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REGULAR ARTICLE

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Contact-dependent inhibition of angiogenesis by cardiac fibroblasts in three-dimensional fibrin gels in vitro: implications for microvascular network remodeling and coronary collateral formation

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Abstract Angiogenesis and coronary artery collateral formation can improve blood flow and thereby prevent myocardial ischemia. The role of perivascular fibroblasts in neovascularization remains incompletely understood. Here we investigated the effects of epicardial and myocardial fibroblasts on angiogenesis in vitro by using a serum-free microcarrier-based fibrin gel angiogenesis system. To clearly distinguish between different cell types, we either stained endothelial cells or fibroblasts in the living with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine-perchlorate (DiI). In cocultures, low numbers of heart fibroblasts stimulated endothelial sprouting, and capillary growth was also induced by fibroblast-conditioned media, indicating a paracrine mechanism. Capillary formation was decreased by increasing the density of fibroblasts in the cocultures, indicating contact-dependent inhibition. Using time-lapse studies, it turned out that close contacts between fibroblasts and endothelial cells resulted in rapid retraction of endothelial cells or, rarely, in cell death. Depending on the local ratio of fibroblasts to endothelial cell numbers, fibroblasts determined the location of capillary growth and the size of developing capillaries and thereby contributed to capillary network remodeling. In contrast to primary heart fibroblasts, NIH 3T3 fibroblasts did not display contact-dependent inhibition of endothelial sprouts. NIH fibroblasts were frequently seen in close association with endothelial capillaries, resembling pericytes. Contact-dependent inhibition of angiogenesis by epicardial fibroblasts could not be reversed by addition of neutralizing anti-TGF-β1 antibodies, by addition of serum, of medium conditioned by hypoxic tumor cells or myocardium, by various cytokines or by growing cocultures under hypoxic conditions. Our results implicate a pivotal role of periendothelial mesenchymal cells for the regulation of microvascular network remodeling and collateral formation.

Key words Angiogenesis · Fibroblast · Heart · Collateral formation · Pericyte · Endothelial cell · In vitro · Fibrin

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Introduction

Ultrastructural investigations have shown that sprouting endothelial cells establish frequent cell-cell contacts to pericapillary fibroblasts (Cliff 1963; Rhodin and Fujita 1989; Diaz-Flores et al. 1992; Hansen-Smith et al. 1996). Fibroblasts most probably give rise to microvascular pericytes (Clark and Clark 1925; Rhodin and Fujita 1989; Nehls and Drenckhahn 1991; Nehls et al. 1992; Stein et al. 1996; for review: Nehls and Drenckhahn 1993), and pericytes were observed to accompany endothelial cells during capillary formation in situ (Verhoeven and Buyssens 1988; Rhodin and Fujita 1989; Schlingemann et al. 1990; Nehls et al. 1992; Hansen-Smith et al. 1996). Pericytes were shown to inhibit in vitro the proliferation and migration of endothelial cells by a cell-cell contact- and TGF-β-dependent mechanism (Antonelli-Orlidge et al. 1989; Sato and Rifkin 1989). In contrast, recent studies provided evidence that fibroblasts may promote angiogenesis. Swiss 3T3 fibroblasts were shown in a collagen gel coculture system to induce capillary formation (Montesano et al. 1993). However, this effect was restricted to this fibroblast cell line, and several primary fibroblast strains were ineffective (Montesano et al. 1993). Other groups reported that primary fibroblasts are capable of stimulating angiogenesis (Sato et al. 1987; Villaschi and Nicosia 1994). It would be compatible with the available data on fibroblasts/pericytes that fibroblasts are proangiogenic and switch to an anti-angiogenic phenotype during their differentiation to pericytes. The developmental stage at which fibroblasts switch to angiogenesis inhibition and become "functional" (inhibitory) pericytes, however, is unknown.

Angiogenesis is characterized by sprouting of new capillaries from preexisting vessels whereas collateral vessels develop by enlargement from preexisting arterioles (Schaper and Ito 1996; Folkman and D'Amore 1996). Hemodynamic forces are considered to play a key role in collateral formation (Hudlicka 1991), but the details of this process are incompletely understood. We do not know, for instance, why collateral formation is rapid and sufficient in some species whereas in others (humans, pigs) collateralization remains poor (Schaper and Ito 1996).

Following the idea that the perivascular mesenchyme of the coronary microvasculature may regulate angiogenesis and possibly also collateral formation, we were interested to investigate the role of heart fibroblasts during capillary development.

We performed numerous time-lapse studies in confrontation cultures in three-dimensional fibrin gels, and provide evidence that cell-cell contacts between heart fibroblasts and endothelial cells inhibit neovessel formation. The findings reported here suggest that periendothelial mesenchymal cells significantly contribute to vascular remodeling.

Materials and methods

Porcine fibrinogen (>70% protein clottable), bovine thrombin, insulin-transferrin-selenium medium supplement (ITS), cytodex-3 microcarriers, platelet-derived growth factor-BB (PDGF, human, recombinant), basic fibroblast growth factor (bFGF, human, recombinant) and the antibodies against von Willebrand factor and smooth muscle-type-α-actin (αsm-actin) were from Sigma (Deisenhofen, Germany). Dulbecco's modified Eagle's medium (DME) and fetal bovine serum (FBS) were from Gibco (Eggenstein, Germany). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-perchlorate (DiI) was from Molecular Probes (Eugene, Oregon, USA). Cellocate coverslips, square size 175 µm, were from Eppendorf (Hamburg, Germany), and 35-mm culture dishes were from Greiner (Frickenhausen, Germany). Aprotinin (TrasylolTM) was from Bayer (Munich, Germany). Recombinant human vascular endothelial growth factor (VEGF) was from Pepro Tech Inc. (Rocky Hill, USA), and recombinant human γ-interferon was from Biomol (Hamburg, Germany). The inhibitory polyclonal antibody to TGF-β1 was from Promega (Madison, USA).

Cell culture

Endothelial cells were isolated from porcine aortas by controlled collagenase digestion and grown in DME supplemented with 10% FBS essentially as described (Nehls et al. 1994; Nehls and Drenckhahn 1995a,b). Trypsinized cells of passages 6–10 were allowed to attach onto cytodex-3 microcarrier beads for 4 h at 37°C and subsequently were grown to confluence for 48 h. Endothelial cells were identified by immunostaining using an antibody against von Willebrand factor.

Epicardial fibroblasts were isolated from porcine epicardial fat tissue and 2×2-mm fragments of epicardial tissue were allowed to adhere to plastic culture dishes for 3 min and were then carefully overlaid with medium. After approximately 5–7 days the first cells were detected leaving the tissue fragments. For coculture angiogenesis assays we used epicardial fibroblasts of passages 12–15. Control experiments were performed showing that earlier passages also inhibited angiogenesis. We routinely used passages 12–15 as these cultures homogeneously contained fibroblasts and were free of endothelial cells. Morphologically, the cells behaved as fibroblasts with lack of contact inhibition and multilayered growth. Fibroblast cultures were negative for von Willebrand factor and contained moderate amounts of αsm-actin. In three-dimensional culture, primary fibroblasts migrated as single cells and did not assemble to multicellular capillary-like structures. Myocardial fibroblasts were isolated by a similar procedure, except using porcine myocardial fragments, which were dissected from deeper layers of myocardium to avoid contamination with epicardial tissue.

NIH 3T3 fibroblasts (Todaro and Aaronson 1969) were obtained from the American Type Culture Collection (Rockville, Maryland, USA; ATCC Number: CRL-1658). The cells were routinely grown in DME supplemented with 10% FBS.

DiI-labeling of living cells

A 2.5-mg/ml stock solution of DiI (Honig and Hume 1986) in 95% ethanol was aliquoted and stored at 4°C until use. Microcarrier beads, covered with endothelial cells or fibroblasts, respectively, were transferred into DME/10% FBS containing 40 µg/ml DiI. Incubation was performed for 60 min at 37°C. Microcarriers were then washed 3 times in DME/10% FBS. Staining and washing steps were performed in 1.5-ml Eppendorf tubes. Microcarriers rapidly sedimented to the bottoms of the tubes. Hence, centrifugation steps were not required. Differences in viability or growth performance between unlabeled cells and labeled cells were not detectable. For cocultures, we embedded endothelial and fibroblast-carriers in a ratio of 1:1 in fibrin gels, and the migration of cells was monitored by using a Zeiss inverse cell culture microscope equipped with inverse fluorescence and rhodamine filter optics.

Serum-free fibrin gel angiogenesis system

The microcarrier-based fibrin gel angiogenesis assay was performed as described (Nehls and Drenckhahn 1995a,b; Nehls and Herrmann 1996), with some modifications. Endothelial cells and fibroblasts were trypsinized and allowed to adhere onto different sets of microcarrier beads. Cells were grown to confluence for 48 h. To safely identify different cell types, either the beads covered with endothelial cells or the fibroblast beads were stained with DiI as described above. Porcine fibrinogen was then dialyzed overnight against PBS (phosphate-buffered saline; pH 7.0; 10 mM phosphate, 140 mM sodium chloride). The fibrinogen stock solution was diluted to 1 mg/ml in PBS, pH 7.0. To facilitate finding of cells in time-lapse studies, sterile Cellocate coverslips were fixed with 10 µl melted agar at the bottoms of 35-mm culture dishes and the fibrin gels were polymerized on top. Forty microliters of packed microcarrier beads (20 µl endothelial cell carriers and 20 µl fibroblast carriers) were pipetted into 2 ml fibrinogen solution. The microcarriers were evenly distributed in the fibrinogen by gently shaking the culture dishes, and polymerization was induced by addition of 0.625 U/ml thrombin. Fibrin gels were allowed to polymerize for 30 min at room temperature and the gels were then equilibrated in medium (DME without serum; supplemented with 1 µg/ml insulin; 1 µg/ml transferrin; 1 ng/ml selenium; 100 U/ml penicillin; 100 µg/ml streptomycin) for 60 min at 37°C. Fresh medium subsequently was added to the gel surface. Growth factors and antibodies, if required, were added to the fibrinogen solution prior to polymerization to avoid poor diffusion from the supernatant into the fibrin clot. Time-lapse studies were performed by taking photographs from selected areas of the fibrin gels at the time intervals indicated. The inclusion of Cellocate slides enabled us to clearly relocate areas of interest within the fibrin gels and to follow the development of capillaries over time.

Quantification of angiogenesis in cocultures

To quantify the effects of fibroblasts on angiogenesis, we counted in cocultures the percentage of DiI-stained endothelial microcarriers which were "positive" (for definition, see below) for capillary growth (MC%). Unstained fibroblast carriers were not included in the count. As a control, we used monocultivated, DiI-stained endothelial cells (20 µl packed endothelial microcarriers/gel). The cocultures contained 20 µl packed (sedimented) endothelial microcarriers

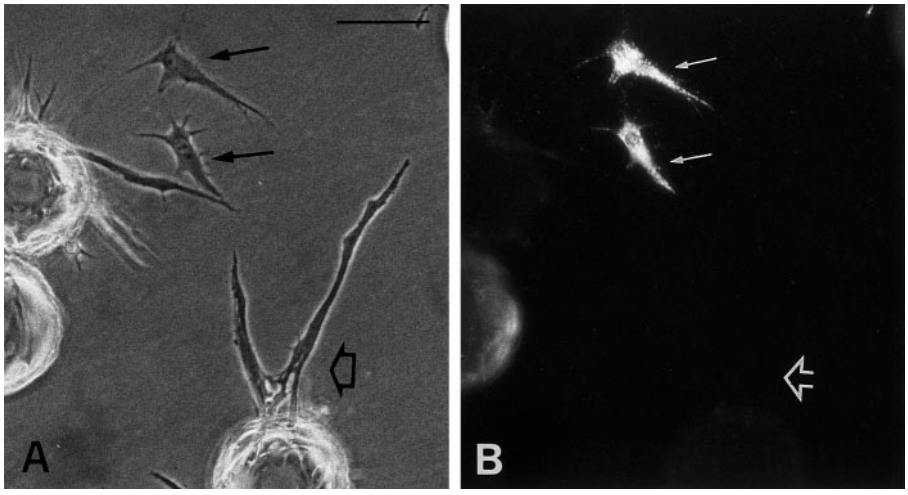

Fig. 1A, B Coculture of DiI-labeled epicardial fibroblasts (*arrows*) and unlabeled endothelial cells in fibrin at day 3. The different mesenchymal cell types were grown on microcarrier beads and embedded in fibrin as described in "Materials and methods." **A** Phase-contrast microscopy; **B** corresponding DiI fluorescence. Note that fibroblasts migrate as single cells through the fibrin gel whereas endothelial cells develop to multicellular capillaries which at their base show formation of lumina (*framed arrow*). One fibroblast sends out a cellular process which contacts the tip of a short endothelial sprout. *Bar* 100 μm

together with 20 μl (1:1) or 100 μl fibroblast microcarriers (1:5). A microcarrier was arbitrarily assigned to be angiogenesis-"positive" when the length of at least one endothelial structure originating from this carrier was equal to or exceeding the carrier diameter (150–220 μm). For each experiment, we determined capillary growth from three different fibrin gels and counted 100 microcarriers per gel. Data are expressed as means±SD of three independent experiments, and statistical analysis was performed by using Student's *t*-test.

Experiments using fibroblast-conditioned media

Conditioned media from epicardial fibroblasts were obtained as follows. Fibroblast cultures were washed 4 times in DME w/o FBS, and 5 ml DME w/o FBS was then added to a 75-cm^2 confluent fibroblast layer. Incubation was continued for 12 h at 37°C. Conditioned media were passed through a 0.2-μm filter to remove cell debris and stored frozen until use. To quantify the effect of conditioned media on capillary growth, the medium supernatant (DME) of fibrin gels containing endothelial microcarriers was supplemented with 30% fibroblast-conditioned medium. Capillary growth was counted at 24 h, 48 h and 72 h after polymerization of the gel. Data are expressed as means of three independently performed experiments, ±SD.

Hypoxia experiments

To grow cocultures under hypoxic conditions, Petri dishes containing the fibrin gel cocultures were transferred into an anaerobic jar (the jar and Anaerocult A oxygen traps were from Merck, Darmstadt, Germany). Cells were exposed to hypoxia for 6 h each day over 4 days. After each period of hypoxia, the medium turns from red to yellow indicating acidification of the medium. In other experiments, only the fibroblasts were exposed to hypoxia for 12 h after which the cells were embedded in fibrin gels together with unexposed endothelial cells.

Conditioned medium from hypoxic or normoxic tumor cells or myocardium was obtained as follows. U-138 MG human glioblastoma cells (Tumorbank, Deutsches Krebsforschungszentrum, Heidelberg, Germany) were grown to confluence in Ham's F10 supplemented with 10% FBS. The culture dish containing 2.2×10^6 cells in 5 ml DME/1% FBS was transferred into the anaerobic jar for 24 h at 37°C. The conditioned medium was then removed, passed through a 0.2-μm filter and stored frozen until use.

Porcine myocardium was obtained from the local abattoir. Larger pieces of myocardium were sterilized in 70% ethanol for 2 min. The margins which were exposed to the ethanol were removed and 2- to 3-mm fragments were transferred into DME w/o FBS. To obtain myocardium-conditioned medium, myocardial fragments were exposed to hypoxia or normoxia for 24 h. The conditioned medium was then passed through a 0.2-μm filter to remove cell debris and stored frozen until use. In coculture experiments, 10% conditioned medium was added to the fibrinogen solution prior to polymerization and the medium supernatant was supplemented with 30% conditioned medium.

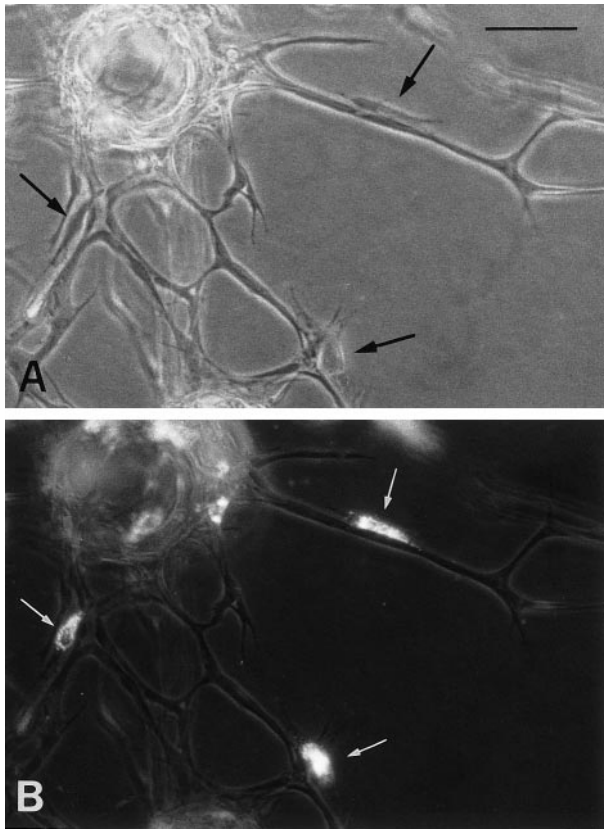

Fig. 2A, B Coculture of DiI-labeled epicardial fibroblasts and endothelial cells at day 6 after polymerization of the fibrin gel. **A** Phase-contrast microscopy; **B** corresponding DiI fluorescence with low-power phase-contrast illumination. Note that fibroblasts (*arrows*) are tightly associated with unstained endothelial capillaries. Fibroblasts have acquired a pericyte-like morphology (compare the multistellate shape of fibroblasts in Fig. 1 with the condensed shape in Fig. 2). *Bar* 100 μm

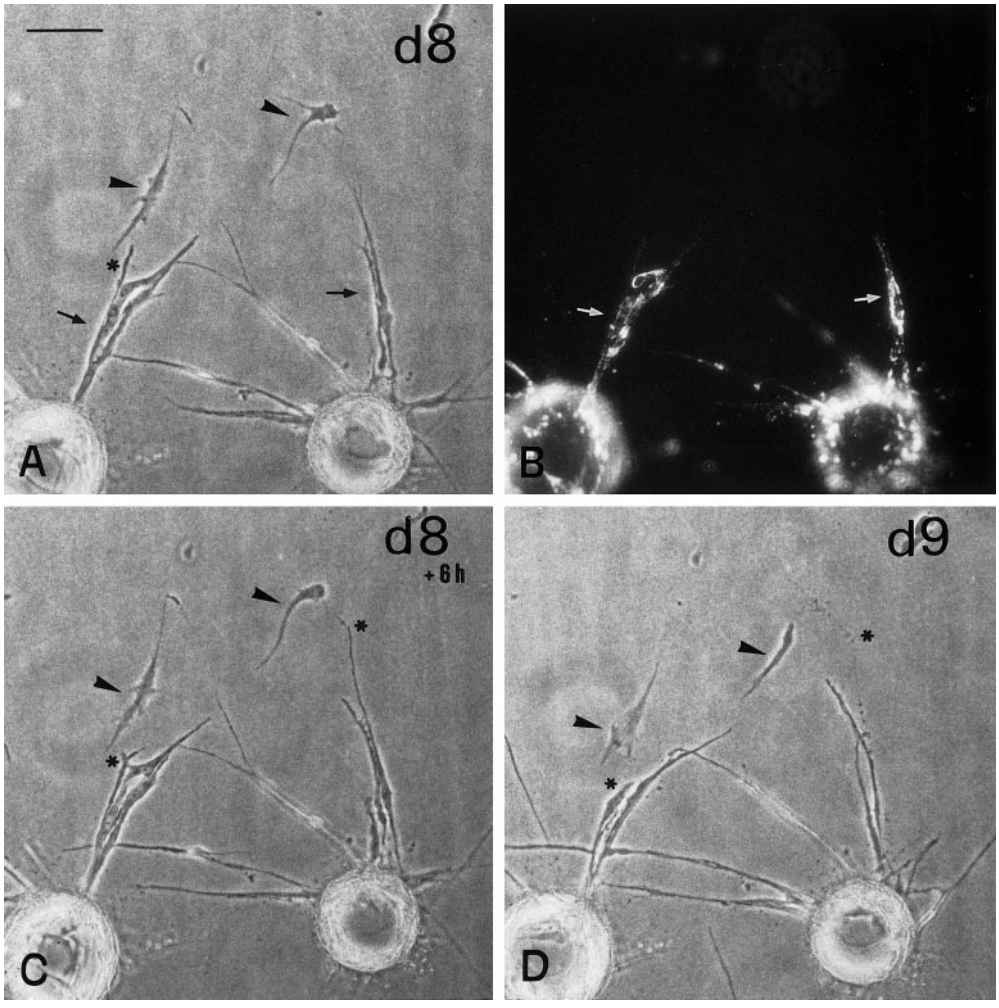

Fig. 3A–D Time-lapse study of cocultured epicardial fibroblasts (*arrowheads* unstained) and endothelial cells (labeled with DiI; *arrows in* **A**). Capillary development was followed from day 8 to day 9 over 24 h. **A, C, D** Phase-contrast micrographs of respective developmental stages. **B** DiI fluorescence, corresponding to **A**. Note that fibroblast-endothelial contacts are only transient and either result in retraction (*asterisks in* **A, C, D**) or degeneration (vesiculation) of endothelial processes (*asterisks in* **C, D**). *Bar* 100 μm

Results

To elucidate the role of fibroblasts during angiogenesis, we embedded in three-dimensional fibrin gels a mixture of microcarriers from which 50% were covered with endothelial cells and the other half with fibroblasts. To clearly distinguish between endothelial cells and fibroblasts in our coculture system, either the endothelial cells or the fibroblasts were labeled in the living with DiI prior to embedding the carriers in fibrin. By inverted fluorescence microscopy, the fluorescent label allowed convenient identification of different mesenchymal cell types. Already some hours after polymerization of the fibrin gel, cells were observed leaving their supporting microcarriers and migrating into the fibrin matrix. Fibroblasts lost the DiI stain more rapidly, as compared with endothelial cells, which were sufficiently labeled for up to 14 days, which might be due to faster migration of fibroblasts in fibrin. However, also the DiI signal of fibroblasts was clearly detectable for a period of up to 10 days.

In our serum-free system, endothelial cells developed by maintaining close contacts to other endothelial cells, forming multicellular capillaries which at their proximal parts showed lumina (Fig. 1). Unlike endothelial cells, fibroblasts migrated as single cells through the fibrin gel and generally did not associate with other fibroblasts to multicellular structures (Fig. 1). Endothelial cells were strongly attracted by other endothelial cells, as indicated by directional migration of sprouts to other sprouts and anastomosation to continuous capillary loops (Fig. 2; described in more detail in Nehls et al. 1998).

Although heart fibroblasts were frequently observed having superficial contacts to endothelial sprouts (Fig. 1), tight associations between heart fibroblasts and endothelial cells (which might be regarded as a beginning differentiation process of fibroblasts to pericytes) were rare (Fig. 2). We hypothesized that this was due to contact-dependent inhibition of endothelial cells by cardiac fibroblasts.

Contact-dependent inhibition of angiogenesis by cardiac fibroblasts

To further investigate the function of cardiac fibroblasts during angiogenesis, we performed numerous time-lapse studies on fibrin gel cocultures. When fibroblasts came

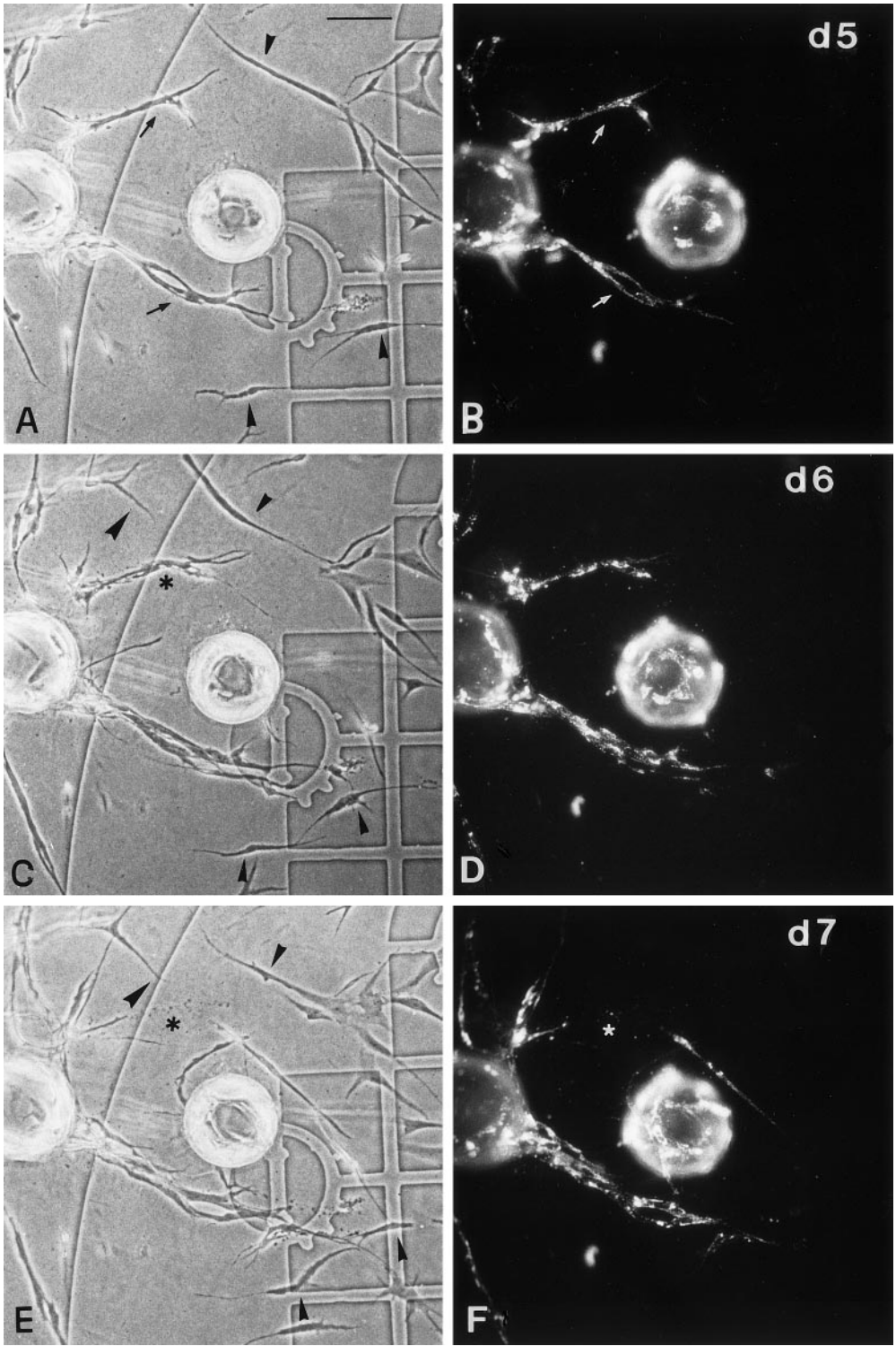

Fig. 4A–E Time-lapse study from day 5 to day 7 of cocultured epicardial fibroblasts (*arrowheads* unstained) and endothelial cells (*arrows in* **A, B** labeled with DiI). **A, C, E** Phase-contrast micrographs of respective developmental stages. **B, D, F** Corresponding DiI fluorescence. Note that there is a "barrier" of fibroblasts which held a constant distance to endothelial cells over 48 h and which probably prevented elongation of endothelial capillaries. Instead, both capillary sprouts shown here underwent thickening (compare day 5 and day 6). One fibroblast (*large arrowhead in* **C, E**) which contacted the upper capillary sprout (*asterisk*) between day 6 and day 7 (cell-cell contact not shown) induced disruption of this capillary structure with partial regression (vesiculation) of endothelial cells. *Bar* 100 μm

into contact with endothelial capillaries, we observed regression or retraction of endothelial sprouts (Figs. 3, 4) under all the conditions tested (see below). Owing to contact-dependent inhibition, fibroblast-endothelial contacts generally were of short duration. Fibroblasts could be frequently shown maintaining a certain distance to endothelial capillaries over several days (Fig. 4). When lying opposite to endothelial capillaries, cardiac fibroblasts appeared to inhibit further elongation of these sprouts. Sprouts which were inhibited to elongate by a "barrier" of fibroblasts occasionally underwent thickening (Fig. 4). In a growing microvascular network, the local distribution of fibroblasts thus may determine the location where continuous capillary loops develop, and fi-

broblasts also may regulate the size and the length of endothelial capillaries.

NIH 3T3 fibroblasts do not display contact-dependent inhibition

In contrast to cardiac fibroblasts, NIH 3T3 fibroblasts associated early and frequently with endothelial capillaries (Figs. 5–7), without suppressing further capillary growth. Branched endothelial capillaries regularly were found to be surrounded by dense cuffs of associated fibroblasts (Fig. 5). NIH 3T3 fibroblasts frequently were seen accompanying endothelial sprouts during elongation and forming the leading tips of sprouts (Fig. 5). It also became apparent that NIH 3T3 fibroblasts were not passively included in bicellular capillaries by ingrowth of endothelial sprouts in groups of fibroblasts but, rather, showed active migration towards endothelial sprouts. One representative example is illustrated in Fig. 6, which shows a fibroblast, lying ahead of an endothelial sprout tip, sending out a slender process which contacts the distal ending of the endothelial sprout (asterisk in Fig. 6, panel C). Thus, there obviously is intense communication between different mesenchymal cell types during angiogenesis. In contrast to cardiac fibroblasts, contacts between NIH 3T3 fibroblasts and endothelial cells did not suppress endothelial cell growth. Even sprouts which were densely invested with fibroblasts continued to elongate (Figs. 5–7), indicating that fibroblast-endothelial contacts do not necessarily result in angiogenesis inhibition, and opening up the possibility that the phenotype of fibroblasts may be modulated by unknown signals towards angiogenesis promotion. From a morphological perspective, it was obvious that the NIH fibroblasts were differentiating more frequently to pericyte-like cells as compared to cardiac fibroblasts (Fig. 2). After initial attachment to endothelial tubules, the NIH fibroblasts frequently assumed a rounded, pericyte-like morphology (Fig. 7).

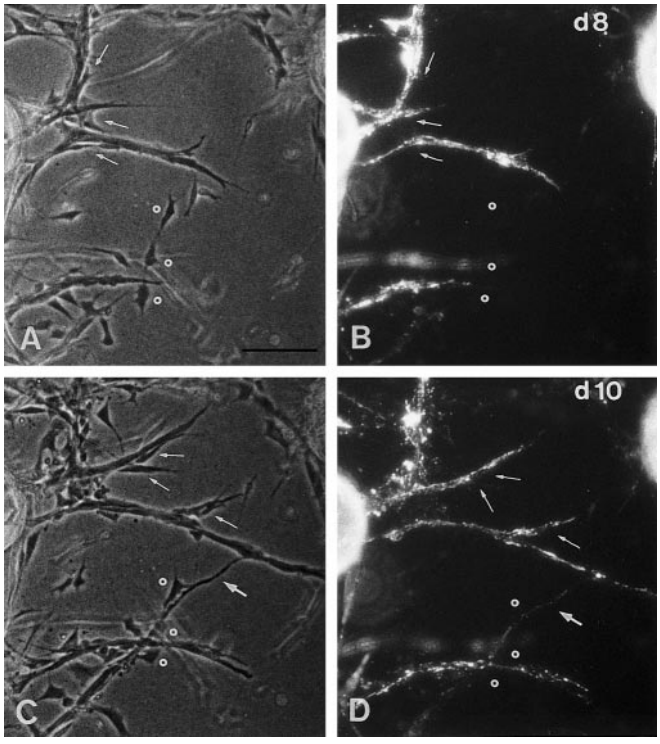

Fig. 5A–D Time-lapse study from day 8 to day 10 of cocultured unlabeled NIH 3T3 fibroblasts and endothelial cells (labeled with DiI). **A, C** Phase-contrast micrographs, **B, D** Corresponding DiI fluorescence. Note that endothelial capillaries are invested by a dense cuff of fibroblasts (*thin arrows in* **A, B**). During elongation of endothelial sprouts, 3T3 fibroblasts accompany endothelial cells (*thin arrows in* **C, D**) and are frequently seen at the leading tips of endothelial sprouts. Within the 32 h covered by this time-lapse study, one thin endothelial branch developed (*thick arrow in* **C, D**). Note that three fibroblasts (*circles in* **A–D**) which before were lying freely within the fibrin matrix now contact this endothelial branch and partially change their shape towards a condensed, pericyte-like morphology. *Bar* 100 μm

Quantification of capillary growth in cocultures

To quantify the effect of fibroblasts on endothelial cell growth in cocultures, we counted capillary sprouts 48 h after polymerization of the fibrin gels as described in "Materials and methods." In cocultures containing fibroblast microcarriers (F) and endothelial cell microcarriers (EC) at a ratio of 1:1, we found initial endothelial sprouting to be stimulated by heart fibroblasts (P=0.012) and even stronger by NIH 3T3 fibroblasts (P=0.006; Fig. 8). However, by increasing the number of fibroblast microcarriers in the fibrin gels (EC/F 1:5), the angiogenic response was significantly suppressed (P=0.023 as compared with EC; Fig. 8), indicating that fibroblasts stimulate angiogenesis by paracrine factors but inhibit endothelial sprouting by a cell-cell contact-dependent manner. To further investigate the paracrine effects of fibroblasts, endothelial cells were grown in the presence of fibroblast-conditioned medium (Fig. 9). It appeared that capillary growth was significantly stimulated by fibroblast-conditioned medium ($P<0.001$).

Attempts to alleviate angiogenesis inhibition by cardiac fibroblasts

In response to physiological demands, fibroblasts may be capable of switching from an antiangiogenic to a proangiogenic phenotype. By suppression of contact-dependent inhibition, we expected that this would lead to increased numbers of fibroblasts resembling pericytes. To test this hypothesis, coculture experiments were performed under hypoxic conditions, or cocultures were treated with conditioned media from hypoxic tumor cells or hypoxic myocardial fragments. Although conditioned media (both from normoxic and hypoxic cells) stimulated the migration of fibroblasts into the fibrin gel, the cells retained their inhibitory phenotype on angiogenesis, and fibro-

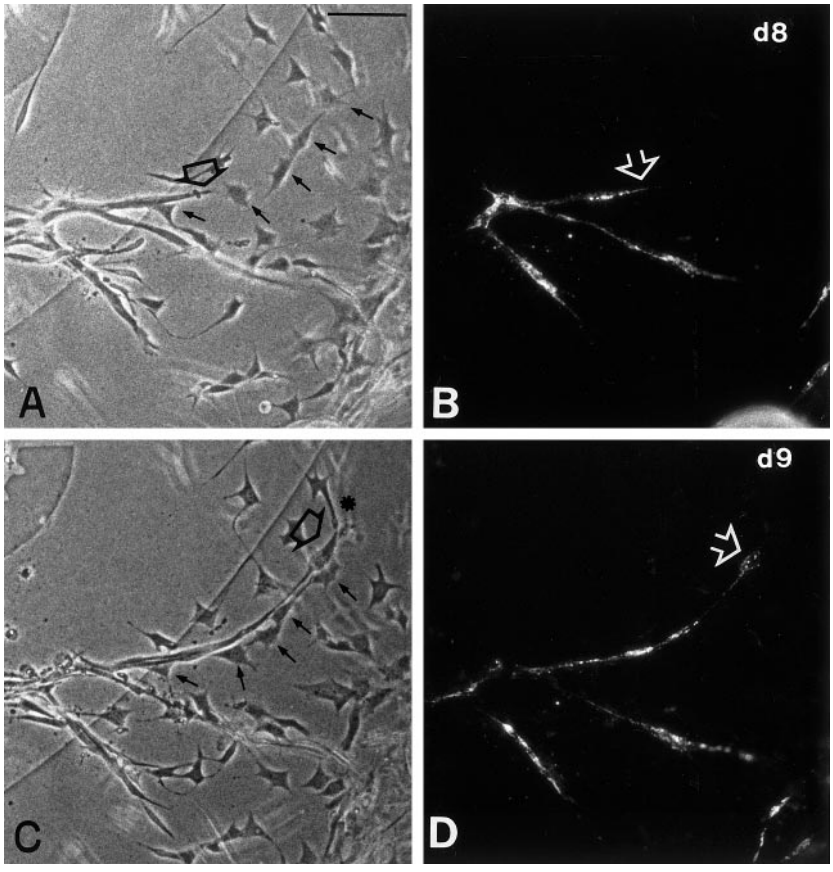

Fig. 6A–D Time-lapse study from day 8 to day 9 of cocultured unlabeled NIH 3T3 fibroblasts and DiI-labeled endothelial cells. **A, C** Phase-contrast micrographs, **B, D** Corresponding DiI fluorescence. The tip of an endothelial sprout which is growing into a group of fibroblasts is indicated by a *framed arrow*. Note that several fibroblasts (*arrows* in **A, C**) become attached to the endothelial sprout. One fibroblast (*asterisk* in **C**) which is located ahead of the sprout tip sends out a cellular process contacting the leading edge of the endothelial sprout. *Bar* 100 μm

blasts were not observed to closely associate with endothelial cells (not illustrated). Similarly, hypoxic conditions did not cause any changes of fibroblast behavior (not illustrated).

To investigate the effect of growth factors, cocultures were stimulated using a variety of factors and cytokines which have been described to modulate fibroblast differentiation (for review, see Sappino et al. 1990) including α-interferon, bFGF, VEGF, TGF-β1, and PDGF-BB. However, none of these factors was effective in alleviating contact-dependent inhibition (not illustrated).

According to the literature, there is ample evidence that pericyte-mediated inhibition of endothelial cell growth is mediated, at least in part, by TGF-β1. However, by using a neutralizing anti-TGF-β1 antibody in concentrations of up to 10 μg/ml, we could not achieve any suppressive effect on fibroblast inhibition in fibrin gel cocultures. However, the latent TGF-β1 is known to become activated upon cell-cell contact between pericytes and endothelial cells (Antonelli-Orlidge et al. 1989; Sato and Rifkin 1989), and these short-range cytokine effects might be largely inaccessible to antibodies.

Discussion

Here we describe that cardiac fibroblasts inhibit angiogenesis in a contact-dependent manner by using a fibrin clot coculture system. We do not know at present whether contact-dependent inhibition requires direct membrane contacts. Although we frequently observed endothelial regression after cell-cell contacts were established, the spatial resolution of the phase-contrast microscopy does not enable this question to be answered, and it is still possible that inhibition occurs before direct membrane contacts are established. However, from our experience with three-dimensional culturing, we estimate that the required intercellular distance for maximum inhibition is <3–4 μm.

NIH 3T3 fibroblasts lacked contact-dependent inhibition and, in contrast to primary heart fibroblasts, NIH fibroblasts were frequently seen in close proximity to endothelial capillaries, resembling pericytes. Retraction of endothelial sprouts upon formation of contacts to heart fibroblasts apparently was the reason why heart fibroblasts were only rarely observed in close apposition to endothelial capillaries. Alleviation of contact-dependent angiogenesis inhibition therefore appears to be an important prerequisite for the differentiation of fibroblasts to pericytes.

In agreement with previous studies (Sato et al. 1987; Villaschi and Nicosia 1994), capillary growth in our assay was stimulated in a paracrine manner and by condi-

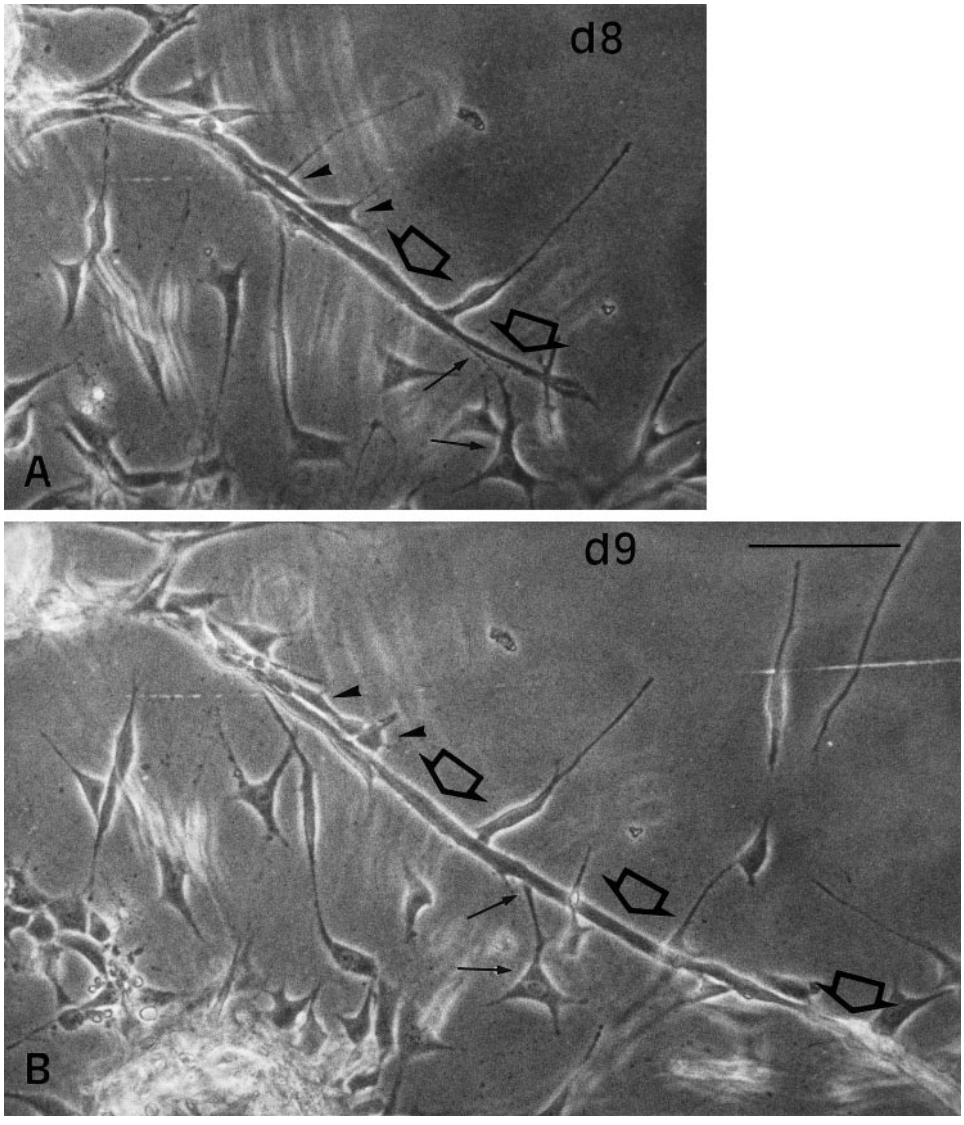

Fig. 7A, B Time-lapse study from day 8 to day 9 of cocultured DiI-stained endothelial cells (DiI fluorescence not shown) and unlabeled NIH 3T3 fibroblasts at higher magnification. Note that the endothelial sprout (*framed arrows* in **A, B**) which elongates during the 24 h of observation has several attached fibroblasts. Some of these change their morphology to a condensed, pericyte-like phenotype (*arrowheads*). One fibroblast (*arrows* in **B**) sends out a slender process which keeps in contact with the endothelial sprout for 24 h. *Bar* 100 μm

tioned media of fibroblasts. With regard to the simultaneous occurrence of contact-dependent inhibition and paracrine stimulation, we obviously face a rather complex and multiply regulated bicellular system. For instance, whether or not fibroblasts contact (and inhibit) endothelial cells probably is regulated by fibroblast- and endothelial-derived factors. We frequently observed that fibroblasts held a certain distance to endothelial tubules over several days, thereby playing a permissive or even stimulatory role in angiogenesis. On other occasions, fibroblasts approached and contacted endothelial capillaries, leading to retraction or disruption of microvascular branches.

NIH 3T3 fibroblasts did not display contact-dependent inhibition, indicating that it might be possible to switch the phenotype of fibroblasts from inhibition to promotion or at least towards a permissive behavior. In spite of the obvious differences between fibroblast cell lines and primary fibroblast cultures, we believe for several reasons that even primary fibroblasts lack under certain conditions contact-dependent inhibition. By studying angiogenesis in the rat mesentery in situ (Nehls et al. 1992), we observed fibroblasts closely associating with endothelial capillaries during the initial stages of capillary growth, indicating that the pericapillary fibroblasts at least were not frankly inhibitory since capillary growth continued for several days more. Additionally, we recently used primary fibroblasts from neonatal mice in our fibrin gel system and realized that these cells also closely associated with endothelial capillaries, resembling the NIH 3T3 fibroblasts (data not shown).

It appears likely that not only the formation of intercellular contacts but also the contact-dependent inhibition by itself is regulated in the developing microvasculature. In an attempt to shift fibroblasts from inhibition to promotion of endothelial growth, we tested a variety of conditions in this study. Unfortunately, we did not yet find the conditions which could render fibroblasts less inhibitory and which might be required to reproducibly grow bicellular capillaries in vitro.

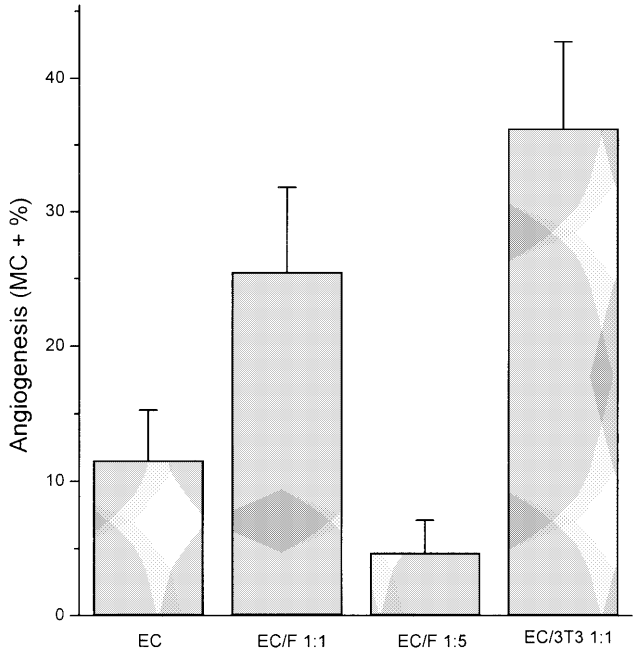

Fig. 8 Quantification of capillary growth in fibrin gel cocultures 48 h after fibrin polymerization. Microcarriers covered with endothelial cells or epicardial fibroblasts were embedded in fibrin in a ratio of 1:1 (*EC/F 1:1*) or 1:5 (*EC/F 1:5*) and compared with monocultivated endothelial cells (*EC*). Note that capillary sprouting is stimulated by low amounts of fibroblast carriers (EC/F 1:1), whereas angiogenesis is suppressed by increasing the density of fibroblast carriers (*EC/F 1:5*), indicating contact-dependent inhibition of capillary growth. NIH 3T3 fibroblasts strongly stimulated sprout formation (*EC/3T3 1:1*)

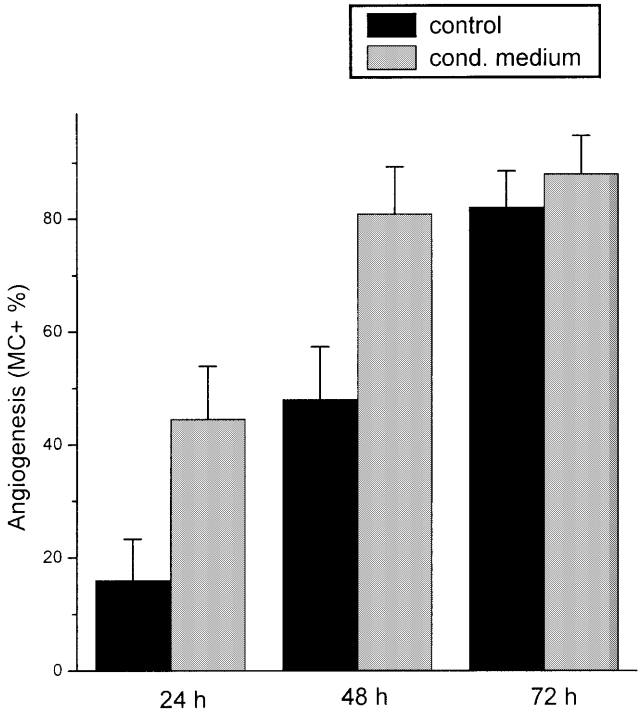

Fig. 9 Stimulation of capillary growth by fibroblast-conditioned medium. Addition of 30% conditioned medium to the supernatants of fibrin gels significantly accelerated endothelial sprouting from microcarriers embedded in fibrin matrices, indicating that fibroblasts stimulate the migration of endothelial cells by paracrine signaling

By considering the physiological function of fibroblasts, inhibition of capillary sprouting might be important for prevention of excess capillary formation, and inhibition also might help in shaping the growing vasculature into a few large conductance vessels instead of numerous low-caliber vessels. Pathologically, exaggerated fibroblast inhibition might contribute to insufficient collateral formation during coronary artery disease. Although the developmental steps in angiogenesis are different from those in collateral formation, there are also similarities. In both cases, endothelial cells grow in close contact to mesenchymal cells, periendothelial fibroblasts or smooth muscle cells of the vessel wall. It might be interesting in this regard that vascular smooth muscle cells isolated from the intima layer of coronary arteries also inhibited endothelial growth in a contact-dependent manner (not shown). During collateral formation, endothelial cells and smooth muscle cells proliferate and reorganize, finally leading to a dilated artery with increased conductance capacity (Schaper and Ito 1996). With regard to the aforementioned observations, cellular contacts between endothelial cells and smooth muscle cells and the angiogenic properties of periendothelial cells in general might be crucial for the development of collaterals and for shaping the growing microvasculature.

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