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CO-CULTURE OF ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS IN A FLOW ENVIRONMENT: AN IMPROVED CULTURE MODEL OF THE VASCULAR WALL?

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Abstract

Numerous studies have demonstrated that the neighboring smooth muscle cells (SMC) influence the morphology, cytoskeleton and growth of co-cultured endothelial cells (EC). Also, flow-induced laminar shear stress has been shown to induce cell elongation, F-actin reorganization and growth inhibition in cultured EC. We investigated the effect of neighboring SMC and collagen matrix on the response of EC to shear stress. The co-culture system was made by growing porcine aortic SMC in a gel of collagen type I and then seeding porcine aortic EC (PAEC) on the top surface. Then the co-culture was exposed to steady, laminar shear stress of 10 and 30 dynes/cm^2 in a parallel-plate flow chamber. EC had a different morphology when cultured on top of collagen gels as compared to cells grown on plastic. When grown in static co-culture with SMC, EC were already elongated and showed a random wavy pattern of orientation. When exposed to 30 dynes/cm^2, the EC aligned with the direction of flow after 24 to 48 hours. We suggest that the elongation and orientation of the EC, when cultured on a collagen matrix under static conditions, may be due to contact guidance on the collagen fibers previously rearranged by the SMC during gel retraction. Shear stress, however, was sufficient to induce cell orientation along the direction of flow.

Key Words: Blood vessel substitute, endothelial cells, smooth muscle cells, co-culture, collagen gel, shear stress, contact guidance.

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Introduction

Progress made in cell culture techniques in the last decades has allowed for investigations which have furthered our understanding of the function of many mammalian cell types. By isolating and culturing vascular cells, i.e., endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts, the pathology of some blood vessel diseases became less of a mystery. Among all vascular diseases, atherosclerosis is probably the one most studied because it has become the main cause of vascular mortality in the western world. The numerous studies focusing on atherosclerosis have been partly carried out using animal models, e.g., pig and rabbit, but also *in vitro* by developing cell culture systems modeling the vascular wall. These culture studies have greatly contributed to the development of an hypothesis describing the genesis of atherosclerosis as well as the role of each component of the vessel wall (Schwartz *et al.*, 1991; Nerem, 1992). As part of these active components, a dysfunctionality of the endothelium is believed to be a key in the initiation of the atherosclerotic plaque. Today, it is still not clear as to why and how this endothelial change occurs in local regions of the vascular system.

In vitro cell culture systems are crude models of the vascular wall because they usually include a single cell type (e.g., the endothelial cell) and little of the very intricate extracellular matrix (ECM) proteins present in the vascular wall. In the last few years, attempts have been made to include SMC co-cultured with EC in several types of culture systems. The most simple co-culture technique involves growing EC and SMC together on the surface of a culture dish, and using such a system, it has been demonstrated that SMC inhibits EC growth by establishing physical contacts with them (Antonelli-Orlidge *et al.*, 1989). More complicated co-culture systems include the use of filters between EC and SMC (Weber *et al.*, 1988) or the presence of a matrix of collagen along with the cells (Weinberg and Bell, 1986). Such systems have demonstrated that not only SMC growth is inhibited by EC (Van Bull-Vortelboer *et*

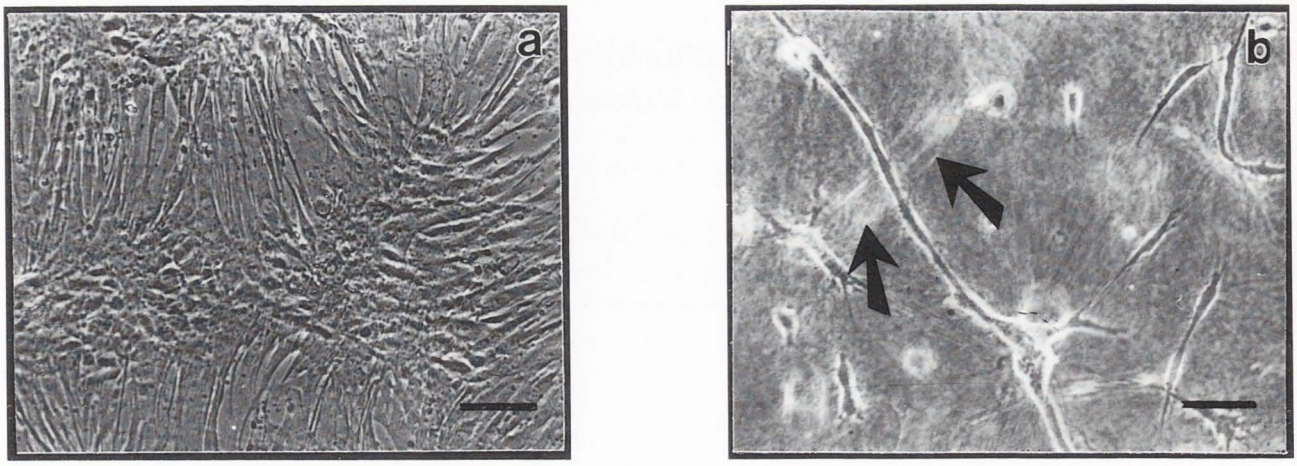


Figure 1. Effect of SMC seeding into a gel of collagen type I. SMC cultured on TCPS tend to flatten and spread onto the surface (**a**). When seeded inside collagen gels, SMC took a more elongated, spindle-like morphology (**b**). SMC exerted a force on the collagen which resulted in fiber reorientation (see arrows in b). Bars = 100 μm.

al., 1986), but also proteoglycan production by SMC (Merrilees and Scott, 1981), SMC cholesterol metabolism (Hajjar *et al.*, 1985) and the secretion of vasoactive substances, e.g., endothelin, by EC (Stewart *et al.*, 1991) are altered in co-cultures.

It has been shown that SMC growth and differentiation is dramatically affected by their culture in a gel of collagen type I (Weinberg and Bell, 1985). Synthetic SMC grown inside a gel of collagen retract the lattice, sometimes by more than 80% in area, differentiate into an elongated cell growing three-dimensionally, and exhibit decreased growth and protein production when compared to cells cultured on top of tissue culture plastic (Thie *et al.*, 1991). Although synthetic SMC seem to undergo a phenotype change to a contractile-like state when cultured inside a gel of collagen, they never fully recover their contractile property in response to agonists. Moreover, contractile SMC have been shown to become synthetic when cultured in collagen gels (Thie *et al.*, 1991).

The localization of atherosclerosis occurs essentially in regions where shear stress (tangential force induced by blood flow) is low and oscillatory, whereas regions of high shear stresses appear to be protected (Nerem, 1992). Recently, many authors have demonstrated the importance of the mechanical environment in the morphology, growth and differentiation of vascular cells in culture (Levesque and Nerem, 1989; Shirinsky *et al.*, 1989; Kanda *et al.*, 1992). Bovine aortic EC subjected to shear stress elongate and orient themselves with the direction of flow (Levesque and Nerem, 1989), reorganize their F-actin filaments from a dense peripheral band to thick stress fibers spanning the entire length of the cells and aligned with direction of flow (Wechezak *et al.*, 1985), and exhibit a decreased growth rate (Mitsumata *et al*, 1991).

Our laboratory has recently developed a new model of the vascular wall which includes SMC cultured inside a gel of collagen type I with EC grown as a confluent monolayer on the top surface. The co-culture is inserted into a chamber where the endothelium is exposed to a steady, laminar shear stress. Results presented here show that endothelium co-cultured with SMC and exposed to low and medium shear stresses of 10 and 30 dynes/cm^2 elongate and orient in a different manner and more rapidly than EC cultured on tissue culture plastic.

Materials and Methods

Cell isolation and culture

EC and SMC were isolated from porcine aorta at the Animal Research Laboratory (Division of Cardiology, Emory University, Atlanta). Briefly, the aortas were cut longitudinally to expose the intimal surface, the endothelium was rinsed with phosphate buffer saline (PBS) and a solution of 0.1% collagenase CLS I (Worthington Biochemical, Freehold, NJ) in serum free Dulbecco modified Eagle's medium (DMEM, Gibco, Grand Island, NY) was spread on the whole surface. The aortas were then incubated at 37°C for 10 minutes after which the endothelium was detached by gentle scraping and resuspended in 5 ml of DMEM with 10% fetal calf serum (FCS) in a T-25 flask (Fisher, Norcross, GA). The media of the aortas was then separated from the adventitia using two forceps and cut into 2 mm x 2 mm pieces. Explants were placed in a T-25 flask containing 3 ml of DMEM with 10% FCS, and the flasks were incubated at 37°C for 3 to 4 weeks to allow cell out-

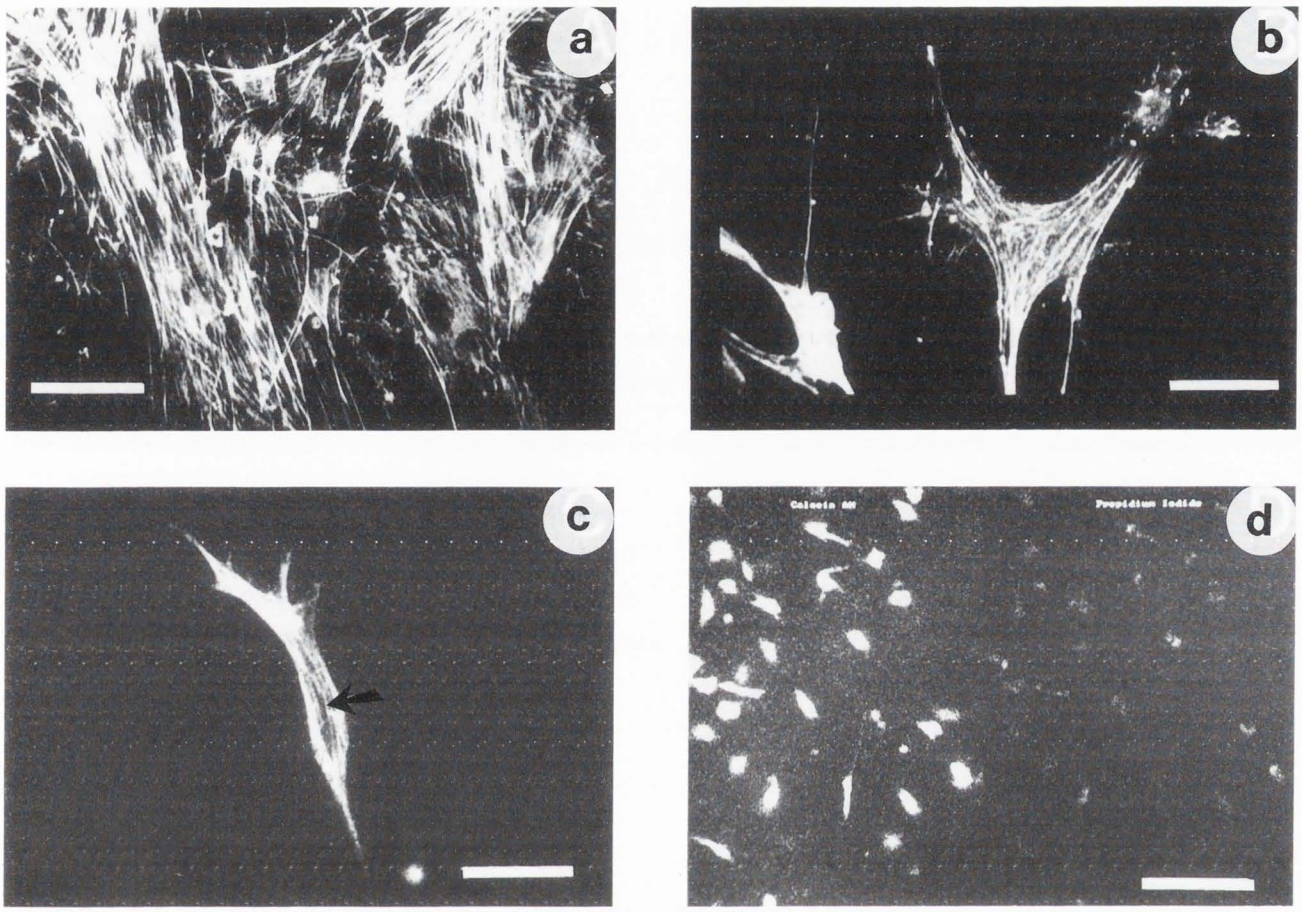
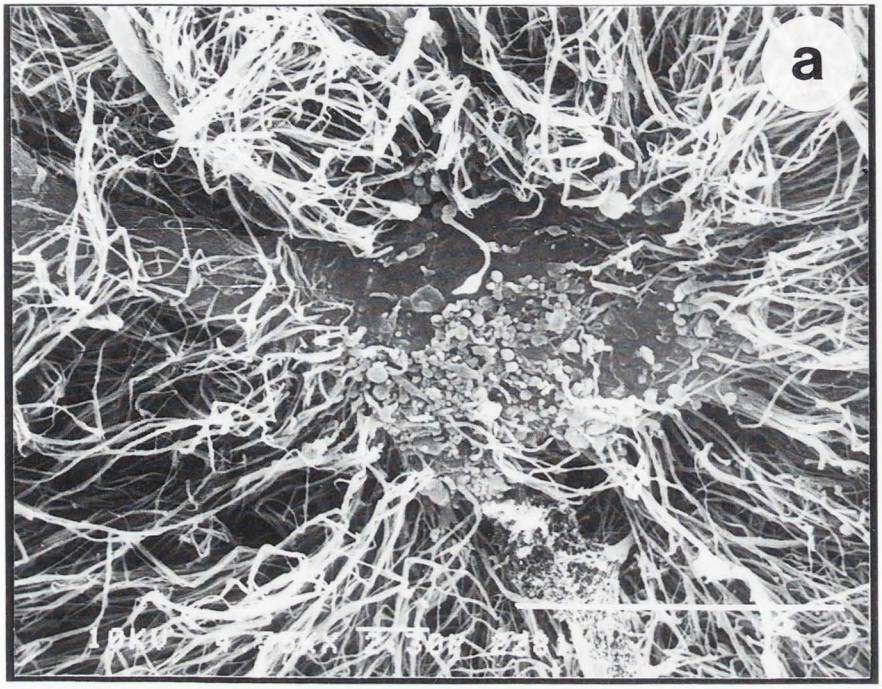


Figure 2. F-actin organization in SMC cultured on TCPS (**a**) or inside collagen (**b** and **c**). The F-actin microfilaments in SMC cultured on plastic were organized into numerous stress fibers (a). In cells cultured inside collagen gels, the F-actin also formed stress fibers (b). Alpha-actin filaments were also found in cells cultured in collagen (c, arrow) but not as distinctly as the F-actin filaments seen in (a) or (b). Panel (**d**) shows that all SMC are stained with calcein AM (left part of panel d) but not propidium iodide (right part of panel d) indicating that these cells were alive. Bars = 25 μm (in a, b and c) and 100 μm (in d).

growth. Purity of the EC cultures was checked by staining with acetylated LDL-dil (Molecular Probes, Eugene, OR) and that of SMC cultures with an antibody against α-SM actin (Zymed, San Francisco, CA). EC and SMC were cultured until passage 5 and 12 respectively in DMEM supplemented with 10% FCS, penicillin-streptomycin (Sigma, St. Louis, MO), and L-glutamine (Sigma). The cells were cultured in 100 mm tissue culture dishes made from tissue culture polystyrene (TCPS, Fisher) until confluence. At this time, they were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA, Gibco).

Culture of the smooth muscle cells inside collagen

The SMC were grown in a lattice made of collagen type I (Collaborative Research, Bedford, MA) in a polymerized form following the technique described by Weinberg and Bell (1985). This technique has the advantage of recreating a three-dimensional, tissue-like substrate on which EC can be seeded. The lattice was made of collagen type I from rat tail (3-4 mg/ml in 0.02 N acetic acid), DMEM 1.5 times concentrated, 10% FCS, 0.1 N sodium hydroxide to neutralize the acetic acid, and a solution of SMC in suspension (10^6 cells/gel). This solution was poured into a rectangular box having a length, width and depth of 7.0, 5.1 and 1.25 cm respectively. Polymerization occurred within 5 minutes at room temperature after which the gels were transferred into the incubator at 37°C. The cell viability was assessed using Calcein AM (Molecular Probes) as live stain and propidium iodide (Molecular Probes) as marker for dead cells. The collagen gels rinsed with PBS and incubated with solutions of 5 μg/ml Calcein AM and 5 μg/ml propidium iodide in culture medium



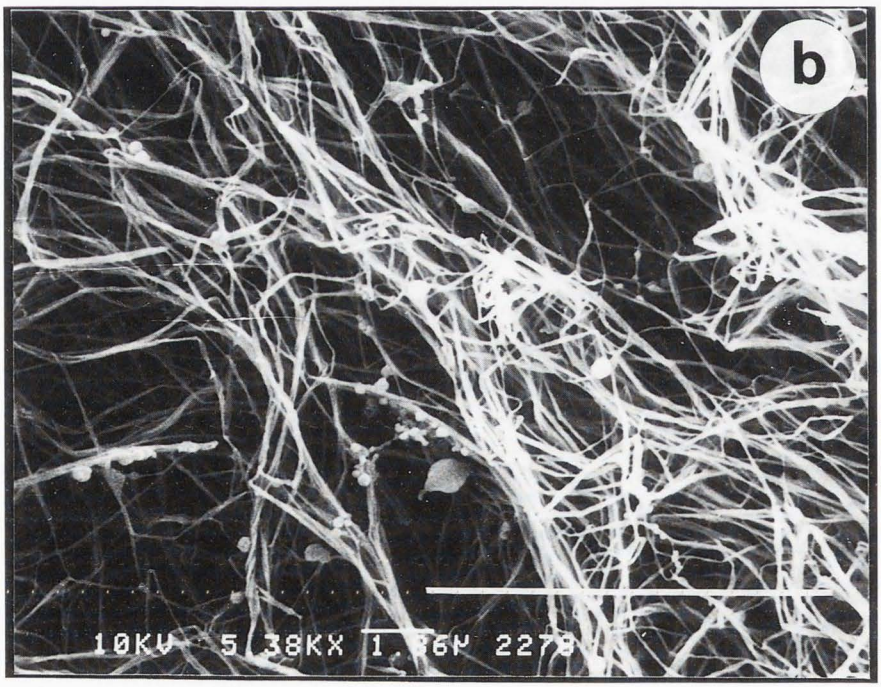


Figure 3 (a and b at left, c on the facing page 119). Scanning electron micrographs of the surface of a retracted collagen gel 5 days after seeding with SMC. Areas with cells show that the collagen fibers run from the cell to the outside everywhere around the cell (a). In areas without cells, the collagen fibers had preferential orientation in one direction (b). The overall surface of the gels was composed of dense packs of fibers oriented in different direction with presence of holes big enough to allow for cell migration (c, arrows). Bars = 10 μm (in a and b) and 500 μm (in c).

for 1 hour at 37°C. After rinsing with PBS, the gels were fixed with 10% buffered formalin, mounted and observed with a confocal microscope (MRC 600, Bio-rad, Cambridge, MA) with a 10X objective.

Seeding of the endothelial cells

When the lattices of collagen and smooth muscle cells were fully contracted, the top surface was seeded with endothelial cells as follows. A 100 mm dish of confluent EC was trypsinized and spun for 5 minutes at 1000 rpm. The pellet was resuspended into 0.8 ml (0.2 ml/gel) of DMEM plus 10% FCS. The seeding density was approximately 5 x 10^4 cells/cm^2. 0.2 ml of cell suspension was deposited on the top surface of the lattice and the cells were allowed to attach for 10 minutes at room temperature. The co-culture was then transferred into the incubator for 20-30 minutes until most of the EC attached to the gel. The degree of attachment was

Figure 3c (see facing page 118 for the legend).

observed using a light microscope with a 4X objective on top. When most of the endothelial cells were attached, the co-culture was fed with 20 ml of DMEM plus 10% FCS and transferred to a 5% CO_2/95% air, humidified atmosphere incubator. The seeding density of EC was always high so that the EC would be immediately confluent. The co-cultures were usually used for flow experiments 48 to 72 hours after seeding.

Flow experiments

The co-culture was inserted into a mold made of 2% agar in order to increase its mechanical stability and so that the top surface, on which the endothelium resided, was as flat as possible. The mold was then inserted into a parallel-plate flow chamber similar to that used by Levesque and Nerem (1985). The height of the channel was chosen to be either 500 μm (for 30 dynes/cm^2) or 1000 μm (for 10 dynes/cm^2). The flow chamber was connected to a flow loop which allowed precise regulation of the flow rate, medium temperature, and pH. The co-cultures were exposed to a steady, laminar shear stress of either 10 or 30 dynes/cm^2 for periods of time up to 72 hours.

Light microscopy

The endothelium was barely observable by phase-contrast microscopy because of the high collagen density in the slab. Silver staining was carried out post-shear in order to increase the visibility of the EC borders. The co-cultures were rinsed in PBS, then in 5% glucose solution for 2 minutes. They were transferred to a 2.5% silver nitrate solution for one minute, rinsed in 5% glucose, then in 1% ammonium bromide for another minute. The co-cultures were then rinsed and fixed in 10% buffered formalin (Sigma) for 30 minutes. The endothelium was observed and photographed using a phase-contrast microscope with 10X or 20X objective and maximum light intensity. Samples were stained for F-actin using fluorescein (FITC-phalloidin; Molecular Probes) and α-actin with an antibody against α-SM actin coupled to FITC (Sigma). The samples were fixed in 10% buffered formalin, permeabilized in 0.1% Triton X-100 (Sigma) and incubated with FITC-phalloidin diluted 1 to 250 in PBS or a 1 to 500 dilution of the α-SM actin antibody. The samples were then mounted between a slide and coverslip and visualized with a confocal microscope (MRC 600, Biorad) using a FITC filter and a 60X oil objective.

Electron microscopy

Co-cultures were prepared for electron microscopy as follows. For scanning electron microscopy (SEM), the gels were rinsed in a buffer solution (sodium cacodylate 0.1 M, pH = 7.4) for 15 minutes and then fixed in 1.5% glutaraldehyde in sodium cacodylate 0.1 M for 4-5 hours. After rinsing with the buffer, the samples were post-fixed in 1% osmium tetroxide in sodium cacodylate 0.1 M for 1 hour, dehydrated in a graded ethanol series, and dried from liquid CO_2 in a critical point drier. The dried samples were mounted on aluminum supports and sputter coated with 10 to 50 nm of gold-palladium alloy. All samples were imaged in the conventional secondary electron mode in a DS 130 SEM (Topcon Technologies, Pleasanton, CA), equipped with

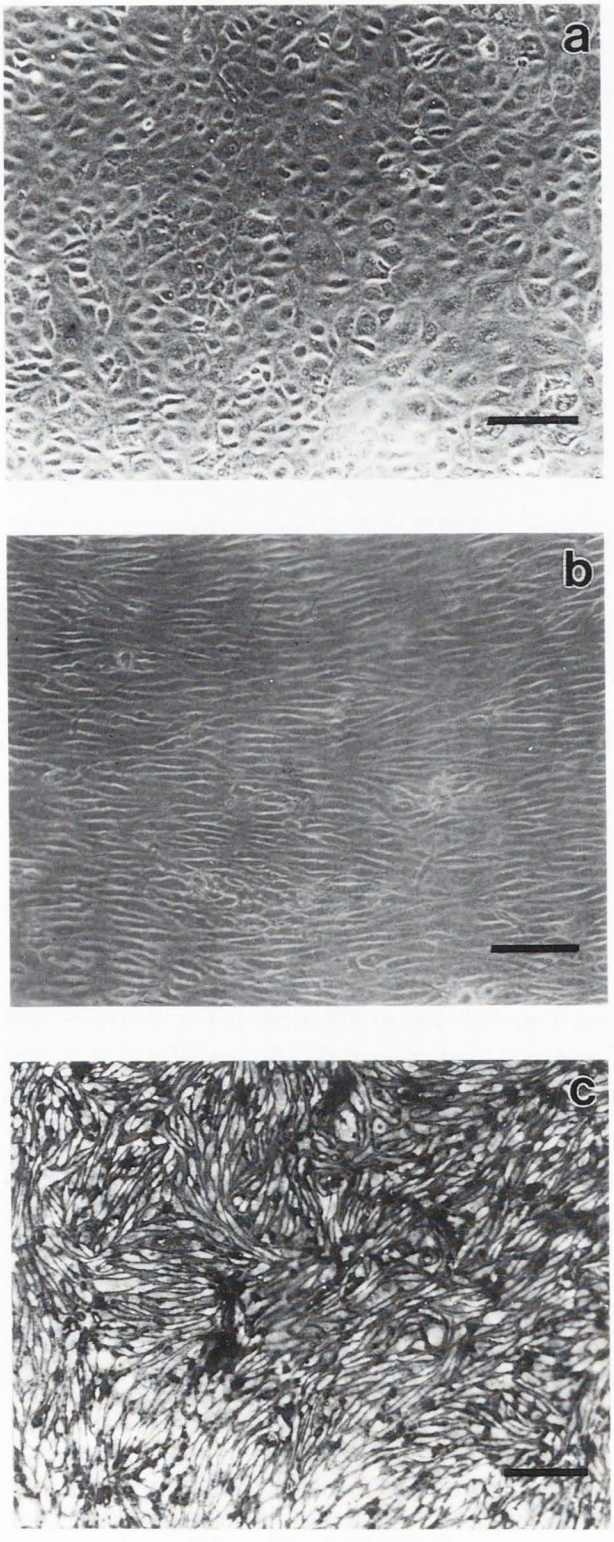


Figure 4. Morphology of porcine aortic endothelial cells cultured on plastic (**a**), collagen gel alone (**b**) and in co-culture with smooth muscle cells in a collagen gel (**c**) all under static conditions. The cells in (c) were silver-stained. Bars = 100 μm.

a LaB_6 emitter, operated at an acceleration voltage of 15 kV. Photographs were taken at different magnifications to observe the complete coverage of the endothelium and the morphology of the cells.

Results

Smooth muscle cell (SMC) growth in collagen gels

When cultured inside a gel of collagen type I (Fig. 1b), the SMC were more elongated than cells cultured on TCPS (Fig. 1a). The cells cultured in collagen formed long filaments growing in three-dimensions (Fig. 1b). Most of the cells were found to be viable by the Trypan Blue exclusion technique after collagenase treatment or using live/dead fluorescent stains (Fig. 2d). The cells bound to the polymerized collagen and exerted a force which resulted in an orientation of the fibers (Fig. 1b) and a dramatic retraction of the whole lattice from 35.7 to 3 cm^2 in *en face* area with a decrease in thickness from 4 to 2 mm. Collagen gels prepared without SMC did not retract at all. The area change with time during retraction followed an exponential curve with a rapid phase lasting 24 hours and a slow phase for the next 48 hours. After 4 days, the area of the gels had reached a plateau. The F-actin organization was also different with the presence of abundant stress fibers in SMC grown on TCPS (Fig. 2a). In SMC grown in collagen gels, F-actin stress fibers were also observed (Fig. 2b). Alpha-SM actin was also found inside cells cultured in collagen (Fig. 2c). The reorientation of the collagen fibers was more clearly defined by observing the electron micrographs of the surface of retracted collagen lattices. The collagen fibers located around the cells (Fig. 3a) usually ran perpendicular to the cell surface, suggesting that the cells bound to them and exerted a traction force all around its surface. In areas without cells (Fig. 3b), the fibers had a specific orientation, usually, preferentially in one direction only. The whole surface of the collagen gel was composed of packed fibers having different orientations usually reflecting the location of cells near the surface. Holes, big enough to allow cell migration, could be observed, but few cells were present at the surface (Fig. 3c).

Co-culture of endothelial cells and smooth muscle cells

The morphology of confluent EC seeded on top of a SMC-collagen lattice was different from that of EC cultured on plastic. The latter were polygonal in shape and had no specific orientation, whereas the former were more elongated and had a pattern of orientation forming waves. To determine if the EC elongation in co-culture was an effect of the collagen alone, the cells were seeded on top of a gel of collagen made with or without

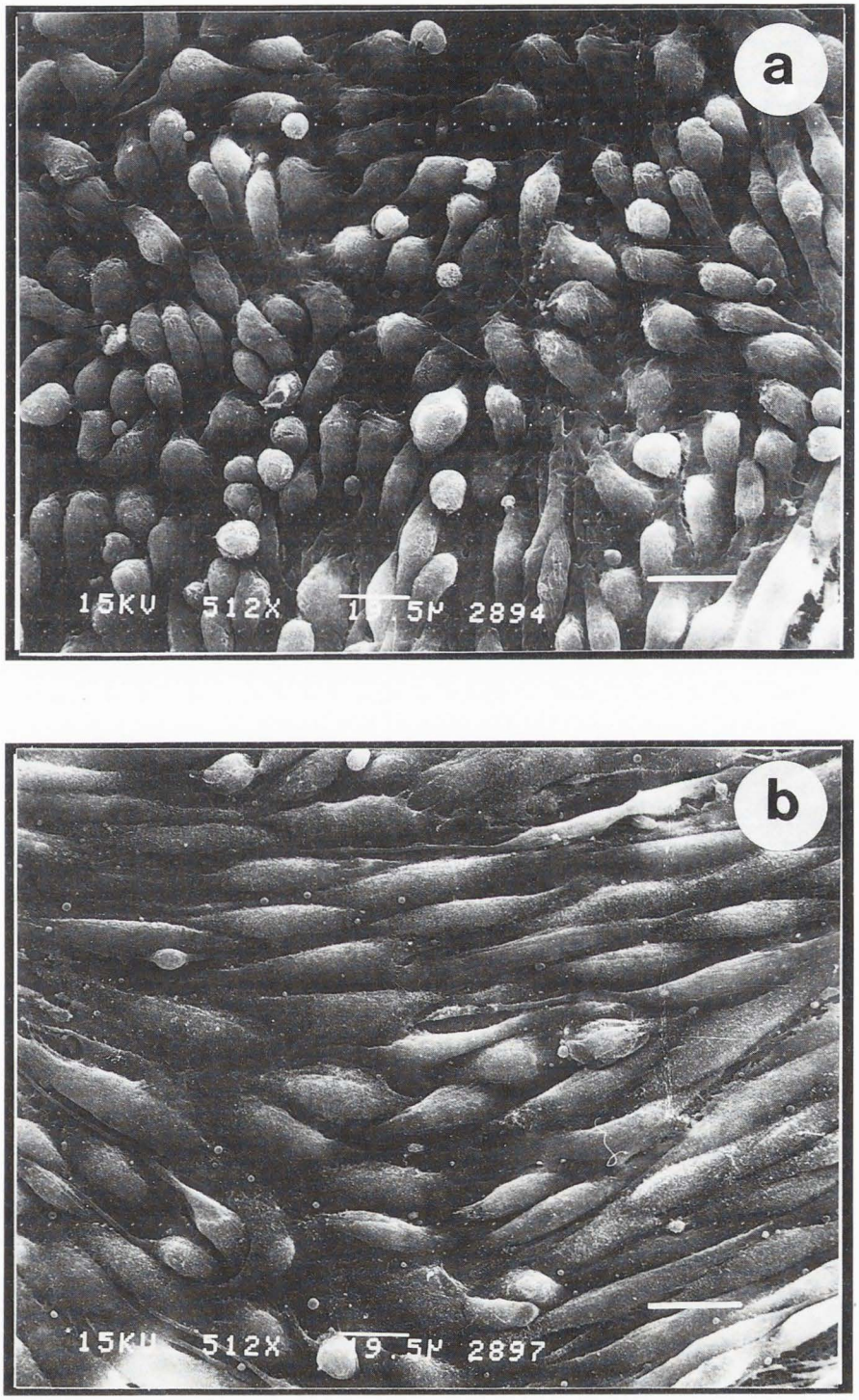


Figure 5. Scanning electron micrographs of porcine aortic endothelial cells in co-culture with smooth muscle cells in a collagen matrix under static conditions (a) and exposed to a shear stress of 30 dynes/cm^2 for 48 hours (b). Bar = 25 μm.

SMC. We observed that EC were more elongated on collagen gels than on plastic and that this elongation was dependent on the shape of the collagen gels (Figs. 4a and 4b). In the case of circular gels (i.e., gels made into a 35 mm tissue culture dish), the presence of SMC inside the gel significantly increased the elongation of EC seeded on the top surface (data not shown). In rectangular gels, EC already were very elongated in the absence of SMC, but the EC orientation pattern was more wavy when SMC were present inside the gel (Fig. 4c). It is interesting to note that although EC in co-cultures formed wavy patterns, the overall orientation was random as for polygonal EC cultured on plastic. When observed with SEM, endothelial cells co-cultured with SMC formed a monolayer which completely covered the entire surface of the lattice (Figs. 5a and 5b). The cells

were elongated in shape and formed contacts with their neighbors. EC were bulging on the surface and many rounded cells could be detected. The nature of these cells is not known, but it may be EC rounding up because of a lack of space to spread. A further slight retraction of the collagen lattice was observed after EC seeding. This retraction seemed to be more pronounced at the center than at the borders, and this resulted in an overall change in shape.

Effect of flow on the co-culture

When porcine aortic endothelial cells (PAEC) co-cultured with SMC in a collagen lattice were exposed to a steady, laminar shear stress of 10 or 30 dynes/cm^2, the cells still were found covering the entire surface of the gel after 72 hours. This demonstrates that our flow system is able to preserve the integrity of the EC monolayer. The cells already were elongated at the beginning of the experiment, hence any further elongation was difficult to detect. Cells started to show orientation parallel to the direction of flow as early as 24 hours after exposure to 30 dynes/cm^2 and 48 hours after exposure to 10 dynes/cm^2. After 48 and 72 hours, most of the cells were aligned with the direction of flow (Fig. 5b). The cells exposed to shear stress were much flatter than those in static culture. No rounded cells were seen in the monolayer, probably because those cells had been swept away by the flow.

Discussion

We chose to design a system where each element to be investigated (EC, SMC, collagen matrix) can be added one by one, so that the effect each of these elements has on the others can be studied separately. Many studies on the effect of shear stress on EC cultured on plastic or adsorbed ECM proteins, including those in our laboratory, show that EC morphology, cytoskeleton, and growth rate are altered by the presence of flow (Levesque and Nerem, 1985, 1989). Bovine aortic EC elongate and align with the direction of flow in response to shear stress. EC also reorganize their F-actin into stress fibers oriented along the major axis of the cell (Wechezak *et al.*, 1985), and EC are shown to be growth-inhibited by flow (Mitsumata *et al.*, 1991). However, although flow seems to bring EC to a state closer to that found *in vivo* (elongation, presence of actin stress fibers, decreased growth rate, ...), EC in blood vessels are usually more elongated and have a lower growth rate when exposed to a similar flow environment than EC *in vitro*. We hypothesize that these differences are due to the absence of SMC and of the appropriate ECM proteins for cultured EC.

SMC cultured inside a collagen matrix are very elongated and have a synthetic phenotype. The presence of abundant endoplasmic reticulum and lysosomes, which sometimes contain extracellular fibers, suggests an active remodeling of the extracellular matrix surrounding the cells. This SMC behavior has been observed when cells are cultured alone and in co-culture with EC, indicating no effect of EC on the phenotype of SMC. EC in static co-culture with SMC in a collagen matrix have been found to be more elongated than EC cultured on plastic. This elongation is partly due to the collagen matrix and partly due to the presence of SMC. To further explain this phenomenon, it is important to note that collagen matrices are substrates very different in nature and properties than either plastic or glass. This is because they are composed of a three-dimensional mesh of fibers which can be rearranged by the cells as it has been shown with both EC and SMC. We observed by electron microscopy that SMC in collagen lattices oriented the collagen fibers into tension lines by a phenomenon previously described (Guidry and Grinnel, 1986). We speculate that it is this SMC-induced collagen re-orientation which causes the elongated EC shape, as well an orientation exhibiting a wavy pattern (Ziegler *et al.*, manuscript in preparation). Contact guidance by oriented fibers already has been demonstrated by Buck (1979) who observed a longitudinally oriented fibrillary pattern of the subendothelial space in EC-denuded rat aortas. Buck (1979) also showed that autologous SMC or chick embryo cells and fibroblasts, seeded on top of the subendothelial basement membrane, were elongated and aligned parallel to the ECM fibers, that is, with the major axis of the vessel. Dunn and Ebendal (1978) also found that cell orientation usually followed that of previously aligned collagen fibers.

If EC elongation and orientation is closely related to the organization of the collagen fibers underneath, then does shear stress induce the EC to reorient these fibers? In others words, can the tension-induced collagen fiber reorientation in the lattice be overcome by the force exerted by flow on the endothelial cells? In blood vessels, the subendothelial matrix is composed of fibers aligned with the major axis of the vessel, that is, parallel to the orientation of the endothelial cells. However, EC lying on the border of valve leaflets have been shown to be aligned perpendicular to the direction of flow, but parallel to the fiber orientation in the subendothelial matrix (Deck, 1986). This may suggest that, if fiber orientation is induced by a high tension force (is this case, due to the high strain in the valve), then EC will stay oriented with the direction of the fibers and shear stress will not exert a strong enough force to induce either fiber or cell realignment with the direction of flow. However, it has been demonstrated in our laboratory, as well as others, that EC exposed to shear stress reor-

ganized their extracellular fibronectin into denser fibrils aligned with the direction of flow (Thoumine *et al.*, 1994). We can speculate that EC in co-cultures, since they were already elongated, start the orientation process as soon as they are subjected to shear stress. This means approximately 48 hours earlier than EC cultured on plastic. The morphology of the endothelial cells in co-cultures as shown by SEM is very similar to the micrographs shown in Allen *et al.* (1984) suggesting that the collagen matrix is mostly responsible for this specific cell shape. The flattening of the cells exposed to flow could be an effect of the shear stress, but also could be due to the pressure imposed, since in our system flow is driven by a hydrostatic pressure of approximately 40 mm Hg. However, pressure magnitudes of 40 mm Hg and less have been found to have no effect on the cell morphology and growth (Tokunaga *et al.*, 1989). Hence, the changes we have observed in EC in co-culture may be mostly due to shear stress. Studies on the effect of collagen matrix, SMC, and shear stress on EC growth rate currently are in progress in our laboratory and should provide us further insight into the growth characteristics of EC in co-culture with SMC in a matrix of collagen.

Acknowledgements

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Discussion with Reviewers

R.M.K.W. Lee: In Figure 3a, you have shown that the collagen fibres are attached to the cells perpendicular to the cell surface, but in **Discussion**, you have quoted the study by Buck (1979) that SMC seeded on top of the subendothelial space oriented themselves parallel to ECM fibers. Can you explain these differences?
Authors: The difference between our results and Buck's work is that in the former, SMC were seeded during polymerization of the collagen, hence the cells were located inside the gel and in the latter, cells were seeded on already formed fibers.

C.J. Doillon: Did you observe a uniform distribution of SMCs within the collagen gel? Did you observe with time and contraction whether the SMCs became close to the periphery of the gel as observed with fibroblasts embedded in contracted collagen gel?
Authors: After polymerization of the collagen, the SMC were uniformly distributed in all directions. Upon contraction of the gels, SMC density at the borders seemed to be higher than that at the center.

R.M.K.W. Lee: The presence of fibers in the SMC, and the attachment of the collagen fibers to the cells, may actually suggest that the SMC were actually producing collagen fibers. Can you rule out this possibility?
Authors: It was demonstrated that SMC cultured inside collagen gels synthesize collagen (Thie *et al.*, 1991). It is possible that the cells also secrete fibers in our system and we did not check whether they actually do.

R.M.K.W. Lee: If the cause of your gel retraction was due to the SMCs, can you prevent it by adding smooth muscle relaxant, or by adding ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA)? Why is the thickness not changed when the surface area changed from 35.7 to 3 cm^2?
Authors: We did not check the effect of smooth muscle relaxant or EGTA on gel retraction by SMC. However, preparing the gels in serum-free medium prevented them from retracting. We actually carried out thickness measurements afterward using an accurate protocol and found that the thickness decreased from 4 to 2 mm (50% reduction compared to 80-90% for the surface area). This difference may be due to the preferential cell orientation parallel to the surface plane.

C.J. Doillon: The authors presume that endothelial cells are rounding up at the surface while the SMCs are elongated within the collagen gel. Have you done some characterization such as LDL uptake and cytoskeleton markers for those cells on or in your collagen gel?
Authors: We stained the co-cultures with AcLDL bound to a fluorescent probe (AcLDL-DiI, Molecular Probes) specific to EC and with an antibody against α-smooth muscle actin (Zymed) specific to SMC. The surface cells were positive for AcLDL and negative for α-smooth muscle actin. On the other hand, the cells located inside the gel were negative for AcLDL and positive for α-smooth muscle actin.

C.J. Doillon: High number of passages of SMC and EC was used for the study. Does this impair the phenotype of these specific cells? Have you done some investigations?
Authors: In the case of SMC, we did not observe any difference in morphology and ability to retract collagen gels between cells at passage 2 and cells at passage 11.

A.R. Wechezak: When SMC on glass or plastic are subjected to shear stress, cells orient perpendicular to the direction of flow. Did the authors ever expose their SMC collagen cultures without endothelial cells to shear stress to observe whether a similar orientation occurred?
Authors: We did not try to expose SMC collagen cultures to shear stress because the SMC were sparsely distributed on the surface of the gel (Fig. 3c).