenic factors. For example, D'Amore's group reported that SMCs, either in direct contact or via SMC-conditioned medium, inhibited EC proliferation [38, 39]. Similarly, when ECs and SMCs were co-cultured in a spheroid co-culture assay, EC responsiveness to VEGF was completely abrogated and the expression of endothelial cell markers for the proliferative phenotype was downregulated [40]. In line with these findings, our results indicated that FBs, but not SMCs, caused a significant increase in EC migration and sprouting in collagen gels. By contrast, in fibrin gels both adipose fibroblasts and vascular

smooth muscle cells not only enhanced EC migration and sprout formation, but also affected the migration strategy of these cells. In the absence of mesenchymal cells, ECs migrated mostly individually and, only occasionally, as collective. In the co-cultures, *viz.* under the influence of the factors released by the bystander cells, EC migration was almost exclusively collective.

In contrast to our results, an earlier co-culture study by Nehls et al. conducted in fibrin gels, reported that neither fibroblasts nor smooth muscle cells were able to significantly enhance capillary-structure formation [41]. An explanation for this difference might be the different setups of the two assays: in a later co-culture study, the same group, using a variation of their original microcarrier-based angiogenesis assay, reported that different types of fibroblasts (heart-derived and NIH 3T3 fibroblasts) did stimulate EC sprouting and capillary growth [13].

It is important to note that the angiomodulatory effects of both fibroblasts and SMCs depended on the type of 3-D scaffold used. While fibroblast cells enhanced BAEC migration independently of the matrix, smooth muscle cells increased EC migration only when co-cultured in fibrin matrices (Fig. 9d). This difference might indirectly reflect the differential activation of the bystander cells by diverse matrix proteins. For example, Song et al. [42] reported that SMCs sandwiched between two collagen layers adopted a quiescent, differentiated 'in vivo-like' phenotype. By contrast, Kodama et al. [43] observed that SMCs readily migrated in fibrin gels. Similarly, the use of fibrin gel as a substrate for the culture of skin substitutes (keratinocytes and dermal fibroblasts) increased the secretion of VEGF, in comparison to culturing these cells in collagen gel [44]. Becker et al. [45] reported that the addition of fibrin glue to a collagen gel resulted in an increased proliferation of fibroblasts. Differences between dermal and gingival fibroblasts in terms of shape and capacity to remodel 3-D matrices were seen only in fibrin gels, but not in collagen gels [46].

As discussed before, prior studies, in which ECs were co-cultured with other cell types (fibroblasts, tumor cells, etc.), have demonstrated differential effects of bystander cells on capillary sprouting and tube formation in vitro. The focus of this paper was not a mechanistic one, *viz.* to elucidate, whether the observed distinct effects of the co-culture might be due to differential production of bFGF, VEGF, and/or other angiomodulatory molecules by fibroblasts and smooth muscle cells. Rather, we aimed at fine-tuning an extant in vitro angiogenesis assay, which will now allow us to study in more detail how matrices can modulate the angiogenic activities (e.g. secretion of growth factors) of the bystander cells. Current experiments in our laboratory focus on studying how different matrices, through activating different integrin receptors, might directly modulate endothelial cell responsiveness to distinct humoral factors secreted by fibroblasts, smooth muscle cells, and tumor cells. Overall, our results stress the matrix-dependence of the interactions between ECs and mesenchymal cells and support the notion that heterotypic cell–cell interactions are matrix-specific.

Acknowledgements This work was supported in part by grants from the Nanotechnology Institute of Southeastern Pennsylvania (NTI), the Calhoun Funds, and the National Aeronautics and Space Administration (NASA, NAG-1436, NNJ04HC81G-01, and NCC9–130).

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