

# Inhibition of Capillary Endothelial Cell Growth by Pericytes and Smooth Muscle Cells

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**Abstract.** Morphological studies of developing capillaries and observations of alterations in capillaries associated with pathologic neovascularization indicate that pericytes may act as suppressors of endothelial cell (EC) growth. We have developed systems that enable us to investigate this possibility *in vitro*. Two models were used: a co-culture system that allowed direct contact between pericytes and ECs and a co-culture system that prevented physical contact but allowed diffusion of soluble factors. For these studies, co-cultures were established between bovine capillary ECs and the following growth-arrested cells (hereafter referred to as modulating cells): pericytes, smooth muscle cells (SMCs), fibroblasts, epithelial cells, and 3T3 cells. The modulating cell type was growth arrested by treatment with mitomycin C before co-culture with ECs. In experiments where cells were co-cultured directly, the effect of co-culture on EC growth was determined by comparing the mean number of cells in the co-cultures to the mean for each cell type (EC and modulating cell) cultured separately. Since pericytes and other modulating cells were growth arrested, any cell number change in co-cultures was due to EC growth. In the co-cultures, pericytes inhibited all EC proliferation throughout the 14-d time course;

similar levels of EC inhibition were observed in SMC-EC co-cultures. Co-culture of ECs with fibroblasts, epithelial cells, and 3T3 cells significantly stimulated EC growth over the same time course (30–192% as compared to EC cultured alone). To determine if cell contact was required for inhibition, cells were co-cultured using Millicell chambers (Millipore Corp., Bedford, MA), which separated the cell types by 1–2 mm but allowed the exchange of diffusible materials. There was no inhibition of EC proliferation by pericytes or SMCs in this co-culture system. The influence of the cell ratios on observed inhibition was assessed by co-culturing the cells at EC/pericyte ratios of 1:1, 2:1, 5:1, 10:1, and 20:1. Comparable levels of EC inhibition were observed at ratios from 1:1 to 10:1. When the cells were co-cultured at a ratio of 20 ECs to 1 pericyte, inhibition of EC growth at 3 d was similar to that observed at other ratios. However, at higher ratios, the inhibition diminished so that by the end of the time course the co-cultured ECs were growing at the same rate as the controls. These results suggest that pericytes and SMCs can modulate EC growth by a mechanism that requires contact or proximity. We postulate that similar interactions may operate to modulate vascular growth *in vivo*.

**M**ATURE capillaries and postcapillary venules are comprised of two cell types—endothelial cells (ECs)<sup>1</sup> and pericytes. Pericytes exist in intimate association with ECs, forming a single layer that covers varying amounts of the abluminal EC surface. This arrangement, unique to small vessels, is characterized by frequent sites of contact between the ECs and pericytes. The interaction between ECs and pericytes has been extensively analyzed at both the light (43) and electron microscopic (9, 34, 37) levels, yet there is little evidence to support specific functional roles for these cell-cell interactions.

The pericytes' position in the capillary and their physical

association with the ECs have led to the postulation of several roles for the pericyte, including regulation of permeability (12), mediation of contractility (44), maintenance of integrity (37), and control of proliferation (10, 28). Although direct evidence is lacking, the localization in pericytes of cGMP-dependent protein kinase (26) and contractile elements including actin (21) and myosin (27) supports their proposed involvement in microvascular contractility. Large, acid phosphatase-containing vacuoles have been observed to be preferentially localized on the interstitial side of pericyte cytoplasm (9, 37), indicating a possible phagocytic function for pericytes (5). In spite of these observations, the specific contributions of pericytes to microvascular function remain uncharacterized.

The absence of pericytes has been correlated with the on-

1. *Abbreviations used in this paper:* EC, endothelial cells; (a)FGF, (acidic) fibroblast growth factor; RPE cells, retinal pigment epithelial cells; SMC, smooth muscle cell.

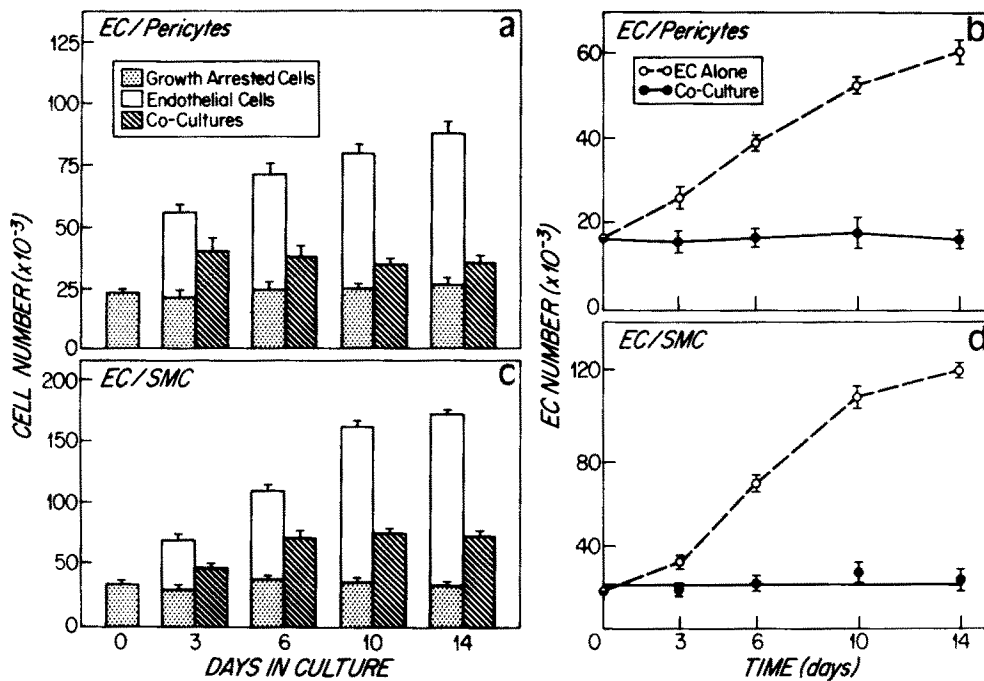


Figure 1. Effect of co-culture of capillary ECs with pericytes or SMCs on EC proliferation. (a and c) Pericytes and SMCs were growth arrested using mitomycin C (10  $\mu$ g/ml; 2 h), washed, trypsinized, and plated (20,000 cells/well) into 24-well dishes (hatched bars). After overnight attachment, equal numbers of ECs were plated into wells containing growth-arrested cells. Controls consisted of growth-arrested cells (stippled bars) and ECs cultured separately (open bars). All cultures were grown in media containing 10% calf serum. At 3–4-d intervals over 14 d, quadruplicate wells of cells from each group were trypsinized and counted. The effect on EC growth was determined by comparing the mean sum of control cell numbers to the mean sum of co-

cultured cells. Since growth-arrested cells were not dividing, all changes in co-cultured cell numbers were due to EC growth. (b and d) The same data are depicted by comparing the number of ECs grown alone (dashed line) to the number of ECs in co-culture (solid line) with modulating cells over 14 d. The number of co-cultured EC was determined by the difference between total number of co-cultured cells and the number of growth-arrested modulating cells.

set of neovascularization. Before the onset of new vessel growth associated with diabetes mellitus, there is a loss of pericytes, termed “pericyte drop-out” (12). Hemangiomas, vascular anomalies characterized by excessive EC proliferation, have few, if any, pericytes or smooth muscle cells (SMCs) (15). Morphogenetic studies of vessel development demonstrate that forming capillaries are characterized by active EC proliferation and migration, and the absence of pericytes (2); whereas, there is a decline in EC proliferation and migration (2, 10) concomitant with pericyte appearance. Such observations suggest a role for pericytes in the regulation of EC growth.

Using an *in vitro* system, we have developed co-culture models to examine this possibility. We have found that pericytes inhibit capillary EC proliferation and we have characterized this inhibition with respect to cell specificity, the role of contact or proximity, and the effect of cell ratios.

## Materials and Methods

### Cell Culture

**Endothelial Cells.** Capillary ECs, isolated from bovine adrenal cortex using previously described methods (17), were the generous gift of Dr. J. Folkman and C. Butterfield. The cells were grown in 35-mm petri dishes coated with 1.5% gelatin (wt/vol [Difco, Detroit, MI] in PBS) in DME (Gibco, Grand Island, NY) containing 10% calf serum (DME/10CS; Sterile Systems, Logan, UT) plus 5  $\mu$ l/ml of crude retina-derived growth factor (containing acidic fibroblast growth factor [aFGF]) (18). The cells were identified as endothelial by staining with antisera to von Willebrand factor (23) and by their uptake of fluoresceinated, acetylated low density lipoprotein (48).

**Pericytes.** Pericytes were isolated from bovine retinas as previously described (18). Briefly, bovine eyes were soaked in 20% betadine (vol/vol in PBS), rinsed in PBS, and the adventitia was removed. For pericyte cultures,

the retinas were removed sterilely and washed free of pigment epithelium. The retinas were minced and then incubated with 0.2% collagenase (type II) with 0.1% BSA for 1 h. Capillary fragments were isolated from the collagenase digest by collecting the supernatant from a 110- $\mu$ m nylon sieve. Pericytes were selected by plating the capillary fragments onto uncoated tissue culture plastic in DME containing 10% FBS (DME/10 FBS; Sterile Systems, Logan, UT). Pericytes were identified and distinguished from ECs by their size and irregular morphology, by their noncontact-inhibited growth pattern, by the lack of staining with antisera to von Willebrand factor, and by their inability to take up fluoresceinated, acetylated low density lipoprotein (48). Pericytes were distinguished from other potential contaminants including SMCs, epithelial cells, and glial cells by their irregular morphology, the lack of “hill and valley” growth pattern (characteristic of confluent SMCs), and the presence of both muscle and nonmuscle actin isotypes (21).

**Epithelial Cells.** Retinal pigment epithelial (RPE) cells isolated from the neural retina and Madin-Darby canine kidney (MDCK) epithelial cells were used for these studies. For cultures of RPE cells, the pigmented layer of bovine retinas was collected into PBS, minced, and incubated for 1 h in 0.2% collagenase in PBS containing 0.1% BSA. RPE cells were washed free of enzyme and grown in DME/10CS. MDCK cells were purchased from American Type Culture Collection (Rockville, MD) and grown in DME/10FBS.

**SMCs and Fibroblasts.** SMCs were explanted from bovine aorta and fibroblasts from human dermis using standard methods and grown in DME/10CS.

**3T3 Cells.** BALB/c 3T3 cells were the gift of Dr. M. Klagsbrun (The Children’s Hospital, Boston, MA) and were grown in DME containing 4,500 mg/liter glucose and 10% calf serum (Colorado Serum Co., Denver, CO).

### Co-culture Studies

EC were co-cultured with pericytes, SMCs, fibroblasts, RPE cells, MDCK cells, and 3T3 cells (modulating cells) using two methods: one in which contact was allowed between the cells and a second in which contact was prevented.

**Co-cultures with Direct Contact.** To measure the effect of the modulating cells on EC proliferation in a co-culture system, the number of modulating cells was held constant so that any increase in total cell number could be attributed to EC growth. For these studies, cultures of the modulating

cells were incubated with mitomycin C (10  $\mu\text{g/ml}$ ; Sigma Chemical Co., St. Louis, MO) for 2 h (37°C) to render them incapable of cell division (46). The growth-arrested cells were then rinsed with PBS, removed by trypsinization (0.05% trypsin, 0.02% EDTA), and plated at a density of 20,000 cells per well into 16-mm, 24-well tissue culture dishes (Nunc, Vanguard International, Neptune, NJ) in their respective growth media.

It is possible that the trypsinization of mitomycin-treated cells altered the modulating cells; i.e., prevented regeneration of particular cell surface molecules that had been removed by trypsinization. To insure that this was not a variable, co-cultures were also established using pericytes that were plated and allowed to attach overnight before mitomycin treatment. Identical results were obtained using both methods. Thus, for consistency and convenience, subsequent co-cultures were established by mitomycin treatment of the stock flasks of modulating cells.

The cells were allowed to attach overnight and the number of cells in quadruplicate wells was determined by removing the cells with trypsin and counting electronically. Capillary ECs, the number determined by the desired final ratio, were plated into wells containing growth-arrested modulating cells in DME/10CS without aFGF. Although ECs respond to and are routinely propagated in the presence of FGF, they will also grow well (though at a lower doubling rate) in the absence of FGF (media containing 10% calf serum). The fact that EC growth is supported in serum that has been FGF-depleted by passage over a heparin-Sepharose column indicates that FGF is not the primary mitogen for ECs in serum (47). Control cells consisted of the capillary EC and growth-arrested cells cultured alone under identical conditions. All cells were refed twice a week. Every 3–4 d for 14 d, quadruplicate wells containing growth-arrested modulating cells alone, ECs alone, and co-cultures of the two cells were trypsinized and counted electronically. The effect of co-culture on EC proliferation was determined by comparing the average number of cells in the co-cultures to the sum of the average number of cells (ECs and modulating cells) in individual cultures.

**Cell Ratio Studies.** To assess the influence of the ratio of ECs to pericytes on the modulation of EC growth, direct co-cultures were established at EC/pericytes ratios of 1:1, 2:1, 5:1, 10:1, and 20:1. To control for the influence of "substrate coverage," the number of ECs was kept constant (20,000 cells/well) while the number of pericytes was varied. All other experimental conditions remained unchanged.

**Co-cultures without Contact.** Additional co-culture experiments were conducted to determine the role of cell contact or proximity. In this system, the cells were co-cultured in the same well, but physical interaction was prevented by growing the ECs in Millicell chambers (Millipore Corp., Bedford, MA), which are inserted into the wells of 24-well plates and sit 1–2 mm above the layer of growth-arrested cells. The bottom of the Millicell chamber is formed by a cellulose membrane containing 0.45- $\mu\text{m}$  pores, which allow for diffusion of soluble effectors. All other experimental conditions were identical to those described for contacted studies.

## Results

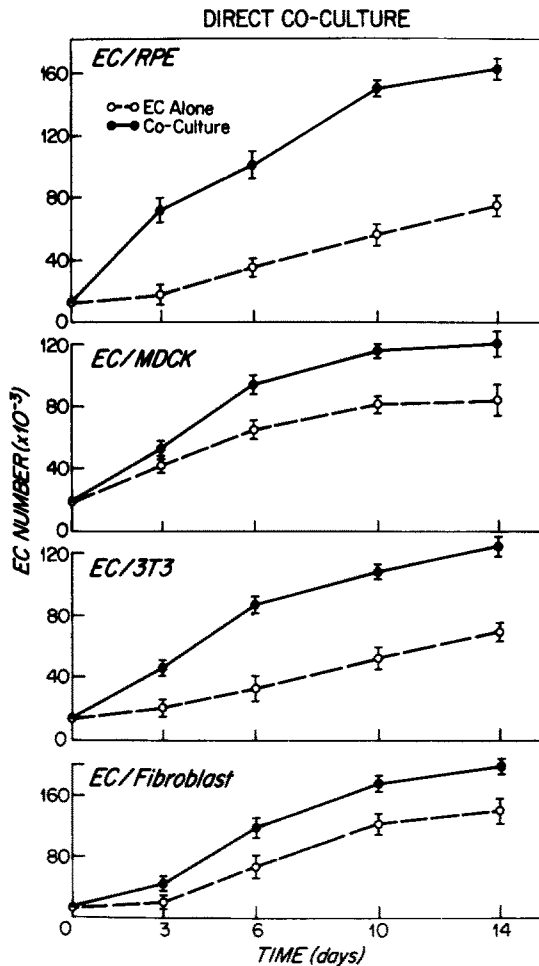
### Co-culture with Contact

We have examined the effect of pericytes, SMCs, fibroblasts, RPE, MDCK, and 3T3 cells on EC proliferation using a co-culture system that allows intercellular contact. Results of these studies are shown in two different forms in Fig. 1. Fig. 1, *a* and *c*, illustrates all of the cell counts including growth-arrested pericytes and SMCs. The effectiveness of mitomycin in growth arresting the cells is evidenced by the fact that there was no increase in the number of the pericytes or SMCs (stippled bars in Fig. 1, *a* and *c*) over the time course of the study. The EC cultured alone (*open bar*) tripled in number over the course of the experiment. Comparison of the number of cells in the co-cultures (*hatched bars*) with the predicted cell number (sum of stippled bar and open bar) reveals an inhibition of EC growth in the co-cultures. The number of ECs co-cultured with pericytes or SMCs was decreased by 33% at d 3 compared to the growth of ECs cultured alone to 42% by d 6, 53% by d 10, and 60% by d 14 (Fig. 1 *a*). The degree of inhibition measured in co-cultures of ECs and SMCs was nearly identical to that of pericyte-EC co-cul-

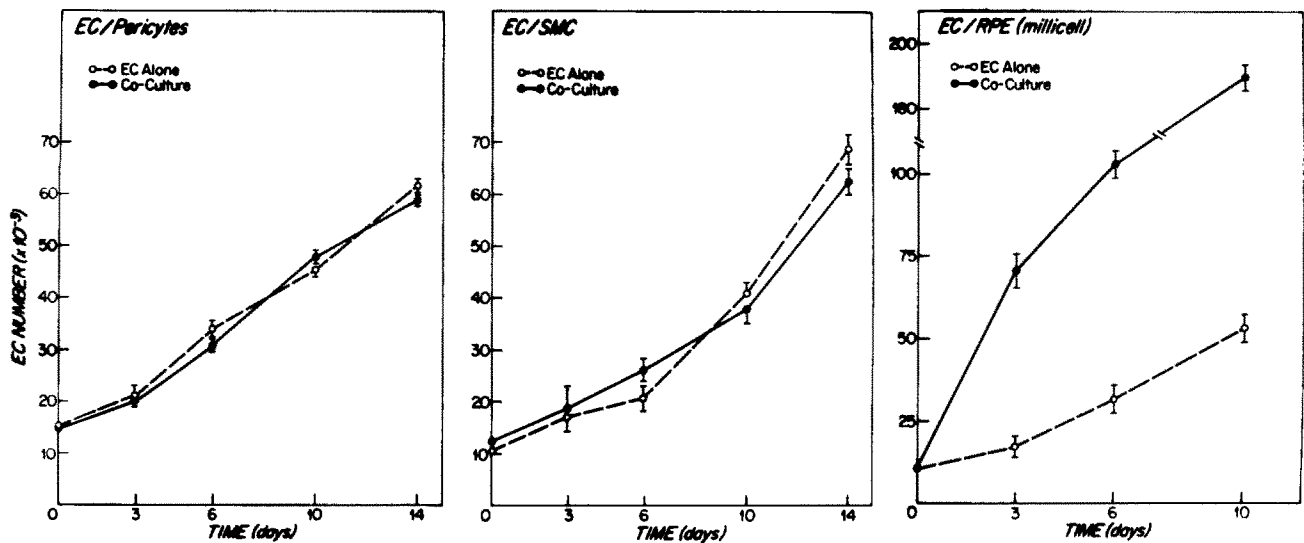
tures: 31% after 3 d, 42% after 7 d, 53% after 10 d, and 63% after 14 d (Fig. 1 *c*).

The extent of this inhibition is more clearly seen in Fig. 1, *b* and *d*, where the number of ECs cultured alone (*dotted line*) is compared to the number of cells in the co-cultures minus the pericyte or SMC number (constants throughout the time course; see Fig. 1, *a* and *c*). Results illustrated in this manner clearly indicate that the co-culture of EC with pericytes or SMCs totally inhibited endothelial growth. The remainder of the co-culture studies will be illustrated using the format in Fig. 1, *c* and *d*, in which the constant (modulating cell number) is subtracted from the co-cultures.

To insure that mitomycin treatment of the modulating cells did not inhibit EC growth, additional studies were performed



**Figure 2.** Effect of co-culture of ECs with fibroblasts and RPE, MDCK, and 3T3 cells on capillary EC proliferation. Fibroblasts, RPE, MDCK, or 3T3 cells were growth arrested using mitomycin C, washed, trypsinized, and plated into 24-well dishes. After overnight attachment, equal numbers of ECs were added to wells containing each type of growth-arrested cell in media containing 10% calf serum. Controls consisted of ECs and growth-arrested cells cultured separately. At 3–4-d intervals over 14 d, quadruplicate wells from each group were trypsinized and counted. The influence of co-culture on EC growth was expressed by comparing the mean sum of ECs grown alone (*dashed line*) to the mean sum of co-cultured EC (*solid line*), which was determined by the difference between the total number of co-cultured cells and the number of growth-arrested modulating cells.



**Figure 3.** Effect of co-culture without direct contact of pericytes, SMCs, and RPE cells with ECs on EC proliferation. Pericytes, SMCs, and RPE cells (modulating cells) were growth arrested using mitomycin C (10  $\mu\text{g/ml}$ ; 2 h), washed, trypsinized, and plated into 24-well dishes. After overnight attachment, equal numbers of ECs were plated into 12-mm Millicell inserts (Millipore Corp., Bedford, MA; cellulose membranes containing 0.45- $\mu\text{m}$  pores), and suspended 1–2 mm above the layer of modulating cells to prevent direct contact between the cell types. Controls consisted of modulating cells alone and ECs alone plated in Millicell inserts. At 3–4-d intervals over 14 d (10 d for the RPE–EC co-cultures; see text for details), quadruplicate wells from each group were counted. The effect of co-culture on EC growth was depicted by comparing the mean sum of control EC (*dashed line*) to the mean sum of co-cultured EC (*solid line*) which was determined by the difference between the total number of co-cultured cells and the number of growth-arrested modulating cells.

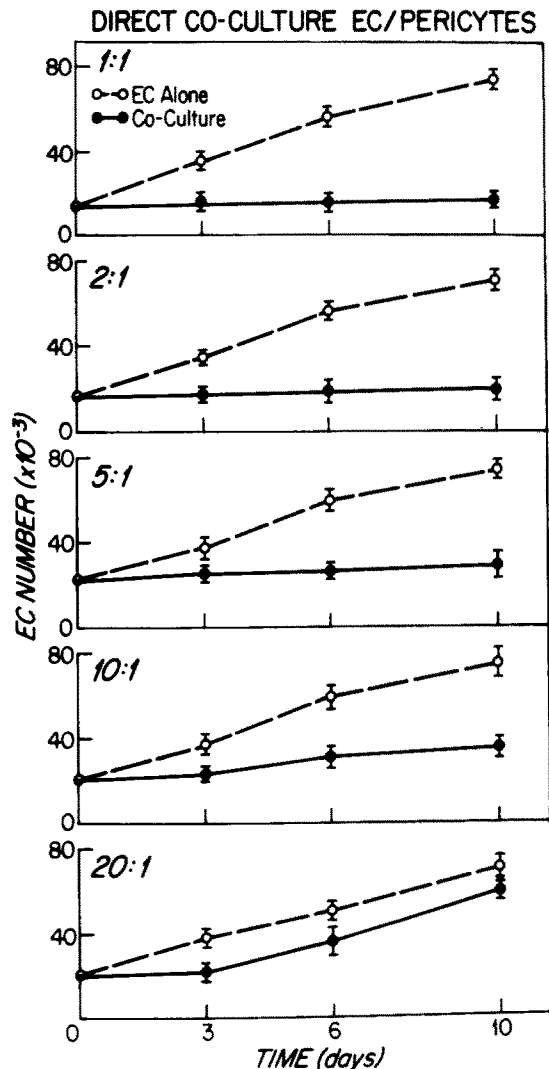
to measure the growth potential of ECs that had been previously co-cultured with growth-arrested pericytes. ECs were removed from these cultures by a selective trypsinization and replated in fresh media with and without purified aFGF. Results of these studies demonstrate that ECs previously growth inhibited by co-culture with pericytes are capable of dividing in response to serum or growth factor (data not shown).

The specificity of the EC inhibition by SMCs and pericytes was assessed by establishing co-cultures of ECs with growth-arrested fibroblasts, 3T3 cells, MDCK cells, or RPE cells. None of these cells inhibited EC growth. On the contrary, after 3 d of co-culture in the presence of these cells, EC growth was stimulated by 30, 88, 32, and 140%, respectively. This stimulation increased after 14 d in co-culture to 40, 120, 86, and 192%, respectively (Fig. 2).

#### Co-culture without Contact or Proximity

When contact or proximity between EC and the modulating cell type was prevented, no inhibition of EC growth was observed in co-cultures of EC with pericytes or SMCs (Fig. 3). After 14 d in co-culture under these conditions, no significant change in EC number was observed, suggesting that the inhibition of EC growth is dependent on contact or proximity

**Figure 4.** Effect of alterations in EC/pericyte ratio on EC growth inhibition by pericytes. The influence of cell ratio on inhibition of EC growth by pericytes was determined by varying the EC/pericyte ratio to 1:1, 2:1, 5:1, 10:1, and 20:1. The EC number was kept constant while the pericyte number was decreased up to 20-fold. The change in EC number was measured over 10 d after direct co-culture as described in the legend of Fig. 1. The influence of cell ratio on EC growth was expressed by comparing the mean sum of control ECs (*dashed line*) to the mean sum of co-cultured ECs (*solid line*), which was determined by the difference between total co-cultured cell number and total growth-arrested pericyte number.



between the cells. In contrast, when direct contact between ECs and RPE cells was prevented, stimulation of EC growth (Fig. 3) was observed to be similar in magnitude to that measured when the cells were co-cultured with direct contact (Fig. 2). The EC stimulation by RPE cells was so great that confluence was rapidly attained, causing the EC monolayer to lift before the final time point (14 d). These results indicate that the stimulation of EC growth by these cells is not dependent on cell contact or proximity.

#### Effect of EC/Pericyte Ratio

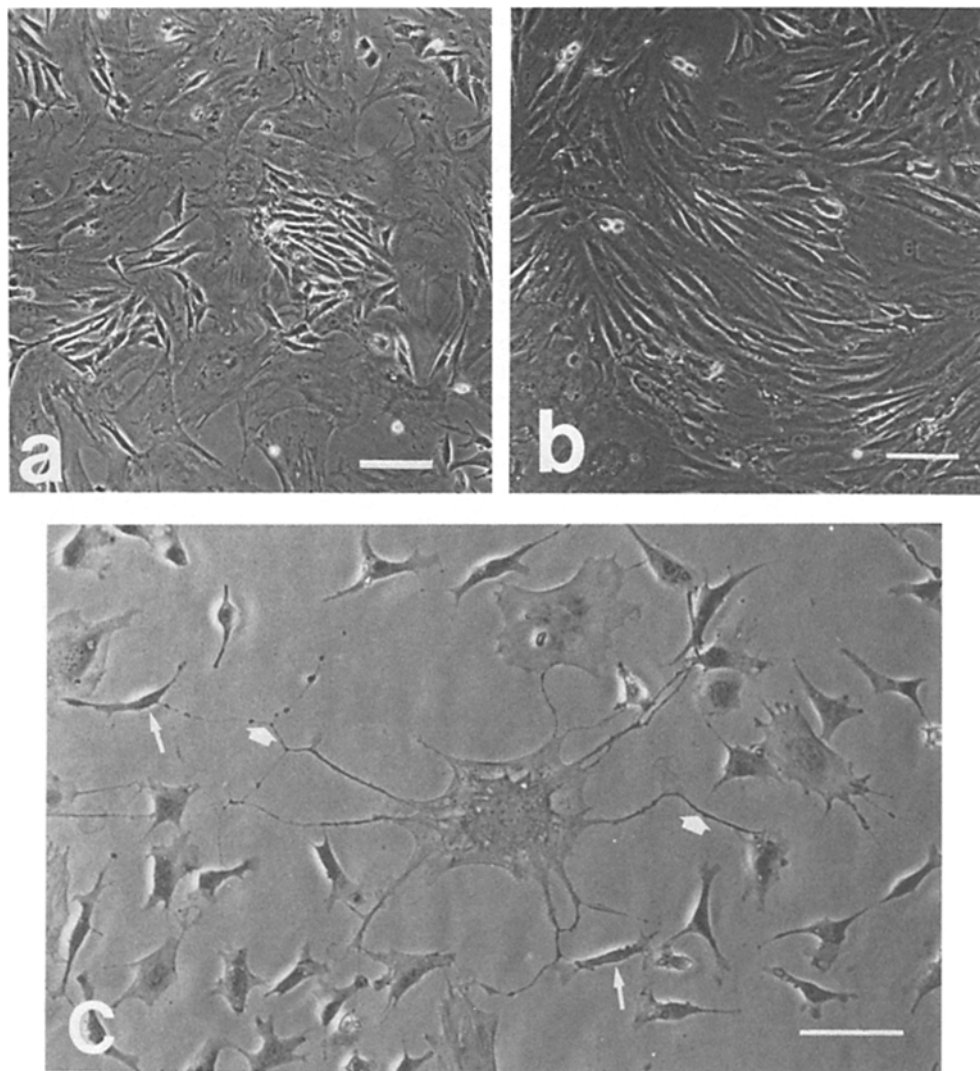
ECs and pericytes were co-cultured for 10 d at EC/pericytes ratios of 1:1, 2:1, 5:1, 10:1, and 20:1. Comparable inhibition (25–35%) of EC growth was observed at all ratios by d 3 and was constant up to d 10 in co-culture for all of these ratios up to and including 10:1 (Fig. 4). However, when ECs were co-cultured with pericytes at a 20:1 ratio, the inhibition of EC growth was similar to other ratios at d 3 (29%) but steadily diminished over the remainder of the time course.

#### Morphology

Observations of changes in EC morphology under various co-culture conditions indicated a correlation between EC growth patterns and the level of proliferation. In direct co-

culture studies, when EC were cultured directly with pericytes or SMCs at a ratio of 1:1 and EC growth was inhibited, the ECs were polygonal in shape, “compact,” and closely packed in discrete colonies with virtually no overgrowth by pericytes or SMCs (Fig. 5 a). In contrast, co-culture of ECs with RPE cells stimulated EC growth. Under these conditions, ECs were elongated, spindle shaped, and overgrew the growth-arrested epithelial cells (Fig. 5 b).

At higher EC/pericyte ratios (5:1, 10:1), the area of substrate covered by pericytes was significantly decreased. In these cases, ECs were less densely packed and discrete EC colonies were scarce. Each pericyte extended processes toward multiple ECs. For example, the centrally located pericyte in Fig. 5 c from co-cultures at ratios of 5:1 has extended processes toward at least 12 ECs. Furthermore, there is a distinct change in EC morphology. ECs that are in proximity to a pericyte process were neither polygonal nor spindle shaped, but were asymmetric and more rounded as indicated by an apparent decrease in spread area and increase in pericellular refractile area (*thin arrows*; Fig. 5 c). The association of ECs with pericytes seemed to occur preferentially along extended processes that are characteristic of pericytes (*wide arrows*; Fig. 5 c). As the number of pericytes was increased, more pericyte cell surface was available to interact



**Figure 5.** Phase-contrast micrographs depicting the morphology of ECs directly co-cultured with pericytes or RPE cells. (a) Equal numbers of ECs and pericytes; (b) equal numbers of ECs and RPE cells, illustrating overgrowth of RPE cell layer by stimulated ECs; and (c) ECs and pericytes at a 10:1 ratio, illustrating a centrally located pericyte extending many processes (*short arrows*) that contact multiple ECs. Note the EC morphology associated with pericyte-dependent growth inhibition, including a distinct pericellular refractile area (*long arrows*). Bar, 10  $\mu$ m.

with ECs. This correlated with shorter and less extensive pericyte processes and fewer ECs associated with each pericyte.

## Discussion

The results of this study demonstrate that pericytes and SMCs inhibit capillary EC proliferation *in vitro*. The inhibitory effect is specific for pericytes and SMCs since epithelial cells, fibroblasts, or 3T3 cells co-cultured with ECs under identical conditions did not inhibit, but significantly stimulated, EC growth. The modulation of EC growth by pericytes and SMCs is dependent upon contact or proximity; co-culture of the cells without contact is not inhibitory. Furthermore, the degree of inhibition is not influenced by altering the number of modulating cells up to 10-fold. Co-culture of EC and pericytes at a ratio of 20:1, however, results in moderate inhibition through the first 6 d of co-culture, followed by resumed EC growth. Pericytes in co-culture with an excess of ECs appear to contact a larger number of ECs than pericytes co-cultured with equal numbers of ECs. These observations suggest that the ability of the pericytes to contact multiple ECs may allow for a constant degree of EC inhibition in the presence of increasing numbers of ECs.

A role for cell-cell interactions in the modulation of growth has been reported in the study of density-dependent growth inhibition in a number of systems. Heimark and Schwartz (20) reported that plasma membrane preparations from confluent large vessel ECs inhibit actively growing ECs, whereas comparable preparations from SMCs did not. Despite the variables associated with membrane isolation techniques, such observations suggest that different mechanisms and/or membrane interactions may occur under homotypic (EC-EC) vs. heterotypic (EC-SMC) conditions to inhibit EC growth. Cell-cell contact has been similarly implicated in the density-dependent growth inhibition of 3T3 cells (49), hepatocytes (35), and fibroblasts (50). The addition of glutaraldehyde-fixed cells, plasma membranes, and, most recently, immobilized plasma membrane glycoproteins isolated from high density cultures of fibroblasts was shown to inhibit the growth of low density proliferating fibroblasts (50). Recent investigations of mechanisms of cellular adhesion have led to the identification and characterization of a specific family of high molecular weight cell surface glycoproteins involved in cell adhesion (cell adhesion molecules) (14). Since contact-dependent cell-cell interaction requires adhesion between the cells, it has been suggested that the same molecules may regulate both cell-cell adhesion and growth (30). Accordingly, fragments of fibronectin, an adhesion molecule, have been reported to inhibit EC growth (22). All of the previously reported studies involve growth suppression by like cell types (homotypic). Our results are unique in that the inhibition of growth occurs between unlike cell types (heterotypic).

Extracellular matrix may also play a role in the inhibition of EC growth observed in these studies. Basement membrane components have been shown to modulate the growth, migration, and differentiation of many cell systems (19, 32, 51). Morphologic studies of developing capillaries correlate the arrival of pericytes with the cessation of EC migration and proliferation, a shift in dominant populations of matrix-associated components and lumen formation (1, 3). Since pericytes synthesize matrix components (8), alterations of

the capillary basement membrane (shared by both cells) may be one mechanism for modulation of EC behavior by pericytes.

Other investigators have used co-culture systems to examine metabolic interactions between vascular cells. These studies indicate that metabolic cooperativity between the cells *in vitro* mimics vascular homeostatic events in intact blood vessels (11, 31). Ultrastructural studies have demonstrated that medial SMCs junction with ECs (24, 25, 40), and, similarly, that pericytes form junctions with ECs (4, 33, 41). Using dye transfer as an indicator of gap junctions, Larson et al. (29) have recently demonstrated that junctional communication between microvascular pericytes and ECs occurs *in vitro* and, thus, may be involved with co-culture-dependent EC inhibition reported here.

Pericytes have been postulated to be structurally and functionally similar to SMCs (37, 38). Pericytes contain contractile proteins including muscle actin (21), and muscle myosin and tropomyosin (27). However, primary cultures of pericytes do differ from primary SMCs in that the pericytes also contain significant levels of nonmuscle isoactin localized along stress fibers. From a functional viewpoint, the cells are also similar; both SMC (6) and pericyte (36) proliferation is inhibited by heparin. The findings reported here indicate another functional similarity between the two cells—the ability to modulate EC growth.

The observation of alterations in the morphology of ECs that were co-cultured with pericytes is in agreement with changes in cell shape observed under other growth conditions. The decrease in spread surface area that we observed in ECs co-cultured with pericytes (Fig. 5, *a* and *c*) is consistent with the findings of Folkman and Moscona (16) who reported that ECs held in a rounded conformation had reduced DNA synthesis. Furthermore, the extended cell shape observed in ECs stimulated by co-culture with RPE cells (Fig. 5 *b*) is reminiscent of that described for cells that are actively migrating and proliferating (2). A change in morphology similar to what we note in the pericyte/EC co-culture has been observed in ECs whose growth has been inhibited by the addition of phorbol esters (13).

A role for cell contact is further suggested by observation of the co-cultures. Under conditions where EC number exceeds pericyte number, pericytes extended numerous “neurite-like” processes toward ECs in their vicinity (Fig. 5 *c*). The ability of the pericyte to extend multiple processes may account for the fact that the inhibition of EC growth was mediated over the range of cell ratios. At the EC/pericyte ratio of 20:1, EC number and/or distance from a pericyte may have increased to an extent beyond the pericyte's capacity for process formation and/or contact. This *in vitro* observation may reflect the variability reported *in vivo* with respect to the degree and spatial arrangement of EC coverage by pericytes in different microvascular beds (for review see reference 39). For example, Tilton et al. (42, 45) found that pericyte coverage of EC surface varies in skeletal muscle capillaries, increasing in the direction of head to foot (23% in abdominal muscle, 32% gastrocnemius) and is highest in retina (46–58%).

There is also reason to suspect that the inhibition of EC by SMCs occurs *in vivo*. Clinical and experimental observations indicate that intimal ECs from host vessels fail to re-endothelialize a graft beyond 2 cm of “pannus ingrowth” (7).



The reason for this phenomenon has previously been unclear. Based on our findings, it seems possible that contact or association between the ECs of the growing pannus front and the underlying SMCs may be responsible for cessation of EC growth in grafts.

Technical limitations prevent the investigation of the effects of cell-cell interactions on the growth and function of the vascular cells in vivo. Recently described techniques for the long-term culture of capillary ECs (17) and pericytes (18) have enabled us to develop co-culture models to examine pericyte-EC interactions in vitro. We have demonstrated that contact or proximity between pericytes (or SMCs) and capillary ECs in vitro modulates EC growth. These model systems will be useful in elucidating the mechanisms involved in this growth inhibition, and will allow characterization of the influence of pericyte-EC interactions on other capillary functions.

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