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An Endothelial Cell-Smooth Muscle Cell Co-Culture Model for Use in the Investigation of Flow Effects on Vascular Biology

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Abstract—Flow and the associated shear stress have been shown to play an active role in the regulation of the structure and function of endothelial cells (EC) *in vitro*. Although cultured EC subjected to flow exhibit an elongated morphology and a decreased cell growth rate rather like those observed *in vivo*, there are differences in morphology and growth rate, as well as other characteristics, between *in vitro* and *in vivo* EC. This suggests that flow is only one of the many factors affecting EC differentiation *in vivo*. In this study, a co-culture model system was designed, which includes smooth muscle cells (SMC), a matrix of collagen type I, and a confluent monolayer of EC, and this simplified model of the arterial wall was subjected to a steady, laminar shear stress of 10 and 30 dyn/cm^2. Under non-flow conditions, EC exhibited an elongated shape, but with a random orientation. In response to flow, there was an alignment with the direction of flow. This alignment occurred more rapidly at 30 dyn/cm^2 than at 10 dyn/cm^2. The collagen matrix was found to be primordial in the maintenance of a quiescent endothelium, even in the absence of SMC and flow, suggesting the importance of an organized extracellular matrix (ECM) in the differentiation of cells *in vivo*.

Keywords—Vascular endothelium, Flow effects, Smooth muscle cells, Co-Culture, Collagen gels.

INTRODUCTION

Although once viewed as a passive, nonthrombogenic barrier, the vascular endothelium is now recognized as a dynamic participant in the biology of blood vessels. As the interface between blood flow and the vessel wall, the endothelium plays a critical role in vascular biology and pathobiology. It is positioned so as to be under the influence of both the flow environment in which it resides and its basement membrane and the underlying smooth muscle cells.

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Many vascular functions have been shown to be endothelium dependent. For example, it is now recognized that the regulation of vascular tone is due to a balance of factors mediated by the endothelium that control vasoconstriction and vasodilation. The endothelium can mediate vasodilation by secreting endothelial-derived relaxing factor (EDRF), a member of the nitric oxide (NO) family, and prostacyclin (PGI_2) or vasoconstriction with the activation of angiotensin I or the secretion of endothelin.

Like the regulation of vascular tone, the control of growth in the blood vessel is also complicated in nature, with EC replication being controlled by a variety of pathways and factors (5,30). The pathway involving soluble factors includes the heparin-binding growth factors (HBGF) (*e.g.*, fibroblast growth factors [FGFs], vascular permeability factor (VPF), platelet-derived growth factor [PDGF], and transforming growth factor-β [TGF-β]), and it is not clear how these factors interact with each other to regulate growth. Many growth factors are secreted by both EC and SMC, and can act on both cell lines, either by a receptor-mediated stimulation or inhibition or through changes in the extracellular matrix composition (*e.g.*, TGF-β). PDGF has been shown to be secreted by SMC *in vivo*, indicating the possible existence of an autocrine system in medial SMC, especially during events like those associated with the genesis of atherosclerosis. Moreover, regular fenestrations and intermittent fragmentation of the elastic laminae have been observed in blood vessels and can facilitate molecular transport across the vessel wall. In addition to cell communication through soluble components, cell-cell contacts have been shown to occur between EC and the SMC of the medial layer. EC-SMC cytoplasmic extensions pass through the fenestrations to form cytoplasmic bridges and might be involved in regulation of cell growth (5). This was demonstrated in cocultures of EC and SMC *in vitro* (2).

In recent years, it has been demonstrated that the hemodynamic environment participates in the regulation of endothelial function. Initial studies reported that the morphology of the endothelium in blood vessels is correlated with the magnitude of shear stress such that elongated cells oriented with the direction of flow are found in re-

gions of high shear stress and polygonal cells in regions of low shear stress (17). Also Herman *et al.* (12), using excised blood vessels perfused in an *in vitro* system, found that the organization of F-actin and the endothelial permeability to cholesterol transport were dependent on the magnitude of shear stress and pressure.

Unfortunately, *in vivo* it is not practical to define and control the endothelial mechanical environment over the time periods required for the biological endpoints of interest. However, EC can be isolated and studied in culture, and in recent years such studies have been extended to include simple, well-defined flow environments. In culture, bovine aortic EC (BAEC) and human umbilical vein EC (HUVEC) have been found to elongate and align with the direction of flow when exposed to a steady, laminar shear stress ranging from 10 to 90 dyn/cm^2 (14,16,18). In addition, the cytoskeleton of BAEC and HUVEC reorganizes as shown by the disappearance of the dense peripheral bands of F-actin located around the cytoplasm of the cells and the formation of stress fibers centrally located and aligned with the direction of flow (24,28). Shear stress also affects a variety of endothelial functions, including growth rate (19,21) and the production of biologically active molecules (7,8,22).

Although cultured EC subjected to shear stress appear to be in a state closer to that *in vivo,* there are differences. *In vitro* flow studies have been performed with EC cultured on plastic or adsorbed extracellular matrix proteins (*e.g.,* fibronectin or collagen type I) and these conditions are markedly different from the *in vivo* environment in which EC reside where there is the presence of a highly organized ECM and neighboring SMC. Thus, the hypothesis guiding the present work is that the response of endothelial cells to flow and the associated shear stress is influenced by the presence of a three-dimensional extracellular matrix structure and neighboring smooth muscle cells. The development of a co-culture model designed to test this hypothesis is described, along with some initial experimental results.

MATERIALS AND METHODS

Cell Isolation and Culture

EC and SMC were isolated from porcine aorta at Emory University (Animal Research Laboratory, Division of Cardiology). Briefly, the aortas were cut longitudinally to expose the intimal surface, and 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's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) was spread over the whole surface. The aortas were then incubated at 37°C for 10 min, after which the endothelium was detached by gentle scraping and resuspended in 5 ml of DMEM with 10% fetal calf serum (FCS, Intergen, Purchase, NY) in a T-25 flask (Fisher, Nocross, GA). The media of the aortas were then separated from the adventitia using two forceps and cut into 2 × 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 outgrowth. 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 Chemical Co., St. Louis, MO), and L-glutamine (Sigma). The cells were cultured in 100-mm tissue culture dishes (Fisher) until confluence, at which time, they were detached using trypsin-EDTA (Gibco).

Culture of the Smooth Muscle Cells Inside Collagen

The SMC were cultured in a lattice made of polymerized collagen following the technique described by Weinberg and Bell (29). This technique has the advantage of recreating a three-dimensional, tissue-like substrate on which EC can be seeded. The lattice was composed of collagen type I (Collaborative Research, Bedford, MA) from rat tail (3–4 mg/ml in 0.02 N acetic acid), DMEM 1.5 times concentrated, FCS, 0.1 N sodium hydroxyde (NaOH, Fisher) to neutralize the acetic acid, and a solution of SMC in suspension (10^4 to 10^6 cells/gel). The gels were poured into molds of different size and shape, according to the parameters being studied. The size of the rectangular molds were either 7.0 × 5.1 × 1.25 cm (L × W × D) for big gels or 5.5 × 1.9 × 0.3 cm for small gels. To prevent the collagen gels from retracting, surgical silk was added to the inside borders of the smaller rectangular mold.

Seeding of the Endothelial Cells

When the lattices of collagen and SMC were fully retracted, the top surface was seeded with EC as follows. Confluent EC in a 100 mm dish were detached using trypsin and the cell suspension was spun 5 min at 1000 rpm. The pellet was resuspended into 0.8 ml (0.2 ml/gel) of DMEM plus FCS. 0.2 ml was deposited on the top surface of the lattice and the cells were allowed to attach for 10 min at room temperature. The co-culture was then transferred into an incubator for 20–30 min, until most of the EC attached to the gel. The degree of attachment was observed using a light microscope (Nikon, Melville, NY) with a 4× objective. When most of the EC were attached, the co-culture was fed with 20 ml of DMEM plus FCS. 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 48–72 h after seeding.

EC also were seeded on top of collagen matrices made without SMC. The collagen matrix was prepared as described previously, except that SMC were omitted. The gels were poured into a rectangular mold 5.5 × 1.9 cm (total volume of 4.0 ml). After a period of 15 min, EC were seeded at a density of approximately 3×10^5 cells/gel (3.2×10^4 cells/cm^2). In a different kind of experiment, SMC in retracted collagen matrices were killed by a 15-min treatment with 2.5 μg/ml saponin (Fisher) in PBS followed by a 30-min exposure to 5 mM NaCN (Aldrich, Milwaukee, WI) in PBS. The matrix was thoroughly rinsed with serum free medium before EC seeding as for the co-cultures.

Light Microscopy

Silver staining of the co-cultures 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 min. They were transferred to a 2.5% silver nitrate solution ($AgNO_3$, Fisher) for 1 min, rinsed in 5% glucose, then in 1% ammonium bromide (NH_4Br, Fisher) for another minute. The co-cultures were then rinsed and fixed in 10% buffered formalin (Sigma) for 30 min. The endothelium was visualized in a phase-contrast microscope (Nikon) with 10 or 20× objective and maximum light intensity. Photographs were taken using tri-X pan 400 ASA (Kodak, Rochester, NY). The shape index (SI $= 4\pi$ area/perimeter^2) and angle of orientation (AO) were measured by outlining the cells on micrographs using a magnetic tablet connected to a computer (Zeiss Videoplan, Thornwood, NY). SI is a measure of the elongation of the cell and is equal to 0 for a straight line and 1 for a circle. Thus, a decrease in the SI value indicates cell elongation. AO indicates the orientation of the major axis of the cell with respect to the direction of flow.

Electron Microscopy

Co-cultures were treated for electron microscopy as follows. The gels were rinsed in a buffer solution (sodium cacodylate 0.1 M, pH = 7.4, Sigma) for 15 min and then fixed in glutaraldehyde 1.5% (Sigma) in sodium cacodylate 0.1 M for 4–5 hr. After rinsing with the buffer, the samples were post-fixed in 1% osmium tetroxide (Sigma) in sodium cacodylate 0.1 M for 1 hr, dehydrated in graded ethanol series and dried from liquid CO_2 in a critical point drier (EM Polaron, Agawam, MA). The dried samples were mounted on aluminum supports and sputter coated with 10 to 50 nm of gold paladium alloy. All samples were imaged in the conventional SE mode at 15 kV of acceleration voltage in a Topcon DS 130 SEM equipped with a LaB_6 emitter. Photographs were taken on Polaroid P-55 films and at different magnifications to observe the complete coverage of the endothelium and the morphology of the cells.

Growth Analyses

The growth of the cells was evaluated using ^{3}H-thymidine incorporation for growth rate and cell count for cell density. The cells were incubated at 37°C with a solution of 2 μCi/ml ^{3}H-thymidine (New England Nuclear/Dupont, Chadds Ford, PA) in medium for 3 hr. After rinsing with Hank's balanced salt solution (HBSS, Sigma), the cells were separated from the collagen by incubating the slab in a solution of 0.3% collagenase CLS II (Worthington) in PBS at 37°C for an hour. The solution was centrifuged at 1000 rpm for 10 min, and the pellet was resuspended in either 0.5 or 1.0 ml of HBSS, depending on the cell density. A cell sample was taken for cell counting using an hemocytometer (Fisher). In initial experiments, viability was measured using Trypan blue staining. The remainder of the cell suspension was washed twice in 5% tricarboxilic acid (TCA), and the pellet was resuspended in 1.0 ml of 0.1 N NaOH, added to 10 ml of scintillation liquid (Beckman Instruments Inc., Norcross, GA), and counted for β-emission. Results were expressed as counts per minute (cpm) per 1000 cells. The thymidine uptake by EC in co-culture was measured using the technique derived from that of Antonelli-Orlidge *et al.* (2). The SMC were growth-arrested by incubating them with mitomycin C 10 μg/ml (Sigma) for 2 hr. The gels were then rinsed with DMEM and seeded with EC previously stained with 2.5 μg/ml DiI (Molecular Probes) for 1 day. A control was made in order to measure the cell number and cpm of growth-arrested SMC only. During analyses, the EC number was determined by counting the number of fluorescent cells and the SMC number by subtracting the number of fluorescent cells from the total number of cells. The value of cpm measured for the co-culture accounted for the EC only and hence could be compared with values obtained in a control made of EC on top of a collagen gel without SMC.

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 (16). The height of the channel was chosen to be either 500 μm (30 dyn/cm^2) or 1000 μm (10 dyn/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 dyn/cm^2 for periods of time up to 72 hr. Measurements were made of the effect of flow on SI and AO of the endothelial monolayer. In addition, the influence of flow on the rate of thymidine incorporation also was measured.

Results

EC Morphology and Growth on Top of Collagen Gels Without SMC

PAEC cultured on plastic assume a polygonal morphology when confluent. Cells seeded on top of rectangular gels without SMC were elongated and aligned parallel to the major axis of the rectangle, except at the borders where cells were aligned parallel to the sides. The morphology of the cells changed according to the shape of the gels (33), and cells seeded on top of circular gels were polygonal and randomly oriented.

The growth of EC on top of collagen gels was measured and compared with that of EC seeded on plastic. EC cultured on plastic had a typical growth curve, with the cell number growing to a density of 5×10^4 cells/cm^2 (Fig. 1). The thymidine uptake was high with a value in excess of 1000 cpm/1000 cells after 24 hr and progressively decreased to values under 200 as the cells reached confluence. When EC were seeded on rectangular, free collagen gels, the cell number did not increase over time for at least 4 days (Fig. 1). Although the cell number did not change, the cell density (cell number per unit area) increased because of the area reduction that took place during the gel retraction caused by the EC. In fact, as shown in the density curves, the densities of cells cultured on plastic and cells cultured on collagen gels followed each other very closely. The stability in cell number on collagen gels was confirmed by the low values of thymidine uptake, which remained at a value on the order of 100 cpm/1000 cells during the entire experiment.

The effect of the shape of the gel was assessed. Although the cell morphology was different, no differences in cell number, cell density, and thymidine uptake were observed between cells cultured on rectangular gels and cells cultured on circular gels (Fig. 2). In all experiments, the cell density on top of collagen gels at confluence was found to be around 3×10^4 cells/cm^2, which is signifi-

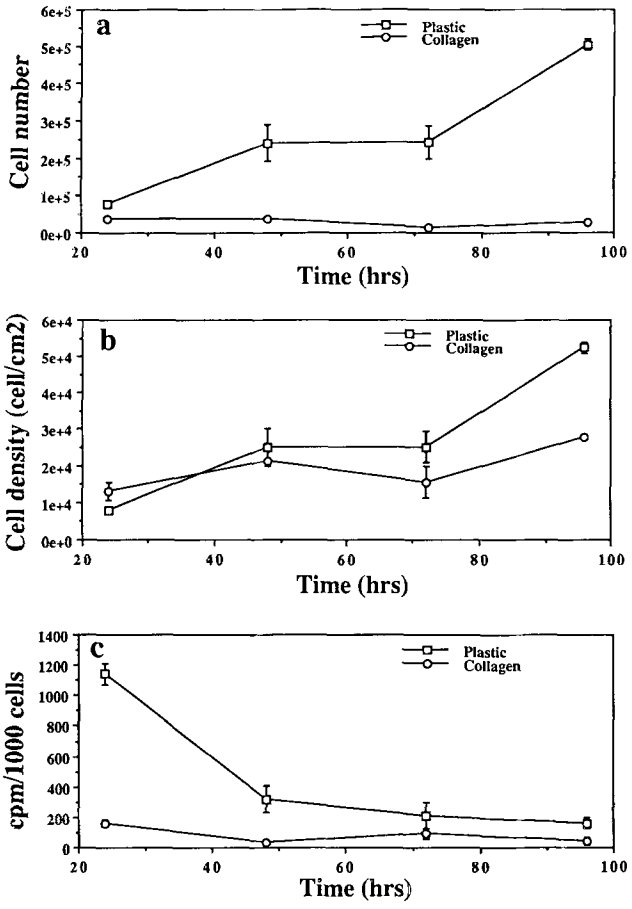


FIGURE 1. Growth of PAEC on plastic or on rectangular free gels of collagen type I. Cell number (a), cell density (b), and thymidine uptake (c) were measured at different time points. Results are presented as the mean ± standard deviation of five experiments.

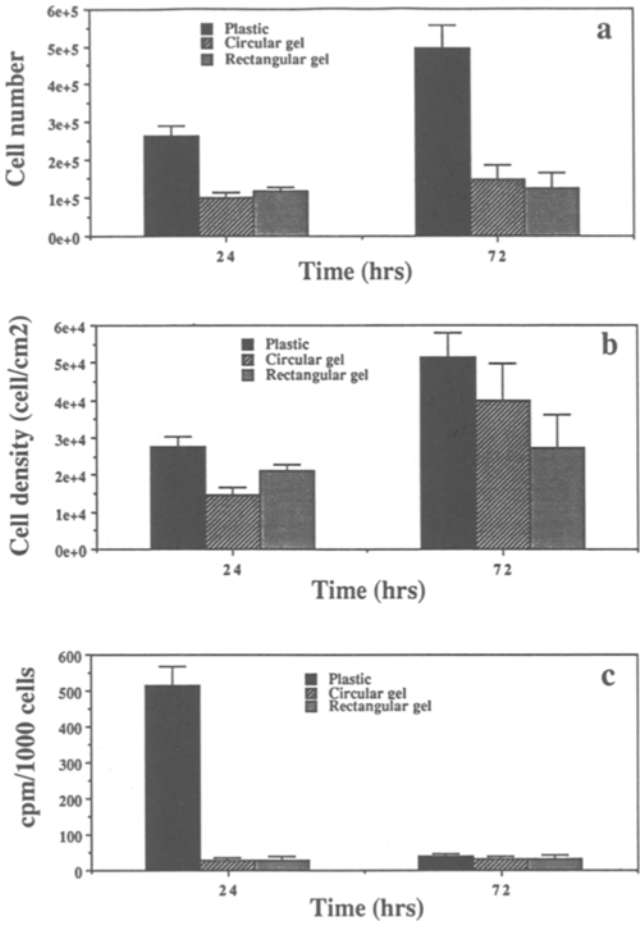


FIGURE 2. Effect of gel shape of the growth of EC. Cells were cultured on plastic and on circular and rectangular free gels of collagen type I. Cell number (a), cell density (b), and thymidine uptake (c) were measured at different time points. Results are presented as the mean ± standard deviation of five experiments. Cells densities and thymidine uptakes at 72 hr are not statistically different.

cantly lower than that of EC cultured on plastic. The cell density on circular gels was 4×10^4 cells/cm^2; however, the difference, compared with rectangular gels, was not statistically significant. Finally, to test the effect of the collagen itself on EC growth, cell number and density, and thymidine uptake were measured for bound and free rectangular collagen gels (Fig. 3). The thymidine uptake was still low in bound gels compared with plastic, but slightly higher than the value found for cells in free gels, again especially after 96 hr. The cell density, however, was significantly lower in bound gels, especially after 96 hr, indicating that cells in bound gels did not reach confluence after 4 days, as opposed to cells cultured on plastic. The low value of cell density in bound gels was confirmed by microscopic observation of subconfluent monolayers on the surface of the gels. These results confirmed the fact that EC growth is inhibited by collagen type I in fibrillar form. The traction force exerted by the cells, which resulted in the retraction of the gels and the area reduction observed, was significant enough to achieve confluence just as rapidly as for cells cultured on plastic.

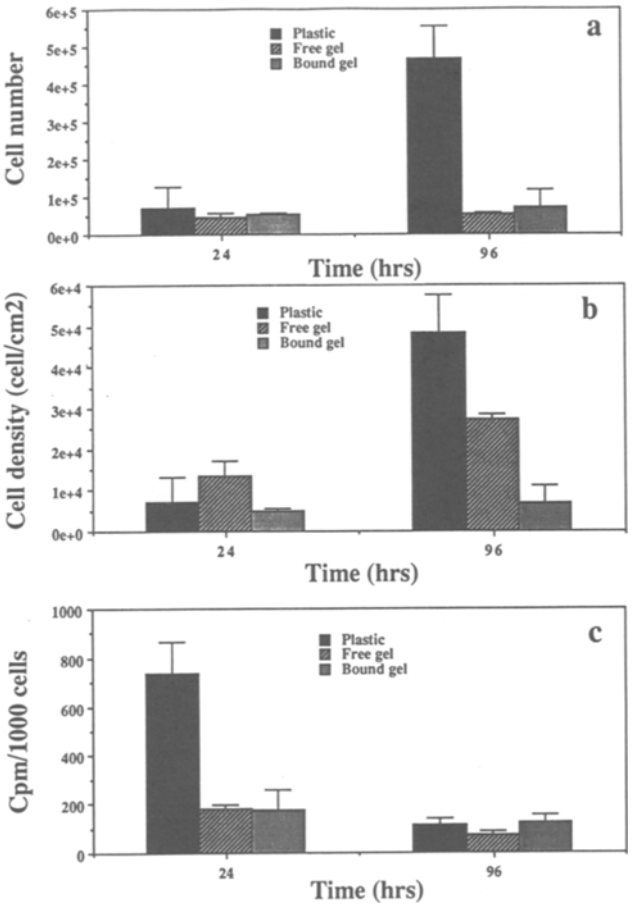


FIGURE 3. Effect of gel restraint of EC growth. Cells were cultured on plastic and on rectangular free and rectangular bound gels of collagen type I. Cell number (a), cell density (b), and thymidine uptake (c) were measured at different time points. The cell density (b) was computed as the ratio of cell number to surface area of the plastic dish (9.62 cm^2) or the gel (10.45 cm^2 for bound gels, from 3.4 to 2.0 cm^2 for free gels). Results are presented as the mean ± standard deviation of three samples in each case. The cell densities for cells in free gels and cells in bound gels are statistically different (p = 0.01), but thymidine uptake rates are not.

EC Morphology and Growth in Co-Cultures

EC co-cultured with SMC were observed to be very elongated and were oriented randomly in a wavy pattern for rectangular free co-cultures (Fig. 4). This was in contrast to rectangular bound co-cultures where EC were aligned with the major axis of the rectangle (32). Killing SMC prior to EC seeding did not affect EC shape and orientation at confluence (*i.e.*, EC were elongated and oriented as those in co-cultures). EC had abundant F-actin microfilaments randomly aligned. When collagen gels with SMC were incubated with mitomycin C (10 μg/ml) for 2 hr and then seeded with EC, the thymidine uptake of the whole population of cells (EC and SMC) was approximately 10,000–15,000 cpm for an average population of approximately 3–4 $\times$ 10^5 EC/gel and 2–3 $\times$ 10^6 SMC/gel. Since mitomycin C completely inhibits SMC growth, the measured cpm accounted for EC only and resulted in a growth rate of 35 cpm/1000 cells (Table 1). Control co-cultures (which were not treated with mitomycin C) had a total cpm of approximately 120,000. This number, although significantly higher than that for co-cultures with growth-arrested SMC, was equivalent to approximately 35 cpm/1000 cells due to the large SMC number in the lattice. Values of thymidine uptake for all culture conditions are reported in Table 1. It can be seen from these values

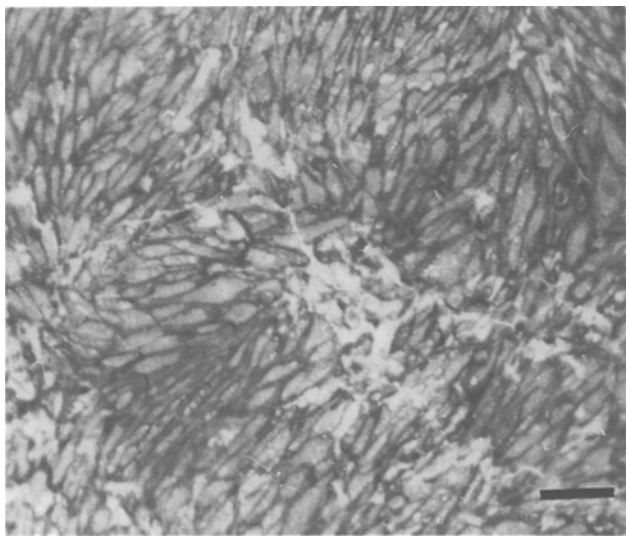


FIGURE 4. A micrograph of the surface of a co-culture of EC and SMC under static conditions showing randomness in cell elongation and orientation. This figure shows similarity with Fig. 4 in Silkworth and Stebhens (23). Bar = 100 μm.

TABLE 1. Effect of culture conditions on the thymidine uptake by porcine EC and SMC.

Culture System	SMC	EC
No growth (starvation or mitomycin-treated)	9 ± 5 (5)	5 ± 2 (2)
Subconfluent SMC on plastic 24 hr after seeding	1462 ± 672 (5)	—
Confluent SMC on plastic 96 hr after seeding	292 ± 50 (5)	—
SMC inside collagen gels 96 hr after seeding	45 ± 13 (5)	—
Subconfluent EC on plastic 24 hr after seeding at 3.2×10^4 cells/cm^2	—	1141 ± 69 (5)
Confluent EC on plastic 96 hr after seeding at 3.2×10^4 cells/cm^2	—	160 ± 37 (5)
EC exposed to 30 dyn/cm^2 for 24 hr	—	109 ± 23 (6)
Subconfluent EC on collagen gels 24 hr after seeding at 3.2×10^4 cells/cm^2	—	159 ± 21 (5)
Confluent EC on collagen gels 96 hr after seeding at 3.2×10^4 cells/cm^2	—	42 ± 22 (5)
Co-culture of EC and SMC	35.2 ± 4.0 (5)	35.5 ± 10.9 (5)
Co-culture exposed to 30 dyn/cm^2 for 24 hr	29.4 ± 6.4 (5)	25.3 ± 9.4 (5)

Results are expressed as mean count per minute per 1000 cells ± standard deviation. The sample number is shown in parentheses.

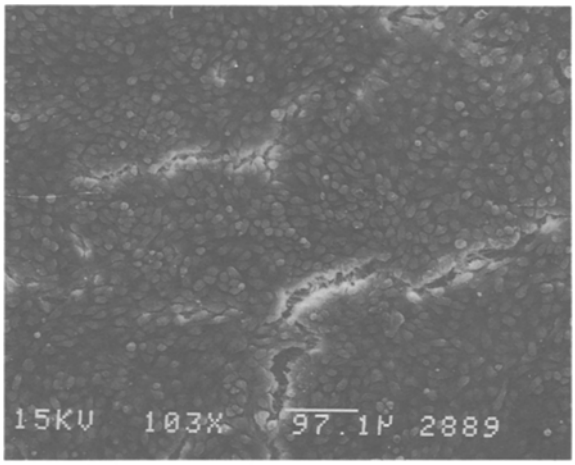
that co-culturing EC and SMC did not result in significantly altered growth rates of either EC or SMC.

EC Morphology and Growth Under Shear Stress

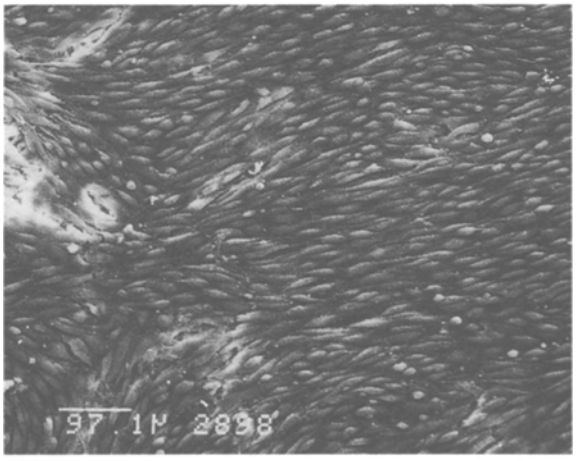
When PAEC co-cultured with SMC in a collagen lattice were exposed to a steady, laminar shear stress of 10 or 30 dyn/cm^2, the cells still were found to cover the entire surface of the gel after 72 hr of exposure. This demonstrates that the flow system, as designed, is able to preserve the EC monolayer. The cells were already elongated at the beginning of the experiment (SI = 0.49), hence any further elongation was difficult to detect. However, SEM pictures showed that cells started to show an orientation parallel to the direction of flow as early as 24 hr after exposure to 30 dyn/cm^2. After 48 and 72 hr, most of the cells were aligned with the direction of flow (Fig. 5). At high magnification, cells in static co-cultures bulged into the lumen (*i.e.*, they exhibited a very round morphology) (Fig. 5). Close examination showed that most EC had an elongated morphology and no cell overlap was found. Upon exposure to flow, the cells not only exhibited an orientation with the direction of flow, but were much flatter (Fig. 5). Cell overlapping and subendothelial spaces were not found, indicating that those cells present were EC and that shear stress did not induce any detachment. In preliminary experiments, EC grown on matrices where SMC were killed before EC seeding aligned in a similar manner as those grown in co-cultures with live SMC. The shape index and angle of orientation were measured for EC in co-culture with SMC and exposed to 10 and 30 dyn/cm^2 for up to 72 hr (Fig. 6). The SI of the EC stayed relatively constant over time, indicating only a small, further elongation of EC with flow. The AO, however, decreased from a random value of approximately 45.24 ± 4.3 to values as low as 10.49 ± 1.18 in cells exposed to 30 dyn/cm^2 for 72 hr, indicating a clear cell alignment with the direction of flow. The magnitude of shear stress seemed to have an effect on the rate of EC orientation. At 10 dyn/cm^2, the AO decreased after a lag time of 24 hr, whereas the AO decrease was immediate in cells exposed to 30 dyn/cm^2. Also, the values of AO for cells exposed to 10 dyn/cm^2 were significantly higher than those for cells exposed to 30 dyn/cm^2.

The F-actin of EC in a co-culture exposed to shear stress was composed of both stress fibers and a more diffuse pattern similar to that for cells in static co-culture. It was difficult to visualize the more diffuse pattern because of the high concentration of stress fibers oriented with the direction of flow. Although it is difficult to quantify from micrographs of stained cells, it appeared that the density of stress fibers was higher in cells exposed to shear stress. No difference was observed in the F-actin microfilament organization of SMC present in the co-cultures exposed to flow compared with those in static conditions.

When co-cultures of EC and SMC were subjected to a shear stress of 30 dyn/cm^2 for 24 hr, the EC number did not significantly decrease, suggesting that EC co-cultured with SMC and subjected to flow remain as an intact monolayer. The thymidine uptake of the cells, which was already very low, slightly decreased, although the difference between static and shear values (Table 1) was not statistically significant. This small decrease in EC growth for a co-culture exposed to flow was in sharp contrast to results obtained for confluent monolayers of PAEC cultured on plastic and exposed to 30 dyn/cm^2 for 24 hr, then analyzed for cell count and thymidine uptake. The cell number did not change in cells exposed to shear stress compared with cells in static culture, which indicates that



(b)



(a)

FIGURE 5. Scanning electron micrographs of PAEC in co-culture with SMC in static conditions (a) and subjected to a shear stress of 30 dyn/cm^2 for 48 hr (b). Bar = 100 μm.

flow did not induce significant cell detachment from the substrate. The value of thymidine uptake decreased from 160 ± 37 cpm/1000 cells for cells in static culture to 109 ± 23 for cells exposed to 30 dyn/cm^2, respectively. Thus, shear stress affected the growth of EC cultured on plastic to a much greater extent than that of EC co-cultured with SMC in a collagen matrix. It is important to note, however, that the thymidine uptake values for cells cultured inside or on top of collagen were so low as to be at the limit of the measurement accuracy using the incorporation of ^{3}H-thymidine. Therefore, any inhibitory effect due to flow and the associated shear stress was more difficult to detect for cells co-cultured.

Discussion

Results presented here show that EC elongation and orientation, when subjected to flow, occurs faster and in a different way for EC co-cultured with SMC on a collagen matrix than when the cells are grown on plastic. In static co-culture, the EC already were elongated, and then, when exposed to flow and the associated shear stress, oriented with the direction of flow. This is consistent with results for bovine aortic EC in which it has been demonstrated that EC cultured on plastic and exposed to shear stress first elongate and then orient with the direction of flow (16). For co-cultures, the PAEC also aligned parallel to the direction of flow, but at a more rapid rate. We can speculate that EC in co-culture, since they were already elongated, started the orientation process soon after they were subjected to shear stress, as much as 48 hr earlier than EC cultured on plastic.

If EC elongation and orientation is closely related to the collagen fibers located underneath the cells, then how can a physical force of low magnitude (*i.e.*, shear stress) induce EC to reorient these fibers? In others words, can the SMC-induced fiber remodeling in co-cultures be overcome by the force exerted by flow on the endothelial cells? First of all, EC exposed to shear stress have been demonstrated to reorganize the extracellular matrix proteins (*i.e.*, fibronectin, laminin, and collagen type IV) into fibrils,

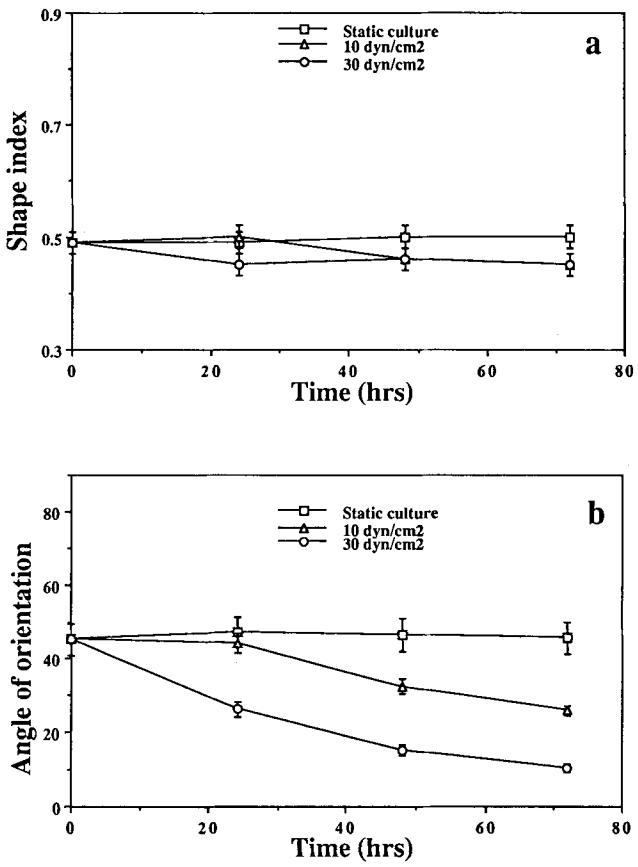


FIGURE 6. Effect of shear stress on the shape index (a) and angle of orientation (b) of PAEC co-cultured with SMC in a matrix of collagen type I. Results are presented as the mean ± 99% confidence interval of five samples.

which for some proteins were aligned with the direction of flow (26). Secondly, studies have shown that cell elongation on top of collagen gels is accompanied by the reorganization of collagen fibers located underneath them (11,15). Hence, in co-cultures EC might be able to orient the collagen fibers located underneath them in response to shear stress. Since EC morphology and alignment in co-cultures in static conditions and exposed to shear stress were the same whether SMC were alive or dead, we can speculate that SMC are only playing an indirect role in EC elongation and orientation, possibly by arranging the collagen fibers in a particular manner. This also implies that the tension induced by SMC and sensed by the basal side of EC might be counteracted by the force exerted by the fluid on their luminal side.

That EC grown on top of gels of collagen type I exhibit a different behavior than EC cultured on plastic has already been demonstrated (3). In this study, the culture of EC on top of gels of collagen type I resulted in a dramatic shutdown of cell growth. We observed that EC seeded on collagen gels achieved confluence by decreasing the overall surface area of the substrate by retraction and not by proliferating. It may be that the cells retract the lattices until they reach a certain level of confluence, and since lattice retraction results in an increase in cell density, the growth signal then is turned off by contact inhibition. However, the persistence of growth inhibition in bound gels (*i.e.*, gels prevented from retraction) implies that low cell densities did not induce any enhanced growth signal. Previous reports have demonstrated a higher rate of cell turnover in EC cultured on adsorbed collagen compared with EC on plastic (10,20). This suggests that the growth inhibition observed in EC on collagen gels was induced by both the three-dimensional structure of the matrix and the organization of the collagen into fibers. These results emphasize the role of tension-dependent interactions between EC and the ECM, which may be involved in the regulation of cell growth and differentiation as has been previously demonstrated with capillary EC (13). When EC and SMC were grown in co-culture, similar values of thymidine uptake were obtained. This implies that either the EC and SMC did not influence each other's growth rate, or the growth of the cells is so low to start with that any effect due to co-culture is masked.

EC cultured on plastic and exposed to shear stress exhibited a decreased growth rate. This decrease, which is on the order of 40%, is consistent with results obtained with BAEC (21,31) and shows that flow has a significant effect on EC growth, independent of the origin of the cells. Experiments using the bromodeoxyuridine incorporation technique suggest that 35 cpm/1000 cells corresponds to a population of approximately 1% dividing cells. Flow thus was able to decrease the number of dividing cells from 4% to 2.5%, suggesting that, although important, it is not enough to bring the cells into a growth state similar to that found *in vivo*.

When EC co-cultured with SMC were exposed to shear stress, no significant further decrease in EC growth rate, compared with static co-culture, was observed. As was pointed out above, the growth rate of EC in static co-culture was found to be 35 cpm/1000 cells. This was at best only slightly lower than that for a collagen gel without SMC, and the measured EC growth rate for both cases represented a factor of five decrease compared with PAEC on plastic. This corresponded to the presence of approximately 1% dividing cells and is a number very similar to that found in the endothelium of blood vessels (9), indicating that ECM proteins in a fibrous form may be a prerequisite for the conservation of a differentiated, quiescent state of the endothelium. With such low growth rates, any effect of flow on the growth rate of co-cultured EC might be difficult to observe.

The flattening of the cells exposed to flow could be an effect of shear stress, but also of pressure since our system does not dissociate the effects of shear stress and hydrostatic pressure (which is on the order of 40 mm Hg). Studies on the effect of pressure on human umbilical vein EC cultured on plastic show that pressures of 80 mm Hg and more induce a disruption of the junctions between the cells which results in a rupture of the monolayer, accompanied by an increase in cell thymidine index. However, pressure magnitudes of 40 mm Hg and less did not affect cell morphology and growth (27). A more recent study on bovine pulmonary artery EC showed that subconfluent monolayers exposed to pressure heads of approximately 10 mm Hg for 7 days exhibited more elongated cells and a higher cell density than control monolayers (1). Although it was not clearly stated in this report, pressure stimuli for periods of time less than 5 days did not seem to affect either cell morphology or cell density. Hence, the alterations observed in EC in co-culture would appear to be mostly due to shear stress.

These experiments reveal the importance of the tension imposed in a three-dimensional matrix on cell elongation and orientation. The subendothelial matrix of blood vessels is composed of fibers aligned with the major axis of the vessel (*i.e.*, parallel to the orientation of the endothelial cells) (4). Whether this fiber orientation is induced by blood flow or by longitudinal tension is not known. Reports state that the morphology of the endothelium in blood vessels is induced by blood flow (17,25). However, unlike most blood vessels, which can be characterized as simple tubes, some parts of the vascular system have a more complicated geometry. For instance, bifurcations and branching are characterized by complicated flows and distributions of wall stress. EC morphology in these areas is different since polygonal cells are found next to elongated cells (25). Flow patterns, and particularly wall shear

stresses, are virtually impossible to characterize in these regions. In this study, we were able to induce different EC morphologies that resemble those found near bifurcations and branches by growing the cells on top of collagen gels under static conditions (Fig. 4). We can speculate that tension patterns due to the combination of the pressure pulse and complicated geometry might induce a particular EC morphology, in addition to any effects of flow. This hypothesis also agrees with observations that EC lying on the border of valve leaflets are aligned perpendicular to the direction of flow, but parallel to the fiber orientation in the subendothelial matrix (6). This also may suggest that, if fiber orientation is induced by a high tension force (in this case, due to the high strain in the valve leaflet), EC will stay oriented with the direction of the fibers, and shear stress will not be sufficiently strong to induce either fiber or cell realignment with the direction of flow. Moreover, the presence of several contradictory stress signals, one being at the basal side and the other at the luminal side, might induce a state of stress in the endothelium.

In summary, we have designed a co-culture system, with EC cultured as a monolayer on top of a three-dimensional matrix of collagen type I with embedded SMC. The morphology, F-actin organization, and growth of EC in such a system is very different from that of cells cultured on plastic, suggesting the important role of an organized, three-dimensional ECM in the differentiation of the endothelium *in vivo*. When exposed to shear stress, EC co-cultured with SMC aligned with the direction of flow, exhibiting only a slight, further elongation. This model succeeded in achieving a quiescent endothelium (*i.e.*, very low cell growth rates on the order of those observed *in vivo*) for periods of time as long as 1 month (at which point experiments were stopped). Such a co-culture model can help us understand the role of each element that is part of the composition and environment of a blood vessel (*e.g.*, SMC, ECM, shear stress, and wall stress) on the differentiation of EC *in vivo*, and it can be used for further studies.

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