THE THREE-DIMENSIONAL MICROCARRIER-BASED ANGIOGENESIS ASSAY: OPTIMIZATION AND APPLICATION

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ABSTRACT

The generation of 3-D vascular networks in engineered tissues can be studied by culturing endothelial cells (ECs) on Cytodex3 microcarrier beads in 3-D matrices in vitro. By optimizing this model we found that the initial EC attachment to the beads depended on EC type and EC/bead culture method, while the EC/bead culture time influenced EC migration . A confluent EC monolayer on the microcarrier bead surfaces formed only when bovine aortic endothelial cells (BAECs) were admixed to the beads under constant agitation. After embedment of these BAEC-coated beads into collagen and fibrin gels, we characterized the outgrowth. Interestingly, migration distance and type were influenced not only be the matrix itself but also by the serum supplementation of the cell culture medium. While at serum concentrations of 0% and 10% the average migration distance was highest in collagen gels, tube formation was enhanced in fibrin matrices. The opposite result was observed at a serum concentration of 0.1%. The average migration distance was reduced and the number of tubes per bead doubled in collagen matrices compared to fibrin matrices. These results stress the importance of a careful selection of cell type, culture method and matrix for in vitro angiogenesis studies. In addition, the results show the existence of a complex interplay of three-dimensional matrix and serum concentration, which can influence the outcome of in vitro angiogenesis assay experiments.

INTRODUCTION

The process of angiogenesis is an area of intense research, especially since it is connected to numerous pathological conditions, such as cancer growth. One important field for research in angiogenesis are three-dimensional in vitro angiogenesis models. These models are developed to mimic the angiogenic process with the final goal to create capillaries and their successors outside the body. To date, various in vitro angiogenesis models exist [1]. In 1995, Nehls and Drenckhahn [2] developed a microcarrier-based angiogenesis assay, in which EC-coated microcarrier beads were embedded into a three-dimensional fibrin matrix. This assay offered a very important advantage over commonly used models: easy and fast quantification. In order to use this assay to access not only tube formation of ECs but also EC migration into the matrix, we slightly modified it (cell type, matrix, bead number). However, first experiments did not yield significant results. Problems experienced comprised incomplete monolayer formation of EC on the microcarrier bead surface and reduced tube formation. In order to successfully expand this assay to the new analysis tool, we had to optimize it before its application.

MATERIALS AND METHODS

Materials: MCDB131, Cytodex3, recombinant human epidermal growth factor (rhEGF), EC growth supplement (ECGS), fibrinogen, thrombin, bisbenzimide were purchased from Sigma. L-glutamine, Antibiotic-Antimycotic Solution and 10xPBS were from Fischer Scientific. Fetal bovine serum (FBS) was from Hyclone. Collagen type I solution was purchased from BD Bioscience.

Cells: Human aortic endothelial cells (HAEC) and primary human microvascular endothelial cells (HMVEC) were cultured in MCDB 131, supplemented with 5% FBS, 10ng/ml rhEGF, 10U/ml heparin, 30ug/ml ECGS, 0.3mg/ml L-glutamine and 0.2% Antibiotic-Antimycotic Solution (adaptation from MGM-2 medium (Clonetics)). Transformed human microvascular endothelial cells (HMEC-1) and bovine aortic endothelial cells (BAEC) were cultured in MCDB131 medium supplemented with 10% FBS, 10ng/ml rhEGF, 0.3mg/ml L-glutamine and 0.2% Antibiotic-Antimycotic Solution (modified from [3]). The cells were cultured in a humidified incubator at 37°C and a CO_2 level of 5%.

Beads: 500mg of Cytodex3 microcarrier beads were swollen for three hours in 100ml 1xPBS . The beads were washed once with 1xPBS, before 25ml 1xPBS were added and the beads were autoclaved at 121°C for 15 minutes. The cooled solution was stored at 4°C.

EC/bead attachment: Four methods to seed ECs on microcarrier beads were tested. For the original method, described by Nehls and Drenckhahn [2], 90,000 ECs and 3,000 beads were suspended in 2ml medium and incubated for four hours statically. After the addition of fresh medium the suspension was gently agitated. For the monolayer method, 6,000 beads were added to a monolayer of ECs and incubated. The beads were dislodged mechanically from the culture flask for analysis. In the suspension method, 60,000 beads were suspended in 7ml medium containing 1,400,000 ECs. This suspension was incubated under static conditions. For the dynamic

method, 3,000 beads, 45,000 ECs and 20ml medium were cultured under gentle agitation. After two days of culture ECs attached to the bead surface were stained with 1μg/ml bisbenzimide.

Gels: For collagen gels, a collagen type I solution with a final concentration of 2mg/ml was prepared according to the manufacturer's protocol. Briefly, 10xPBS, 1N NaOH, water and collagen were added to a tube, mixed and stored one ice until use. For fibrin gels, a fibrinogen solution with a final concentration of 6mg/ml was prepared. Briefly, fibrinogen powder was added to pre-warmed, pH-adjusted 1xPBS. The solution was shaken for 45 minutes at 37°C, sterile filtered and stored on ice until use.

Assay set up: One volume gel solution and one volume serum free cell culture medium were mixed together. In case of the fibrinogen solution the cell culture medium contained additionally 0.5U/ml thrombin. Small amounts of this mixed gel/medium solution were added to the wells of a well plate and allowed to form a gel (bottom layer). The EC-coated beads were transferred into one volume serum free medium, which was then mixed with one volume gel solution. Small amounts of this suspension were added on top of the bottom layers and incubated at 37°C. After 20 minutes cell culture medium with different supplementation was added to the sandwich-like structures. Medium was changed every second day.

Assay analysis: Single microcarrier beads were randomly selected immediately after their embedment. Microphotographs of these selected beads were taken after six days with a Nikon TE-2000U microscope (4x objective). Microphotographs were characterized by counting the number of beads, which showed tubular morphogenesis, by counting the number of tubes per bead and/or by migration distance measurements. Only structures which originated from the microcarrier bead surface and were longer in length than the diameter of the beads were classified as tubes. Migration distance measurements were performed using a radial grid, consisting of 36 radii, which was overlaid on the bead. On each radial line, the distance of the EC that had migrated furthest away was recorded. All 36 distances were averaged and the radius of each bead was subtracted to yield the migration distance for each bead.

Statistics: Average migration distance, number of cells and number of tubes are given in mean±standard error of the mean. Statistical analysis (Single factor ANOVA; $p<0.05$) was performed using Microsoft Excel.

RESULTS

EC attachment on Cytodex3 microcarrier beads

In order to generate beads that were fully covered with a confluent EC monolayer, four different EC lines were tested. Table 1 shows that very little attachment was found with HAECs, HMVECs and HMECs-1. BAECs attached extremely well and were the only EC line able to form a confluent monolayer on the bead surface.

cell line	total # of cells attached
BAEC	72.2±6.7
HAEC	0.7±0.3
HMEC-1	5.3±0.7
HMVEC	2.8±0.5

Table 1: Cell attachment to microcarrier beads (cell line) Cells were cultured with beads using the original method. After two days cells attached to microcarrier beads were counted (n=12-18).

Different EC/bead culture methods were tested for their potential to establish a confluent BAEC monolayer on the bead surface. Three of the four EC/bead culture methods (except the suspension method) resulted in comparable cell attachment and monolayer formation (Figure 1). However, bead aggregates frequently

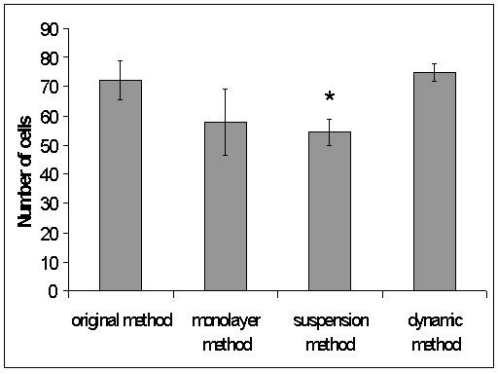


Figure 1: BAEC attachment to microcarrier beads (EC/bead culture method)
BAEC and beads were cultured for two days to test different EC/bead culture methods. Number of cells per bead was counted (n=10). Label $\star$: significantly different from original method ($p<0.05$).

formed in the original method. The monolayer method resulted in damages of the EC monolayer caused by the mechanical dislodgement process. Only the dynamic method showed uniform EC attachment to the beads a few hours after set up and resulted in a confluent BAEC monolayer after only two days of culture (Figure 2). To test the influence of the EC/bead culture time on mi-

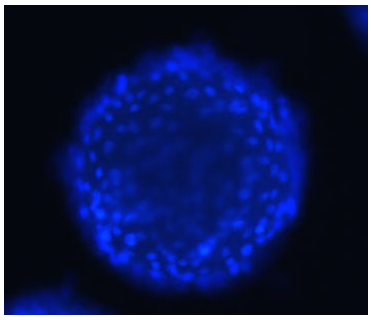
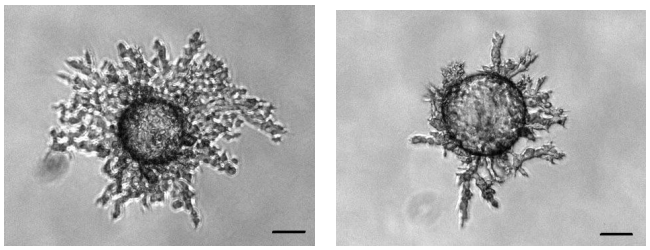


Figure 2: BAEC coated Cytodex microcarrier bead
BAECs and beads were cultured completely dynamic for two days.

gration, BAECs and beads were cultured for two or four days before they were embedded into the matrix. Migration distance and tube formation were affected by culture time. Only 29% of BAEC-coated beads, cultured for two day, showed single tubes originating from the bead surface. BAECs migrated a distance of 101.1μm into the gel (Figure 3(a)). 59% of BAEC-coated beads, cultured for four days, showed single tubes. The average migration distance was 66.8μm (Figure 3(b)). Therefore,



(a) 2 days (b) 4 days

Figure 3: Effect of culture time on BAEC outgrowth
BAEC-coated beads were cultured for six days in a fibrin matrix. Bar: 100μm

BAEC-coated Cytodex3 microcarrier beads cultured for four days under agitation were chosen for further studies.

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

To test the effect of the matrix and the serum concentration on EC migration another study was conducted. The average migration distances and tube numbers in collagen and fibrin gels were studied and are summarized in table 2. Microphotographs are shown in Figure 4. While the average migration distance increased continuously for BAECs in collagen gels (0.1% FBS: three-fold, 10% FBS: 7-fold; negative control: 0% FBS), the average migration distance in fibrin matrices increased more for 0.1% FBS (26-fold) than it did for 10% FBS (15-fold). However, the addition of serum enhanced the EC migration distances in fibrin gels more than it did in collagen matrices.

Serum conc.	Matrix	Migration distance	# of tubes per bead
0%	collagen	47.2±5.1	0.2±0.1
	fibrin	10.8±2.8	0.6±0.2
0.1%	collagen	171.8±14.8	1.7±0.3
	fibrin	264.4±13.2	0.8±0.2
10%	collagen	350.5±13.2	none
	fibrin	156.5±15.9	0.15±0.1

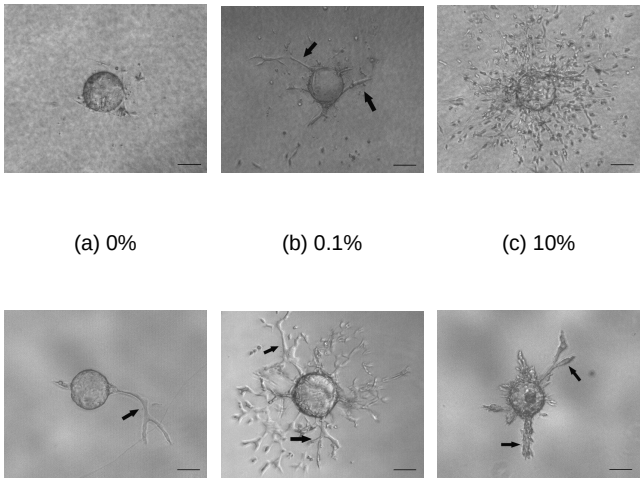
Table 2: Tube formation and migration distance under different serum supplementations
BAEC-coated beads were embedded into collagen (1mg/ml) and fibrin (3mg/ml) gels and cultured in MCDB131 supplemented with 0%, 0,1% and 10% FBS. Tubes were counted and migration distances were measured after six days.

Tube formation occurred in both matrices but not under all serum concentrations. In collagen gels BAEC migrated individually but also formed tubes at 0% and 0.1% FBS. At 10% FBS tube formation could not be observed (Figure 4(c)). Fibrin gels supported tube formation under all serum concentrations and many tubes participated in network formation (especially at 0.1% FBS). Tubes were generally thin structures consisting of aligned ECs (Figure 4(a)-(e)). However, tubes formed at 10% FBS in fibrin gels were thick structures (Figure 4(f)). At 0% and 10% FBS the number of tubes per bead was higher in fibrin gel, while at 0.1% FBS the total number of tubes per bead was higher in collagen gel. Surprisingly, at this serum concentration (0.1%) the total number of tubes was highest for both matrices.

DISCUSSION

The microcarrier-based angiogenesis assay developed by Nehls and Drenckhahn [2] was originally introduced as a system for fast quantification of angiogenic sprouting. Since a modified version of this assay did not yield satisfactory results, the assay was optimized before its application.

EC attachment on Cytodex3 microcarrier beads



(a) 0% (b) 0.1% (c) 10%

(d) 0% (e) 0.1% (f) 10%

Figure 4: Tube formation of BAEC under different serum concentrations
Six days after setup microphotographs were taken. Structures identified as tubes are marked with an arrow. Subfigure (a)-(c): collagen gel; Subfigure: (d)-(f): fibrin gel; Bar: 200μm.

A confluent monolayer is a critical requirement for angiogenesis, since ECs have to be in close contact before blood vessel formation can occur [4]. Only one EC line (BAEC) yielded reproducibly a confluent monolayer on the bead surface. This observation showed that different EC lines could attach very differently to Cytodex3 microcarrier beads. We found that also the EC/bead culture method influenced EC attachment. For this reason the presence of a confluent monolayer in in vitro angiogenesis studies should always be confirmed first. The EC/bead culture time additionally played a role in EC migration. This observation stresses the importance of a confluent EC monolayer on the microcarrier beads for tube formation in angiogenesis studies.

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

The effect of the serum concentration is rarely studied but influences many aspects of cell behavior. We found that the serum concentration affects 3-D EC migration and tube formation. Surprisingly, these effects were also dependent on the 3-D matrix. Individual cell migration was primarily supported by collagen type I gels, while tube formation was enhanced in fibrin matrices. These findings are confirmed by Kroon et al. [5]. They observed an inhibition of tube formation of human microvascular endothelial cells in pure and mixed (with fibrin) collagen type I gels. Interestingly, they reported tube formation of BAEC in collagen gels, but only after stimulation with growth factors. Collen et al. investigated fibrin matrices containing 10% collagen [6]. They observed that capillary-like structure formation was largely driven by Matrix metalloproteinases (MMPs), while pure fibrin matrices were degraded primarily via the plasminogen activator/plasmin system[1]. This finding could be an explanation for the observed decline in tube numbers in collagen matrices at 10% but also at 0% FBS. However different sets of integrin receptors can also play a role [7].
Interestingly, the addition of a relatively low serum concentration (0.1%) to the culture medium resulted for both matrices in the highest number of tubes per bead in this study. This is a surprising finding. One explanation may be found in a decline of proteolytic enzymes (can be produced by the cells and can be also present in FBS) at low serum concentrations resulting in enhanced collective EC migration and tube formation. So far, one can only make assumptions about involved mechanisms. In general, our findings suggest the existence of a complex interaction between EC behavior in 3-D matrices and the serum concentration.

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