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ORIGINAL PAPER

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A microcarrier-based cocultivation system for the investigation of factors and cells involved in angiogenesis in three-dimensional fibrin matrices in vitro

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Abstract Angiogenesis in situ includes coordinated interactions of various microvascular cell types, i.e., endothelial cells, pericytes and perivascular fibroblasts. To study the cellular interactions of microvascular cells in vitro, we have developed a microcarrier-based cocultivation system. The technical details of this method include seeding of endothelial cells on unstained cytodex-3 microcarriers and seeding of pericytes, fibroblasts or vascular smooth muscle cells on microcarriers which have been labeled by trypan blue staining. A mixture of both unstained and trypan blue-stained microcarriers was subsequently embedded in a three-dimensional fibrin clot. The growth characteristics of each cell type could be conveniently observed since the majority of cells left their supporting microcarriers in a horizontal direction to migrate into the transparent fibrin matrix. As differently stained microcarriers were randomly arranged in the fibrin matrix, the characteristic patterns of the microcarriers allowed location of particular points of interest at different developmental stages, facilitating the observation of cellular growth over the course of time. One further advantage of this microcarrier-based system is the possibility of reliably quantifying capillary growth by determination of average numbers of capillary-like formations per microcarrier. Thus, this model allows convenient evaluation of the effects of non-endothelial cells on angiogenesis in vitro. By using this coculture system, we demonstrate that endothelial capillary-like structures in vitro do not become stabilized by contacting vascular smooth muscle cells or pericytes during the initial stages of capillary formation.

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Introduction

Angiogenesis, the development of new capillaries from a preexisting microvascular network, is important for various physiological and pathological conditions such as organ development, tumor growth or the proliferative retinopathy in diabetes mellitus (Yanoff 1966; Paweletz and Knierim 1989; Blood and Zetter 1990; Bicknell and Harris 1991; Klagsbrun and D'Amore 1991; Folkman and Shing 1992; Fidler and Ellis 1994).

Investigation of the basic mechanisms of capillary development requires appropriate in vitro systems. In the living organism, angiogenesis occurs within the interstitial extracellular matrix and this may be the reason why two-dimensional models which have been frequently used as convenient in vitro assay systems do not appropriately reproduce in situ conditions (Madri et al. 1988; Montesano et al. 1991; Williams 1993; Nehls et al. 1994). For example, transforming growth factor-β (TGF-β) was reported to inhibit endothelial proliferation in two dimensional systems, whereas in a three-dimensional system TGF-β appeared ineffective (Madri et al. 1988). As a consequence of these limitations, three-dimensional systems have been developed. In the most commonly used of these in vitro systems, endothelial cells are grown on the surfaces of fibrin or collagen gels (Montesano et al. 1987, 1993; Pepper et al. 1990; Williams 1993). Following stimulation by angiogenic factors, the cells can be induced to invade the underlying matrix and to differentiate into capillary-like structures. Although these systems represented a major step forward in angiogenesis research, it soon became clear that the quantification of capillary-like sprouts in three-dimensional matrices is difficult. Moreover, due to the complexity of three-dimensional systems it is almost impossible to safely identify particular cell types in three-dimensional cocultures.

We recently developed a microcarrier-based in vitro assay (Nehls and Drenckhahn 1995) which allows rapid and reliable quantification of capillary growth in three dimensions. According to this method, endothelial cells

are grown on microcarriers which subsequently are embedded in fibrin. Following stimulation by angiogenic factors such as basic fibroblast growth factor (bFGF; Klagsbrun and D'Amore 1991) and vascular endothelial growth factor (VEGF; Ferrara and Henzel 1989; Gospodarowicz et al. 1989; Plate et al. 1992), or by morphogenic factors such as fibronectin, we observed endothelial cells assembling to multicellular capillaries, which radiated from microcarriers in a spoke-like fashion to invade the surrounding fibrin gel. This model allowed us to conveniently quantify capillary-like structures by counting the numbers of capillary-like structures per microcarrier. Since a single fibrin gel contains hundreds of microcarriers, a sufficient number of random samples is available in this assay for a reliable statistical evaluation. Only recently, we found that the mechanical properties of fibrin clots are decisive for capillary morphogenesis. The mechanical properties of fibrin clots, in turn, are dependent on several chemical parameters of the polymerization milieu. Fibrin clots which have been polymerized at pH 7.6 are more malleable than the more rigid fibrin gels polymerized at pH 7.2. Interestingly, spontaneous and bFGF/VEGF-stimulated capillary formation occurred in malleable gels, but not in the rigid gels. A preliminary account of this work will be presented in abstract form (Nehls and Herrmann 1995, in press).

In previous studies, we and others found some preliminary evidence supporting the hypothesis that microvascular pericytes evolve from perivascular fibroblasts during the process of neovascularization (Cliff 1963; Nakayasu 1988; Archer and Gardiner 1981; Rhodin and Fujita 1989; Díaz-Flores et al. 1991; Nehls and Drenckhahn 1991; Nehls et al. 1992; Stingl and Rhodin 1994; for review see Nehls and Drenckhahn 1993). There is increasing evidence that pericytes play an early role during angiogenesis (Verhoeven and Buyssens 1988; Wakui 1988; Schlingemann et al. 1991). For example, we detected pericytes accompanying early endothelial sprouts during angiogenesis in the rat mesentery (Nehls et al. 1992). We thus became interested in studying the differentiation and the function of periendothelial cells during capillary formation in vitro. The first approach was to cultivate endothelial cells together with other cell types on the surface of fibrin gels. These gels were then dissected and the cell-coated fragments were reembedded in a second fibrin gel. By using this technique, we found that neither fibroblasts, pericytes nor vascular smooth muscle cells were capable of modulating the morphological differentiation of endothelial cells during the initial phase of sprout formation (Nehls et al. 1994), indicating that unknown factors not included in our in vitro model might regulate the coordinated growth of these cells in situ. However, although this cocultivation method allowed us to look at the differentiation of capillary-like structures in general, due to the complexity of the growth response it was not possible to safely distinguish endothelial from non-endothelial cells or to follow individual cells over the course of time. In our search for an improved cocultivation system, we found it most advantageous to grow distinct types of vascular cells on differently stained microcarriers which were then embedded in fibrin matrices. We wish to describe here the details of this novel cocultivation method. We think that this simple technique will markedly facilitate the investigation of cellular interactions in three-dimensional environments in vitro.

Materials and methods

Materials

Bovine fibrinogen (>95% protein clottable), bovine thrombin, trypan blue, cytodex-3 microcarriers and the antibodies to smooth muscle α-actin and von Willebrand antigen were from Sigma, Deisenhofen, Germany. Dulbecco's modified Eagle medium (DME), and newborn calf serum (NCS) were from Gibco, Eggenstein, Germany. VEGF (human, recombinant) was kindly provided by Dr. Werner Risau, Bad Nauheim, Germany. Fluorescent Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) was from Paesel and Lorei, Frankfurt am Main, Germany. Aprotinin (Trasylol) was from Bayer, Munich, Germany. Collagenase A from *Clostridium histolyticum* (>0.15 U/mg) and bovine bFGF were purchased from Boehringer, Mannheim, Germany.

Isolation of endothelial cells

Calf pulmonary arteries were obtained from a local slaughterhouse, ligated at one end and washed twice with sterile phosphate-buffered saline (PBS, 140 m*M* NaCl, 10 m*M* phosphate, pH 7.4). Arteries were then filled with collagenase A (0.1% w/v in PBS) and incubated for 10–15 min at 37° C to detach endothelial cells from the intima layer. The isolated endothelial cells were then centrifuged at 400×g for 5 min, washed twice in PBS, resuspended in DME supplemented with 20% NCS and seeded in culture flasks (Greiner, Frickenhausen, Germany) coated with gelatine. Endothelial cells were characterized according to staining for von Willebrand antigen, uptake of Dil-Ac-LDL and absence of staining for smooth muscle α-actin. Only cell cultures from passages 4–7 containing >98% endothelial cells were used for the experiments.

Isolation of pericytes, fibroblasts and vascular smooth muscle cells

Pericytes were isolated from bovine retinas, fibroblasts from the avascular centers of bovine corneas and vascular smooth muscle cells from bovine pulmonary arteries as described (Nehls and Drenckhahn 1994). The cells were characterized by immunostaining using antibodies to smooth muscle-type α-actin and von Willebrand factor. All cell types were negative for von Willebrand factor. Smooth muscle cells and, after some passages, pericytes were positive for α-actin, whereas fibroblasts were negative.

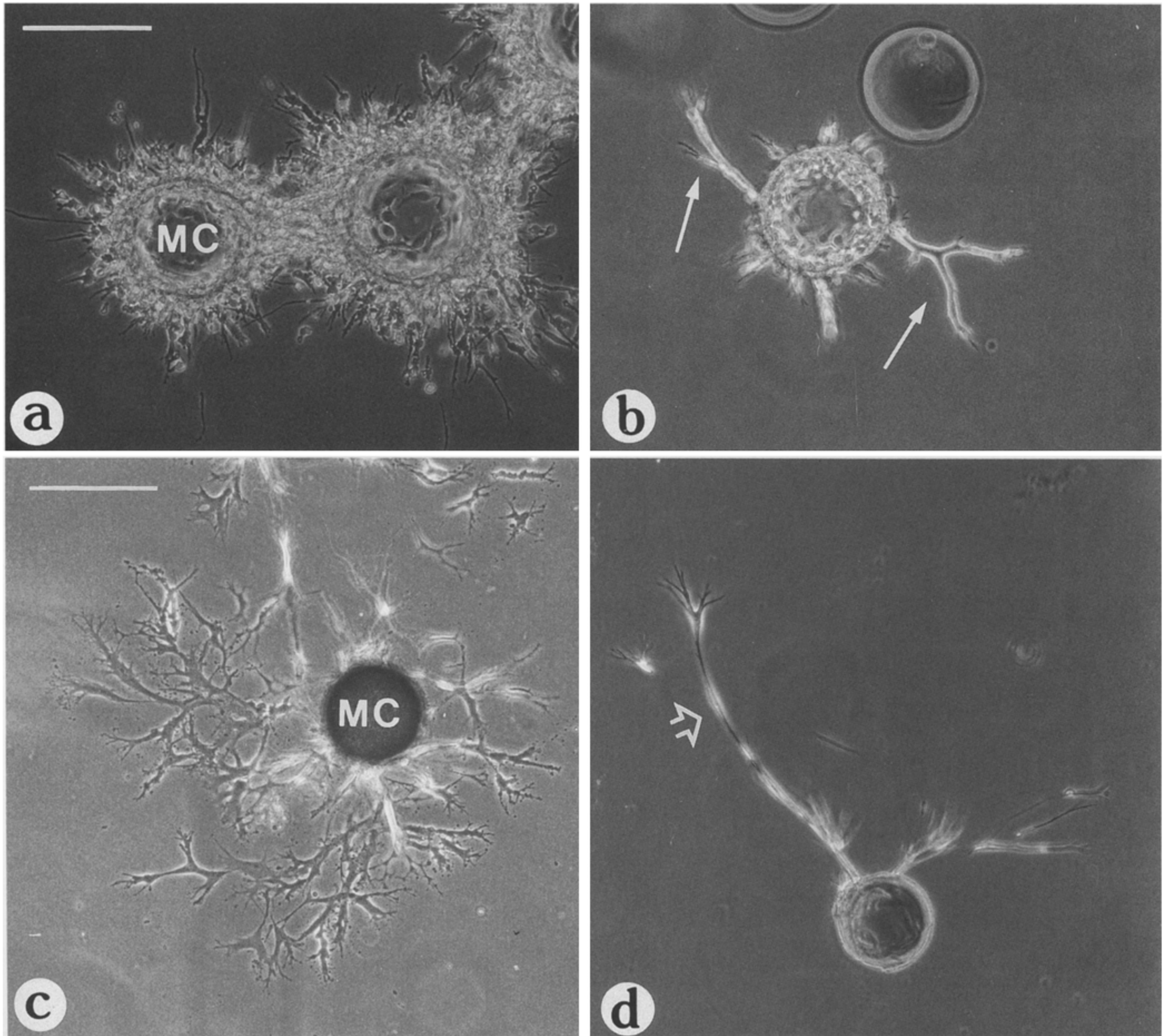
Microcarrier cell culture

Gelatine-coated cytodex-3 microcarriers (MCs) were prepared according to the recommendations of the supplier. Freshly autoclaved MCs were suspended in DME+20% NCS and endothelial cells or periendothelial cell types were added to a final concentration of approximately 30 cells/MC. For the coculture experiments, the MCs to be coated with periendothelial cells were stained with trypan blue (0.5 mg/ml in PBS) for 30 min at 37° C prior to cell seeding, whereas endothelial cells were grown on unstained MCs. The cells were allowed to attach to the MCs during a 4-h incubation at 37° C. The MCs were then suspended in a larger volume of medium and cultivated for 2–4 days at 37° C in a 5% CO_2 atmosphere. MCs were gently agitated to prevent aggregation of individual MCs.

Preparation of fibrin gels and coculture of cells

Fibrin gels were prepared as described (Nehls et al. 1994), with some modifications. Briefly, bovine fibrinogen, dissolved in PBS, was extensively dialyzed against PBS, pH 7.4 and non-soluble material was removed by filtration through 0.2-μm filters. To prevent excess fibrinolysis by fibrin-embedded cells, aprotinin (Trasylol) was added to the fibrinogen solution and to growth media at 200 U/ml. In order to obtain malleable fibrin gels which would promote capillary morphogenesis (Nehls and Herrmann 1995, in press), we adjusted the pH of the fibrinogen solution in some experiments to pH 7.6 before polymerization was induced by addition of thrombin. Immediately following polymerization, physiological pH values were restored in these gels by repeated washes with PBS. For the experiments which required bFGF and VEGF, the respective component was added to the fibrinogen solution at the desired concentration (20 ng/ml bFGF; 50 ng/ml VEGF) prior to polymerization. The fibrinogen solution (1.5 mg/ml) was transferred to 35-mm culture dishes. Cell-coated microcarriers were added to the fibrinogen solution at a density of approximately 150 MCs/ml, and clotting was induced by addition of thrombin (0.6 U/ml). After clotting was complete, gels were equilibrated with DME (pH 7.4; supplemented with 10% NCS) and, following 60 min incubation, fresh medium was added to the fibrin matrices. For coculture experiments, unstained MCs coated with endothelial cells were mixed 1:1 with trypan blue-stained MCs which had been coated with either fibroblasts, pericytes or smooth muscle cells.

Fig. 1a–d Morphological differentiation of distinct vascular cell types monocultivated in fibrin clots. Cells were grown on microcarriers (*MC*), which were subsequently embedded in fibrin, and photographs were taken at 48 h (**a**, **b**, **d**) or 96 h (**c**). The formation of capillary-like structures by endothelial cells is highly dependent on the mechanical properties of the surrounding fibrin gel: rigid gels, polymerized at pH 7.2, support migration of cells but do not promote development of capillary-like formations (**a**), whereas malleable gels, polymerized at pH 7.6, inhibit cell migration and simultaneously stimulate capillary-like morphogenesis (**b**; *arrows* indicate capillary-like structures invading the fibrin matrix). Pericytes (**c**), embedded in fibrin, develop into highly branched structures, whereas vascular smooth muscle cells (**d**) exhibit elongated capillary-like cellular processes (*arrow*). *Bars*: **a**, **b** 150 μm; **c**, **d** 200 μm



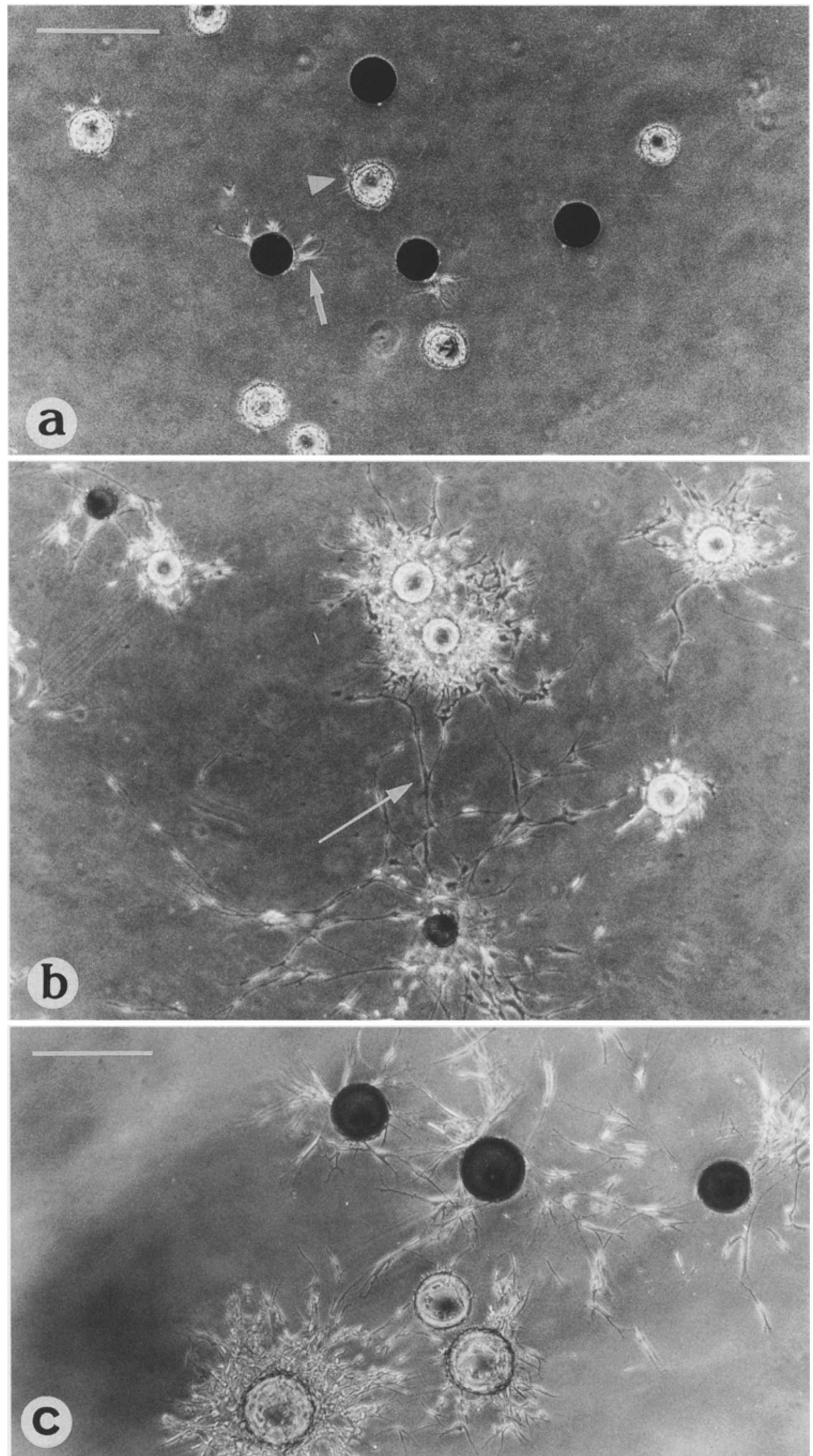


Fig. 2a–c Coculture of endothelial cells and vascular smooth muscle cells in a fibrin gel (polymerization at pH 7.4). Endothelial cells were seeded on unstained microcarriers and smooth muscle cells were seeded on microcarriers stained by trypan blue. Both carrier types were embedded in fibrin, and 24 h after polymerization, the smooth muscle cells display cellular processes protruding into the fibrin matrix (*arrow* in **a**). Endothelial cells, in comparison, show lower migratory activity (*arrowhead* in **a**). After 4 days, the smooth muscle cells have developed into a network of interdigitating cellular processes (*arrow* in **b**), which, however, is not used by endothelial cells as a template for cell migration. Endothelial cells preferably grow as clusters of cells surrounding individual microcarriers (**b**, **c**). *Bars*: **a**, **b** 500 μm; **c** 280 μm

Results

Morphological differentiation of monocultivated vascular cells in fibrin clots

Endothelial cells were isolated from bovine pulmonary arteries and grown to confluence on cytodex-3 microcarriers. Cell-coated MCs were suspended in a solution of bovine fibrinogen which was induced to polymerize by the addition of thrombin. During the process of polymerization, endothelial cells appeared to degranulate their Weibel-Palade bodies, as indicated by the emergence of pericellular granular material. Approximately 12 h after the addition of thrombin, the cells began to migrate into the surrounding fibrin matrix. In the absence of stimulators of angiogenesis, the endothelial cells migrated in an incoherent manner and only rarely assembled into multicellular capillary-like structures. On addition of both bFGF and VEGF to fibrin gels of intermediate rigidity (polymerized at pH 7.4), a marked increase in capillary-like formation occurred (Nehls and Drenckhahn 1995). This increase appeared to be largely due to enhanced proliferation of endothelial cells. In contrast, spontaneous capillary formation was observed in malleable fibrin gels which were created by polymerization at pH 7.6, whereas rigid gels which were polymerized at pH 7.2 stimulated only the migration of cells but not capillary morphogenesis (Fig. 1a, b). The angiomorphogenic effects of bFGF and VEGF appeared to be completely dependent on the mechanical properties of the extracellular matrix; in rigid gels bFGF/VEGF stimulated solely the

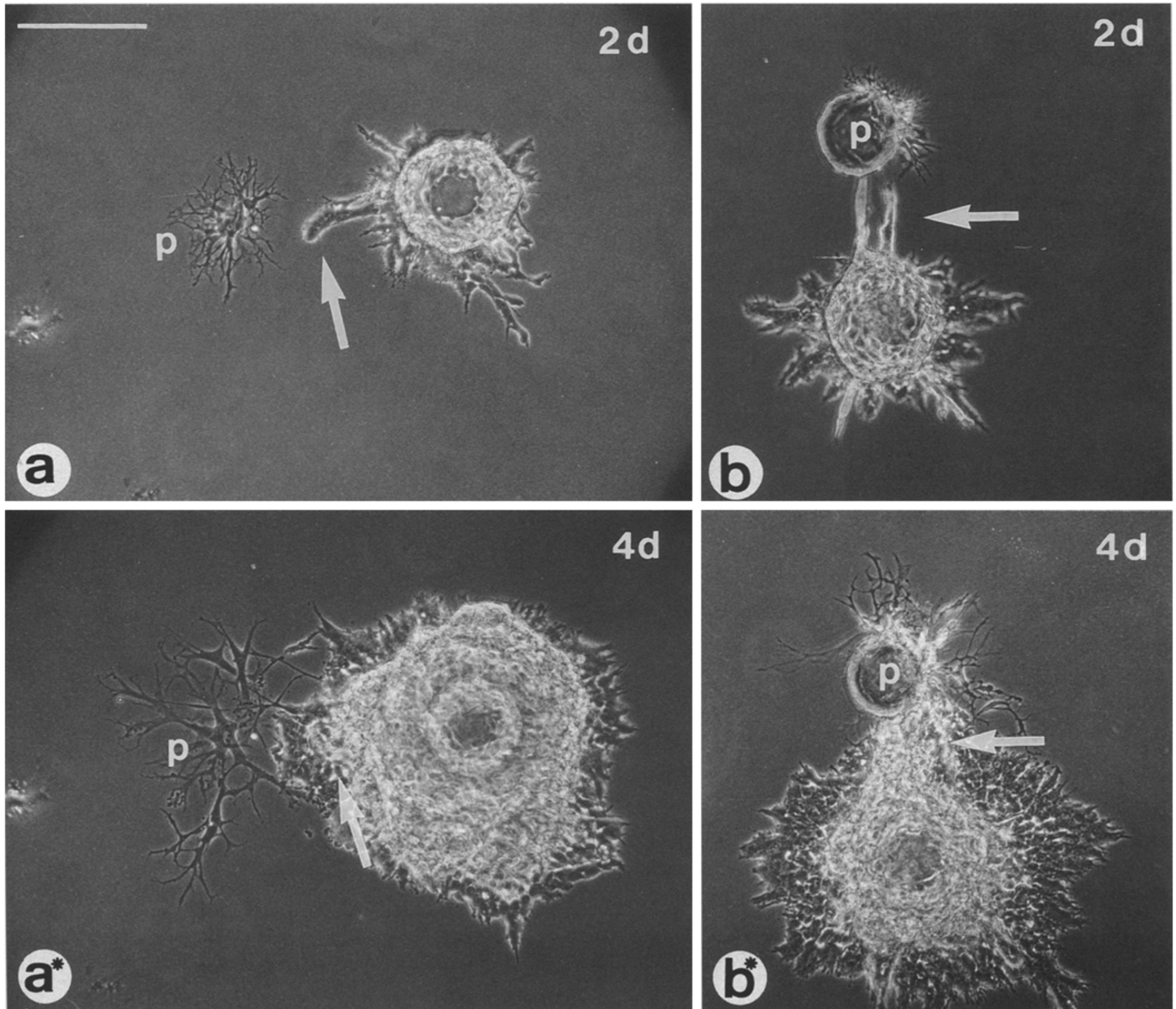


Fig. 3a, b, a*, b* Contact of pericytes (*p*) with endothelial-derived capillary-like structures (*arrows*) in malleable gels (polymerization at pH 7.6). The cells were photographed at 48 h after fibrin polymerization (**a**, **b**), and the same microcarriers were photographed 2 days later (**a***, **b***), by which time capillary-like structures were no longer detectable. The pericytes obviously did not stabilize these early endothelial capillaries. *Bar* 150 μm

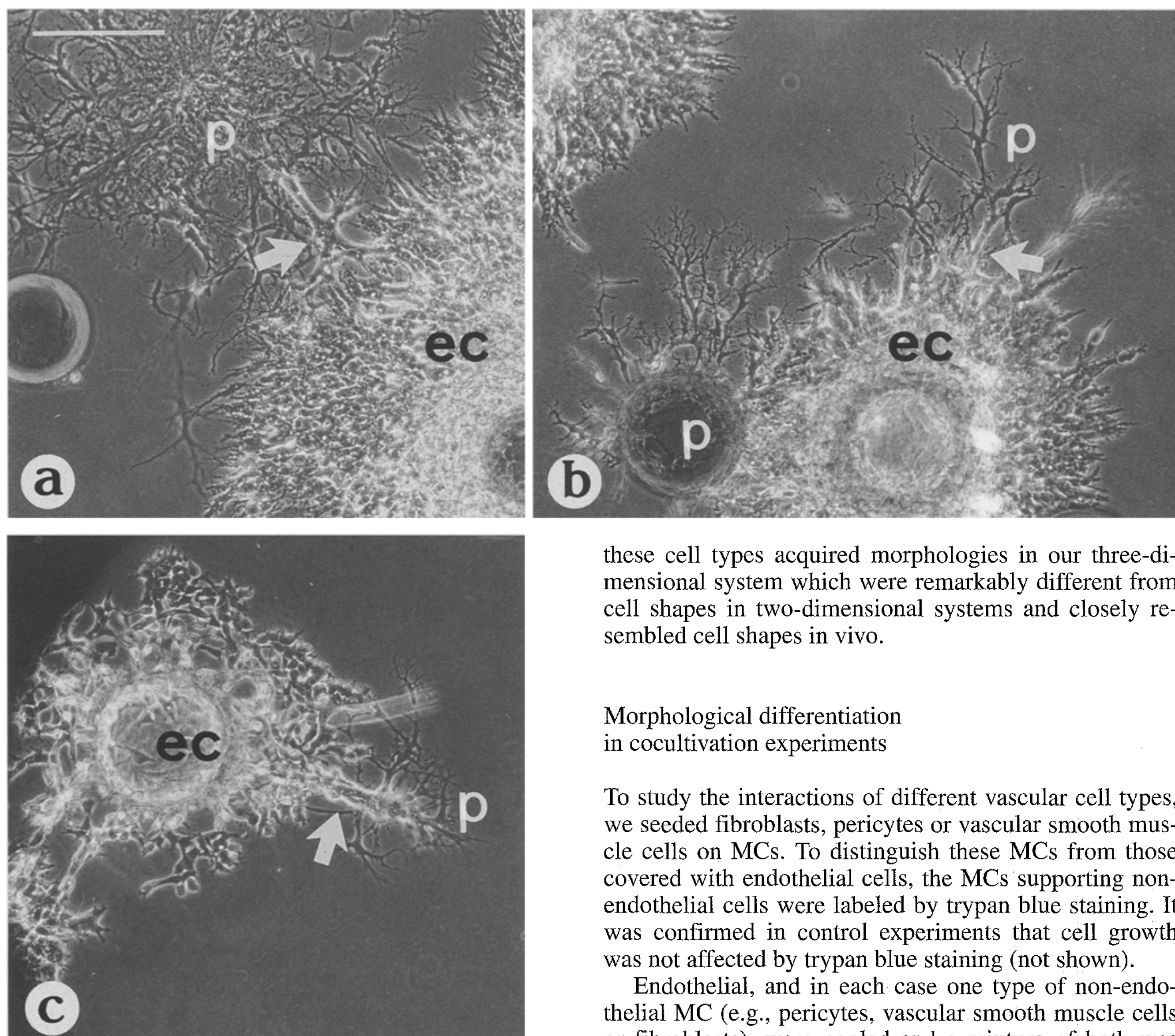


Fig. 4a–c Three examples of the formation of capillary-like structures (*arrows*) in cocultures of endothelial cells (*ec*) and pericytes (*p*) in fibrin gels (polymerization at pH 7.6). At more advanced stages of cell growth (6–10 days), capillary-like structures were occasionally observed in regions where endothelial cells and pericytes established close contacts, suggesting that both cell types participated in the formation of capillary-like sprouts. In all three examples, due to migratory activity, pericytes are also found at some distance from microcarriers. *Bar* 150 μm

proliferation and the migration of endothelial cells but not the association of cells into multicellular structures, whereas in malleable gels the formation of impressive capillary-like networks was observed (not shown).

Fibroblasts displayed the most rapid migration into fibrin clots (not shown). Vascular smooth muscle cells had an intermediate invasion capacity and pericytes migrated quite slowly into the fibrin matrix. Smooth muscle cells usually acquired an elongated, capillary-like morphology, whereas pericytes showed a bizarre, multiply branched appearance (Fig. 1c,d). It was obvious that these cell types acquired morphologies in our three-dimensional system which were remarkably different from cell shapes in two-dimensional systems and closely resembled cell shapes in vivo.

Morphological differentiation in cocultivation experiments

To study the interactions of different vascular cell types, we seeded fibroblasts, pericytes or vascular smooth muscle cells on MCs. To distinguish these MCs from those covered with endothelial cells, the MCs supporting non-endothelial cells were labeled by trypan blue staining. It was confirmed in control experiments that cell growth was not affected by trypan blue staining (not shown).

Endothelial, and in each case one type of non-endothelial MC (e.g., pericytes, vascular smooth muscle cells or fibroblasts), were pooled and a mixture of both was embedded in fibrin. Since blue and white MCs were found in characteristic random arrangements in the fibrin matrix, we were able to easily locate individual MCs of interest at different stages of development.

In cocultivation experiments, we could detect coordinated, capillary-like growth of endothelial cells and non-endothelial cells only on some occasions. In general, we did not observe fibroblasts, pericytes or vascular smooth muscle cells stimulating the development of endothelial capillary-like structures during the early stages of capillary formation. The elongated cellular processes of vascular smooth muscle cells obviously were not used by endothelial cells as guidance structures for migration and capillary morphogenesis (Fig. 2). When spontaneously developing capillary-like formations in malleable gels (after polymerization at pH 7.6) came into contact with pericytes, the pericytes did not lead to further elongation of these sprouts and, furthermore, pericytes did not show any tendency to occupy their physiological position on the outside of the nascent capillary (Fig. 3). However, in

advanced stages of cell growth, we occasionally observed capillary-like endothelial formations which developed in close contact with pericytes (Fig. 4). We have not yet investigated whether these structures included cell-cell contacts between endothelial cells and pericytes. The formation of cell-cell contacts and the development of bicellular capillaries (comprising endothelial cells and pericytes) was not stimulated by addition of bFGF/VEGF to cocultures.

Discussion

The effects of periendothelial cells (fibroblasts, pericytes, vascular smooth muscle cells) on capillary formation can broadly be attributed to two different mechanisms. First, periendothelial cells may produce humoral factors which may stimulate or inhibit endothelial growth and capillary differentiation. Montesano et al. (1993) provided one example of this mechanism by showing that fibroblasts can secrete factors which paracrinely stimulate endothelial cells to develop into capillary-like structures. These results appear to conflict with our data since we could not find an increase in formation of capillary-like structures by cocultivating endothelial cells with fibroblasts. However, Montesano et al. (1993) offered an explanation for this discrepancy. From several fibroblast subtypes under investigation, only one line (Swiss 3T3) was suited to stimulate in vitro angiogenesis by soluble factors. Thus, this mechanism apparently cannot be generalized and appears to require specialized types of cells or selected growth conditions.

The second mechanism for the modulation of endothelial differentiation by periendothelial cells requires cell-cell contacts. Microvascular pericytes were described as inhibiting the proliferation of endothelial cells in vitro (Orlidge and D'Amore 1987). TGF-β was identified as the responsible mediator for this effect and both the inhibitory effect and the conversion of latent TGF-β to active TGF-β appeared to require cell-cell contacts between pericytes and endothelial cells (Antonelli-Orlidge et al. 1989; Dennis and Rifkin 1991). In other studies it was shown that TGF-β modulates the morphological differentiation of endothelial cells in vitro (Madri et al. 1988; Pepper et al. 1990). In a recent study on angiogenesis in the rat mesentery, we observed that endothelial sprouts in situ tend to differentiate in close contacts with pericyte-like cells, which were often seen even to precede outgrowing endothelial cells (Nehls et al. 1992). Thus, it appears reasonable to assume that pericytes have a role in the regulation of capillary morphogenesis.

In this study, we described a microcarrier-based cocultivation system which markedly facilitates the observation of cellular interactions in three-dimensional matrices in vitro. So far, it was only infrequently that we observed coordinated growth of endothelial cells and pericytes in our three-dimensional system: several factors might interfere with intercellular signaling, which most likely is required for the formation of complete capillaries comprising both endothelial cells and pericytes. It appears conceivable, for instance, that the cells were overstimulated by serum-derived growth factors. Therefore it seems important to develop a serum-free cocultivation system in which subtle cellular signals will not be drowned by the noise of serum-derived factors.

In conclusion, we are presenting a novel cocultivation system which allows investigation of cellular interactions in three-dimensional matrices. We think that the technical simplicity of this model in conjunction with other advantages, i.e., convenient quantification of angiogenesis and easy identification of cell types, will facilitate the investigation of cellular interactions of distinct microvascular cell types.

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