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Hydraulic Conductivity of Smooth Muscle Cell-Initiated Arterial Cocultures

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Abstract

The purpose of the study was to examine the effects of arterial coculture conditions on the transport properties of several *in vitro* endothelial cell (EC) – smooth muscle cell (SMC) – porous filter constructs in which SMC were grown to confluence first and then EC were inoculated. This order of culturing simulates the environment of a blood vessel wall after endothelial layer damage due to stenting, vascular grafting or other vascular wall insult. For all coculture configurations examined, we observed that hydraulic conductivity (L_p) values were significantly higher than predicted by a resistances-in-series (RIS) model accounting for the L_p of EC and SMC measured separately. The greatest increases were observed when EC were plated directly on top of a confluent SMC layer without an intervening filter, presumably mediated by direct EC – SMC contacts that were observed under confocal microscopy. The results are the opposite of a previous study that showed L_p was significantly reduced compared to an RIS model when EC were grown to confluency first. The physiological, pathophysiological and tissue engineering implications of these results are discussed.

Keywords

Endothelial Cells; Smooth Muscle Cells; Porous Membrane; Coculture; Hydraulic Conductivity

Introduction

In a recent paper ^[1], we examined the effects of arterial co-culture conditions on the transport properties of *in vitro* endothelial cell (EC) – smooth muscle cell (SMC) constructs in which EC were cultured on top of a porous filter and SMC were plated either on the bottom of the filter or the bottom of a companion well. A unique feature of the study was that EC were grown to confluence first and then SMC were inoculated. It was observed that heterotypic interactions between EC and SMC affected the measured hydraulic conductivity (L_p) of the co-cultures and that the co-culture L_p were always lower than predicted by a resistances-in-series model accounting for the L_p of EC and SMC measured separately. The heterotypic interactions were mediated by soluble factors as no direct contacts between EC and SMC through the filter pores were observed.

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The aim of the present study was to examine the effects of heterotypic interactions between EC and SMC on Lp in co-cultures with SMC plated first in order to see if the order in which cells are cultured affects their transport properties. The motivation for this study comes from *in vivo* observations that the order in which EC and SMC are established in the vessel wall varies with physiological and pathophysiological conditions. In healthy arterial walls, the porous internal elastic lamina (IEL) physically separates the intimal EC and medial SMC^[2]. In this case, EC and SMC can establish heterotypic contact through soluble factors and by extending cell processes through the pores of the internal elastic lamina^[3–5]. In the event of intimal denudation, that occurs in vascular grafting and stenting, the close proximity between EC and SMC can be reestablished by re-endothelialization in the presence of preexisting medial SMC without the intervening IEL^[6]. There are cases where both cell types are intact in the arterial wall and come into direct contact. For example, during intimal hyperplasia or fibroatheroma formation an increasing number of medial SMC come into direct contact with EC as the IEL is degraded^[7].

Earlier work with coculture systems^[8] has clearly demonstrated how EC can influence SMC function. Coculturing bovine aortic endothelial cells (BAEC) and bovine aortic smooth muscle cells (BASMC) on opposing sides of a porous membrane (bilayer coculture), as well as through shared media alone (conditioned-media coculture), was shown to cause significant changes in SMC proliferation rates, density, transmembrane projections, and protein synthesis compared to SMC monocultures^[8].

In another coculture study^[9], human saphenous vein EC and SMC that were in direct contact, without an intervening porous membrane, were shown to cause significant changes in gene expressions in both EC and SMC compared to EC and SMC monocultures. The genes whose expression changed in direct cocultures were described as key angiogenic factors^[9].

Bilayer coculturing of EC and SMC with laminar fluid shear stress imposed on the EC surfaces also elicited changes in EC gene expression^[10]. Endothelial cell gene expression for adhesion molecules was inhibited by shear stress whereas static (no-flow) conditions induced EC gene expression for adhesion molecules. Incorporating a porous membrane between EC and SMC in both static and laminar fluid shear stressed conditions was essential to induce the changes in gene expression.

Smooth muscle cell proliferation can be reduced by omitting serum supplements from culture media and a temporary serum-free culture period of 2–5 days induces SMC to adopt a contractile (quiescent) phenotype^[11, 12]. Serum-dependent SMC proliferation can also be restored by reintroducing serum into culture media. The serum-dependent modulation of porcine arterial SMC phenotypes was shown to dramatically affect EC monolayer formation when EC were cultured directly above existing SMC^[13]. That study demonstrated that EC could not develop into a monolayer when paired with proliferative SMC. In contrast, EC monolayer formation was only achieved after switching the proliferative SMC phenotype to a quiescent SMC phenotype through a short period of serum starvation. In the present study, we have incorporated 3 days of serum starvation of SMC cultures prior to inoculating EC in order to suppress the proliferative SMC phenotype in co-cultures allowing for the

development of confluent EC monolayers. In our previous study, serum starvation of SMC was not required as confluent EC monolayers were grown to confluency first and retained their confluency during subsequent coculturing with SMC^[19]. The common aspect of both studies is that EC were cultured for a total of 5 days before measurements of L_p .

Varying both serum content and culture time in bilayer cocultures and monocultures were also shown to cause changes in human aortic SMC cytokine secretions^[14]. Cytokine secretions from cocultured SMCs were found to be significantly different from monocultured SMCs when supplied with higher fetal bovine serum concentrations and cultured for longer periods of time. Those findings illustrated that serum supplementation and time-dependent arterial coculturing methods stimulate heterotypic activities. In directly cocultured porcine arterial ECs and SMCs (without an intervening porous membrane), serum content, and culture time were also demonstrated to be factors regulating thrombogenicity^[15].

Hydraulic conductivity of BAECs grown on Transwell Permeable Supports and supplied with 10% fetal bovine serum in culture media has been measured extensively in previous studies^[16–21] conducted in our laboratory, and there has been a quantitative consistency in these measurements over the last 20 years^[22]. It is also well established that changes in hydraulic conductivity correlate with changes in permeability of endothelial layers to large solutes such as albumin and LDL^[23]. Thus hydraulic conductivity measurements are a consistent indicator of the integrity of the transport barrier of the endothelium. The present study determines L_p for several monoculture and coculture configurations of BAECs and BASMCs with the BASMCs plated first. Our results reveal that the proximity of cocultured BASMCs to BAECs on the porous membrane, along with serum content modulate arterial L_p . Arterial cocultures that mimic the intimal arrangement of ECs and SMCs after intimal damage (direct contact) feature an intact monolayer of ECs with a circular morphology pattern, and lead to elevated L_p values compared to EC monoculture controls. This suggests that although EC appear to be intact after recovery from intimal damage, their transport properties are likely to be impaired.

Materials and Methods

Materials

Transwell polyester permeable supports (0.4 μ m pore diameter) were purchased from Corning, Inc., NY. BAECs were purchased from VEC Technologies, Inc, NY and primary BASMCs from Cell Applications, Inc., CA. Fibronectin (FN) – 0.1% from bovine plasma; Triton X-100; Trypsin-EDTA; Penicillin Streptomycin (PS); 200mM L-Glutamine (LG); 30% Albumin solution from Bovine Serum (BSA); and Phenol Red Minimum Essential Medium (MEM) were purchased from Sigma-Aldrich, Inc., MO. Fetal Bovine Serum (FBS) and Paraformaldehyde (PFA) were purchased from Thermo Fischer Scientific, Inc. Phenol Red Free Minimum Essential Medium (PRF-MEM) and Calcium and Magnesium Free Phosphate Buffered Saline (CMF-PBS) were purchased from Mediatech, Inc., VA. VE-Cadherin Primary Antibody (PAb), and Anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor 488 Conjugate) Secondary Antibody (SAb) were purchased from Cell Signaling Technology Inc. MA.

Defined Cell Culture Media

Serum-free MEM (SF-MEM) consisted of MEM with 1% LG and 1% PS. Direct adaptation to SF-MEM was used for temporarily suspending the proliferation of BASMC cultures. 10% FBS MEM (10FB-MEM) consisted of MEM with 10% FBS, 1% LG, and 1% PS. 10FB-MEM was used for culturing cells in tissue culture flasks and on Transwell Permeable Supports. 2.5% FBS MEM (2.5FB-MEM) was made by combining 1 part 10FB-MEM with 3 parts SF-MEM. 2.5FB-MEM was also used for culturing cells on Transwell Permeable Supports. Experimental MEM (E-MEM) consisted of PRF-MEM with 1 % BSA, 1% LG, and 1% PS, and was used when measuring L_p .

Cell Culture in Flasks

Cryogenically frozen vials of either passage 2 BAEC cultures or passage 3 BASMC cultures were thawed for 2 minutes in a 37°C waterbath and the cell suspensions were transferred to separate sterile T-75 tissue culture flasks. 10FB-MEM was added to the flask (15 mL/T-75 flask) and the cultures were incubated until 80% confluent. Each culture was then passed into three new sterile tissue culture flasks and these subcultures were grown until 80% confluent. BAEC and BASMC cultures were each passed a total of three times and then inoculated on Transwell inserts or companion wells. Passage 5 BAEC and passage 6 BASMC subcultures were inoculated on Transwell inserts or companion wells in specific monoculture and coculture arrangements and supplied with 10FB-MEM, 2.5FB-MEM, or SF-MEM.

Cell Culture with Transwell Permeable Supports

Transwell insert membranes—Transwell inserts containing 10 μm thick polyester (PET) membranes with a total growth area of 1.12 cm^2 were used. While the PET membrane is about 10X thicker than normal IEL, the 0.4 μm diameter membrane pores and membrane pore density of 4.0e6 pores/ cm^2 fall within the normal ranges found in IEL^[24].

Coating with Fibronectin—BAEC and BASMC cultures were inoculated on membranes that were pre-coated with FN diluted to 30 $\mu\text{g}/\text{mL}$ with MEM. 224 μL of diluted FN was pipetted on either the apical or basal side of the Transwell insert membrane. Diluted FN was also placed on top of existing BASMC cultures, which were on the apical side of the membrane, prior to inoculating BAEC cultures directly above BASMC cultures. FN coated Transwell inserts in companion plates were incubated for 2 hours and then excess FN was removed. Coating with fibronectin is used to promote adhesion of EC or SMC to the Transwell filter surface. The fibronectin layer itself is very thin (nanometers) and not expected to contribute directly to hydraulic conductivity.

Cell culture media for Transwell Permeable Supports—The Transwell insert to companion well volume ratio was 1:3 with 0.5mL of culture media in the Transwell insert and 1.5mL in the companion well. To culture cells with either SF-MEM or 10FB-MEM, the defined media was placed in both compartments. To create 2.5FB-MEM culture media, SF-MEM was placed in the companion well and 10FB-MEM was placed in the Transwell insert.

BAEC and BASMC plating densities, locations, and culture times—BAEC and BASMC cultures were inoculated with a 1:1 plating density ratio of 1.25×10^5 cells/cm². BAECs in monoculture and coculture cases were always the most apical culture on the Transwell membrane. BASMCs in monoculture or coculture cases were inoculated either on the apical or basal side of the Transwell membrane or on the bottom surface of the companion well. The total BASMC culture time in all monocultures and cocultures was 11 days. The total BAEC culture time in all monoculture and coculture arrangements was 5 days.

Monoculture and Coculture Notations—The apical and basal locations of the Transwell membrane will be denoted as (a) and (b), respectively. The bottom surface of the companion well is denoted as (c). BAEC culture inoculums are abbreviated as (EC), and BASMC culture inoculums are abbreviated as (SMC). Monoculture formats are described by [Location (Inoculum)]. Coculture formats are described by [Location (First Inoculum); Location (Second Inoculum)]. Monoculture and coculture notations and formats are presented in Figure 1.

BAEC and BASMC monocultures and cocultures

EC and SMC Monocultures and Cocultures

[a(EC)] monocultures: A BAEC culture was inoculated on an apical FN coated Transwell membrane. The culture was supplied with either 10FB-MEM or 2.5FB-MEM and incubated for 5 days.

[a(SMC)] and [b(SMC)] monocultures: A BASMC culture was inoculated on either an apical or inverted basal FN coated Transwell membrane. Inverted Transwell membranes were incubated for 30 minutes while BASMCs settled and attached to the basal side of the membrane. Upright Transwell membranes with either apical or basal BASMC cultures were supplied with 10FB-MEM for 3 days. That culture media was then replaced with SF-MEM and the culture was incubated for 3 days. SF-MEM was then replaced with either 10FB-MEM or 2.5FB-MEM and the culture was incubated for 5 days.

[a(SMC);a(EC)] cocultures: A BASMC culture was inoculated on an apical FN coated Transwell membrane and supplied with 10FB-MEM for 3 days. BASMC culture media was replaced with SF-MEM and the culture was returned to the incubator for 3 days. SF-MEM was then removed and the apical BASMC culture was coated with FN. A BAEC culture was then inoculated above the apical FN coat. The coculture was supplied with either 10FB-MEM or 2.5FB-MEM and incubated for 5 days.

[b(SMC);a(EC)] cocultures: A BASMC culture was inoculated on an inverted basal FN coated Transwell membrane. The inverted membrane was placed in an incubator for 30 minutes while BASMCs settled and attached to the basal side of the membrane. The upright Transwell membrane with basal BASMC culture was then supplied with 10FB-MEM for 3 days. BASMC culture media was then replaced with SF-MEM and the culture was incubated for 3 days. SF-MEM was then removed and the apical side of the membrane was coated with

FN. A BAEC culture was then inoculated on the apical side of the membrane. The coculture was then supplied with either 10FB-MEM or 2.5FB-MEM and cultured for 5 days.

[c(SMC);a(EC)] cocultures: A BASMC culture was inoculated on the bottom surface of a companion well, separated by 1 mm from the porous membrane, and supplied with 10FB-MEM for 3 days. BASMC culture media was then replaced with SF-MEM and the culture was incubated for 3 days. A BAEC culture was then inoculated on the apical side of an FN coated Transwell membrane. BASMC culture media was then removed and the BAEC culture was paired with the BASMC culture in the companion well. The coculture was supplied with either 10FB-MEM or 2.5FB-MEM, respectively, and incubated for 5 days. These cocultures were dissociated on day 5 in order to measure endothelial L_p .

Measuring L_p of Transwell cultures—Following the prescribed monoculture and coculture times, the Transwell insert was transferred to a bubble tracking apparatus. E-MEM was added above and below the Transwell insert culture to eliminate the development of an osmotic pressure gradient. Fluid flux (J_v) across Transwell insert cultures in response to a transmembrane pressure differential of $P = 10 \text{ cmH}_2\text{O}$ was measured in pairs with a bubble tracking apparatus described previously [25], and those measurements were used to calculate L_p using equation (1).

$$L_p = J_v / \Delta P \quad (\text{Eqn. 1})$$

Resistances-in-Series Models of Coculture L_p — L_p measurements for BAEC and BASMC monocultures were combined in a resistances-in-series (RIS) model and then compared with coculture measurements. Differences between coculture measurements and RIS predictions are attributed to heterotypic interactions between EC and SMC. The resistance values for individual components are given by equations 2–4 and the RIS predictions by equations 5 and 6. Since a PET membrane was included as a substrate in each monoculture, the hydraulic resistance of the membrane ($R_{[PET]}$) was duplicated in summations of monoculture resistances. Therefore, a constant value for the hydraulic resistance of one membrane, $R_{[PET]} = 1.196\text{e}4 \text{ cmH}_2\text{O}/\text{cm/s}$ ($L_{p[PET]} = 8.361\text{e}-5 \text{ cm/s/cmH}_2\text{O}$), was subtracted from each RIS model (equations 5,6).

$$R_{[a(EC)]} = 1 / L_{p[a(EC)]} \quad (\text{Eqn. 2})$$

$$R_{[a(SMC)]} = 1 / L_{p[a(SMC)]} \quad (\text{Eqn. 3})$$

$$R_{[b(SMC)]} = 1 / L_{p[b(SMC)]} \quad (\text{Eqn. 4})$$

$$L_{p[a(EC)]+[a(SMC)]-[PET]} = 1 / (R_{[a(EC)]} + R_{[a(SMC)]} - 1R_{[PET]}) \quad (\text{Eqn. 5})$$

$$L_{p[a(EC)]+[b(SMC)]-[PET]} = 1 / (R_{[a(EC)]} + R_{[b(SMC)]} - 1R_{[PET]}) \quad (\text{Eqn. 6})$$

Immunofluorescence

Immunofluorescence solutions—Paraformaldehyde fixative was diluted with CMF-PBS to 1% PFA and filtered through a 0.45 µm syringe filter. Fixative was freshly made on the day of use. Triton X-100 was diluted with CMF-PBS to a 0.2% Triton X-100 permeabilizing solution. Blocking buffer consisted of BSA and Triton X-100 diluted in CMF-PBS to 10% BSA and 0.1% Triton X-100. Primary antibody (PAb) was diluted 15:1000 in blocking buffer. Secondary antibody (SAb) was diluted 2:1000 in blocking buffer.

Immunofluorescence of VE-cadherin in Transwell cultures—All rinses with CMF-PBS were immediately removed after addition. All Transwell filters remained in the same companion well throughout the immunofluorescence procedure. All solutions were added to the insert first and then, when required, to the companion well. All solutions were carefully vacuum aspirated from the companion well first and then from the insert without touching the membrane. Rinses with CMF-PBS were 0.5 mL/insert and 1 mL/companion well. All steps were carried out in room temperature (RT) conditions in a laminar flow hood.

Remaining cell culture media was aspirated; the culture was quickly rinsed once and immediately, 0.5 mL of fixative was added to the apical side of the insert. The culture was then fixed for 10 minutes. After fixative was removed the culture was rinsed once and 0.5 mL of permeabilizing solution was added to the apical side of the insert. The culture was permeabilized for 10 minutes; the permeabilizing solution was removed and the culture was rinsed once before 0.5 mL of blocking buffer was added to the apical side of the insert. The culture was blocked for 60 minutes; after blocking buffer was the culture was rinsed once and 200 µL of diluted PAb was added to the apical side of the insert. The culture was incubated at RT with PAb for 3 hours; after PAb was removed the culture was rinsed five times before the remaining steps that were carried out in a dark environment.

200 µL of diluted SAb was added to the apical side of the insert; the culture was incubated at RT with SAb for 60 minutes, removed, and the culture was rinsed four times. 0.5 mL of rinse solution was added to the apical side of the insert and the insert was transferred to a clean glass slide set on a Nikon TE 2000 microscope stage equipped with epi-fluorescence microscopy and MetaVue Imaging Software (Universal Imaging Corp. PA). The culture was imaged in the center field and then in four peripheral fields with a 10x objective. Five more similar fields were imaged with a 20x objective.

Cell Tracker Green Staining and Confocal Imaging—Co-cultured BAECs and SMCs were briefly washed with PRF serum-free medium. The Transwell inserts were then inverted and the SMCs were incubated in PRF serum free media containing 5µM of Celltracker green for 15 min. The excess media was then aspirated before incubating the BAECs for 15 minutes with cell tracker green. The co-cultured cells were quickly washed with PBS before incubation in growth media containing 10 % FBS, 1% penicillin and streptomycin for 30 min at 37°C. After incubation, the inserts were flushed for 15 minutes (3×, 5 min each) by adding 1ml and .5ml of PBS to the apical and basal side of the inserts respectively to remove any free dye from the filter pores. They were then fixed in 3.7% fixative for 10 minutes. The cultures were washed twice, and mounted on glass coverslips

for imaging. Confocal z-stacks of the cell tracker green stained co-cultures were obtained on a LSM 510 confocal laser scanning system, using the Plan-Neofluar 40×/1.3 Oil DIC objective and analyzed using the Zeiss LSM software.

Morphometric Analysis

BAEC shape factors—The shape factors of BAECs in VE-cadherin immunostained monocultures and cocultures were calculated using the instructions that were provided in the MetaVue software. Briefly, a trace region tool was used to outline individual BAECs. MetaVue's Region Statistics of the outline generated calibrated pixel areas and perimeters for each cell. The shape factors for BAECs were then calculated with the formula $(4\pi \cdot \text{area})/(\text{perimeter})^2$, where values ranged between zero and one; a value of one is a perfect circle and a value near zero is a flattened or elongated object.

Statistical Analysis

Hydraulic conductivity measurements and shape factors are presented as the mean $\pm$ the standard error of the mean (SEM). Each coculture experiment was normalized against its paired EC monoculture experiment to account for the variations of L_p from plate to plate of the cultures. When comparing normalized coculture experiments to controls (EC monocultures) a one sample t test on the null hypothesis was performed, i.e. we tested whether the averaged normalized values were different from 1. This approach has been used in other studies [26, 27]. A two sample t test was performed when comparisons were made between different coculture formats. Comparisons were considered statistically significant if $p < 0.05$. Where multiple comparisons were made the Bonferroni correction was used.

Results

Monoculture L_p

The L_p measurements of BAEC monocultures supplied with 10FB-MEM were paired with L_p measurements of BAEC monocultures supplied with 2.5FB-MEM and are presented in Figure 2a. The 2.5FB-MEM monocultures had a significantly lower L_p than the 10FB-MEM monocultures. The L_p measurements of BASMC monocultures supplied with 10FB-MEM were paired with the L_p measurements of BASMC monocultures supplied with 2.5FB-MEM and are presented in Figure 2b for both [a(SMC)] and [b(SMC)] configurations. The apical cultures of BASMCs [a(SMC)] in 2.5 FB-MEM had a significantly higher L_p than cultures in 10FB-FB-MEM. Differences in serum content did not significantly alter L_p when BASMCs were cultured on the basal side of the insert [b(SMC)]. L_p values were lower in the [a(SMC)] configuration than the [b(SMC)] configuration.

Coculture L_p

The L_p of each coculture was normalized to the L_p of its paired BAEC monoculture. The normalized L_p values of each coculture configuration are presented in Figure 3 for both 10FB-MEM and 2.5FB-MEM. L_p values for [a(SMC);a(EC)] and [c(SMC);a(EC)] were significantly higher than [a(EC)] at both serum concentrations. Figure 3 also shows that L_p of [b(SMC);a(EC)] was significantly lower than L_p of [a(SMC);a(EC)] in both serum

concentrations, and that L_p of [c(SMC); a(EC)] was also lower than L_p of [a(SMC);a(EC)] in 2.5FB-MEM.

Resistances-in-series models of coculture L_p

Resistances-in-series model predictions of L_p (Eqns. 5 and 6) for each coculture configuration were normalized to paired endothelial monoculture L_p and are displayed next to the experimental values of L_p in Figure 4 both for both serum concentrations. Differences between the model predictions and the experimental measurements indicate the presence of heterotypic interactions in the coculture experiments. At 10FB-MEM, [a(EC)] + [b(SMC)] – 1[PET], and [a(EC)] + [c(SMC)] – 1[PET] RIS L_p values were significantly lower than the actual [b(SMC);a(EC)], and [c(SMC);a(EC)] L_p measurements, respectively. At 2.5FB-MEM, the [a(EC)] + [a(SMC)] – 1[PET] RIS L_p values were significantly lower than the actual [a(SMC);a(EC)] L_p .

Immunostaining of VE-cadherin

BAEC VE-cadherin was immunostained for each monoculture and coculture. Center field 10x objective images for each monoculture and coculture are presented in Figure 5. VE-cadherin was well expressed in each culture configuration and was localized at the cell border as expected. VE-cadherin staining was used as an indicator of EC confluency and morphology. Endothelial cells imaged in coculture [a(SMC);a(EC)] with 10FB-MEM appeared more circular, and EC imaged in [c(SMC);a(EC)] with 2.5FB-MEM appeared more elongated when compared to any of the other cultures.

EC morphometry

The sample mean shape factor for random samples of 10 BAEC cells from each culture configuration are presented in Figure 5B. The mean shape factor for EC in the [a(SMC);a(EC)] coculture in 10FB-MEM was closest to one (most circular), and the mean shape factor for EC in the [c(SMC);a(EC)] coculture in 2.5FB-MEM was closest to zero (most elongated), when compared to any other culture condition. In addition, the shape factors for both of those cocultures were significantly different in comparison to EC alone in 10FB-MEM.

Fluorescent Images of SMC and EC interaction

Fluorescent staining of coculture configuration [b(SMC);a(EC)] is shown in Figure 6. Figure 6A–B shows a representative images of cell tracker green localization in BAECs, [a(EC)] on the apical side, and BASMC, [b(SMC)] on the basal side of the side of the PET membrane (blue), respectively. The cross sectional view of each culture format in the Z-plane of the confocal stack is demonstrated in Figure 6C. The EC and SMC layers are clearly labeled with cell tracker green. The intervening filter that fluoresces in blue shows no sign of connecting processes that would be stained in green. In our previous paper ([1], Fig. 9), we showed a positive control in which a filter was incubated in cell tracker green and then imaged. It showed clear evidence of green staining in the filter pores. Figure 7 displays the same type of staining for coculture configuration [a(SMC);a(EC)]. Here it is clear that there is intimate contact between the EC and SMC.

Discussion

Serum concentration modulates monoculture L_p

As we showed in our previous study ^[1], EC L_p was significantly lower in 2.5FB-MEM than in 10FB-MEM (Figure 2a). The mechanisms underlying this phenomenon including reduced EC motility and turnover and weakened intercellular junctions in high serum have been discussed in detail previously ^[1].

SMC monocultures were inoculated on either the apical or basal side of porous membranes to identify the influence that membrane location may have on SMC L_p . During apical and basal SMC culture periods, proliferation was temporarily suspended through direct adaptation to SF-MEM and then supplied with either 10FB-MEM or 2.5FB-MEM to regulate SMC proliferation rates. The L_p of [a(SMC)] in 2.5FB-MEM was significantly higher than [a(SMC)] in 10FB-MEM (Figure 2b) suggesting that the proliferation rate of apical SMC cultures affects their L_p response. SMC Proliferation rates are known to be higher in high serum, contributing more cell mass to reduce L_p ^[8, 14]. However, L_p of [b(SMC)] cultures supplied with either 10FB-MEM or 2.5FB-MEM were not different from each other, and were higher in magnitude than the L_p of [a(SMC)] cultures (Fig. 2b).

The transition to much higher L_p values for the b(SMC) configuration compared to the a(SMC) configuration (Fig. 2b) may be explained by several possible mechanisms. A previous mathematical model^[24] of fluid flowing through the pores of an internal elastic lamina (like the supporting filter) demonstrated that the laminar profile of fluid flow in the pore becomes complex at the exit site of individual pores draining into a layer of SMC. This may contribute to loosen cell connections to the membrane and lower resistance. It is also very likely that gravitational settling prevents SMC from accumulating in the b(SMC) case and this may neutralize the increased proliferation rates expected in 10FB-MEM.

Cocultures exhibit elevated L_p

In healthy arteries, the intimal and medial regions, which consist of an endothelial monolayer and dense multilayered SMC, respectively, share the same porous internal elastic lamina^[2]. Our coculture [b(SMC);a(EC)] best mimics this organization. During events that cause endothelial denudation such as vascular grafting, balloon angioplasty and stenting, endothelial cells must re-grow over an existing vascular smooth muscle layer. Coculture configuration [a(SMC);a(EC)] best models that intimate contact of EC and SMC. Coculture [c(SMC);a(EC)] is not physiologic but serves as a control emphasizing possible heterotypic interactions through soluble factors, while eliminating the possibility of direct contact between EC and SMC.

The L_p of each coculture was significantly higher than the L_p of the monoculture control (Fig. 3). This is in distinct contrast to observations reported for cocultures of the same cells and the same membranes when the EC were plated first ^[1]. In those cases, [a(EC);b(SMC)] and [a(EC);c(SMC)], coculture L_p were significantly lower than monoculture control L_p .

Comparison to resistances-in-series models

Resistances-in-series model predictions of L_p (Fig. 4) were always lower than L_p for a(EC) monocultures (normalized values less than 1) because of the added resistance of the SMC layer and they were much lower than the measured L_p for the corresponding coculture. For the case of [c(SMC);a(EC)], the RIS model prediction corresponds simply to a(EC) and the comparison to the coculture data is given in Fig. 3. In all cases except [b(SMC);a(EC)] for 2.5FB-MEM, the RIS model prediction is significantly lower than the coculture data by factors greater than 4 in the cases of [a(SMC);a(EC)] and [c(SMC);a(EC)]. Since the [a(SMC);a(EC)] coculture configuration most closely represents the intimal organization of EC and SMC after endothelial damage, intimal hyperplasia and fibroatheroma, it is tempting to conclude that this heterotypic configuration diminishes the transport barrier for blood vessels, tending to sustain disease states.

Coculture arrangements regulate patterns of BAEC morphology

It is clear in Figure 5 that coculturing of EC and SMC led to rather distinct EC morphologies that are indicative of heterotypic interactions between the cell types. The more rounded morphology of the [a(SMC);a(EC)] configuration was associated with the highest heterotypic interactions at both serum concentrations as quantified in Figure 4. The direct contact between EC and SMC in this arrangement (Fig. 7) would appear to be the vehicle for this enhanced interaction. The heterotypic interactions in the [b(SMC);a(EC)] and [c(SMC);a(EC)] configurations were mediated by soluble factors only.

To properly investigate the soluble factors involved would require the use of proteomics tools that were beyond the scope of the present study. However, several candidate soluble factors have been suggested by previous studies. It was demonstrated that directly coculturing ECs on top of preexisting SMCs led to different VEGF, PDGF-BB, TGF- β and bFGF secretions from both ECs and SMCs^[9]. Another coculture study^[14] of ECs and SMCs reported changes in endothelial secretions of IL-1 and MCP-1. Many of these growth factors and cytokines are known to increase the permeability and hydraulic conductivity of endothelial layers and could play a role in the elevation of hydraulic conductivity that we have observed.

Study Limitations

The limitations of the coculture methods that we have employed were discussed at length in our previous study^[11] and will not be repeated in detail here. An additional limitation of the present study was that 3 days of serum starvation of SMC were required before coculturing the EC in order to allow the EC to develop a confluent monolayer. In both studies, the EC were cultured for a total of 5 days and the last 3 days were in the serum-containing media. Since the EC present the controlling resistance to water flux, any differences in the resistance of the SMC layer due to different growth times should not be significant. The other limitation of both studies is that all of the transport experiments were conducted under static conditions without physiological shear stress applied to the EC surface. Most in vitro transport studies have been conducted under static conditions, but two recent studies of EC monoculture transport under shear stress conditions have shown that after an initial rise in permeability in response to shear stress, permeability values return to static baseline levels

within 12 h of sustained shear [28, 29]. Bilayer coculturing of EC and SMC with shear stress imposed on the EC surface did elicit changes in EC gene expression for adhesion molecules^[10]. It is not known, however, if genes for intercellular junction proteins that control permeability would be affected.

Concluding Remarks

The most important general observation of this study is that when SMC are plated first and then EC are grown to confluency, the L_p of the coculture is elevated relative to the EC monoculture control. In our previous study ^[1], we observed that when EC were grown to confluency first and then SMC were cocultured, L_p was always reduced relative to the EC monoculture control. The physiological relevance of these results is that they model both pathophysiological and normal physiological processes. Therefore we might expect that when damaged endothelial layers re-grow over an existing smooth muscle layer as in vascular grafting, balloon angioplasty or stenting, the L_p of the healed layer will be elevated. Conversely, during arteriogenesis, a heterotypic tissue structure develops as SMCs are recruited to form a dense sub-endothelial medial region near a preexisting intact endothelium ^[30–33]. In this normal physiological process we would expect the L_p of the artery to be normal.

The implication of our two studies for tissue engineering of a vascular graft incorporating EC and SMC would simply be that EC should be grown to confluency first followed by plating of SMC. This should result in a construct with the lowest hydraulic conductivity / permeability characteristics which in general are desirable. Coculturing of SMC or stem cells with EC are current methods being explored for tissue engineering of vascular grafts ^[34, 35]. Our studies suggest that EC should be grown to confluency first followed by plating of SMC or stem cells in order to optimize the mass transport characteristics of the resulting constructs. Ye et al. (2015)^[36] developed a co-cultured graft in which SMC were plated before EC. Our study suggests that a more successful approach would be to plate the EC first. Delayed addition of stem cells in coculture with EC resulted in improved vascularization^[35], providing indirect support for the conclusions of the present study.

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Figure 1. Schematic of BAEC and BASMC monoculture and coculture formats on Transwell membranes and companion wells. The horizontal dashed line indicates the location of the porous Transwell membrane. The round cells represent EC and the elongated cells represent SMC. Monoculture formats include [a(EC)], [a(SMC)], and [b(SMC)]. Coculture formats include [a(SMC);a(EC)], [b(SMC);a(EC)], and [c(SMC);a(EC)].

EC Monoculture L_p **a.**

b.

Figure 2.

Hydraulic conductivity of monocultures. (a) 5 day apical EC ([a(EC)]) monocultures supplied with 10FB-MEM and 2.5FB-MEM. (b) 11 day apical SMC ([a(SMC)]) and SMC ([b(SMC)]) monocultures supplied with 10FB-MEM and 2.5FB-MEM, respectively. * $p < 0.05$ compared to 10FB-MEM; $n = 6$.

Figure 3.

Hydraulic conductivity of cocultures. [a(SMC);a(EC)], [b(SMC);a(EC)], and [c(SMC);a(EC)] normalized to the mean L_p of paired endothelial monocultures [a(EC)], as indicated by the dashed line (1.0). Each culture format was supplied with 10FB-MEM and 2.5FB-MEM, respectively. Statistical significance ($p < 0.05$) of coculture L_p normalized to paired endothelial L_p were denoted by a * symbol (* vs [a(EC)]). A Δ symbol denotes statistically significant normalized L_p values of each culture that were statistically significant compared to the normalized L_p of [a(SMC);a(EC)] coculture (Δ vs [a(SMC);a(EC)]). All experiments were $n = 6$.

Figure 4.

Hydraulic conductivities of membrane cocultures [a(SMC);a(EC)], [b(SMC);a(EC)], and resistances-in-series model predictions of membrane cocultures [a(EC)] + [a(SMC)], [a(EC)] + [b(SMC)] normalized to the mean L_p of paired endothelial monocultures [a(EC)], as indicated by the dashed line (1.0). Each culture was supplied with 10FB-MEM or 2.5FB-MEM, respectively. Statistical significance of resistances-in-series L_p compared to related membrane coculture L_p was denoted by □ and ◇ symbols (□ vs [b(SMC);a(EC)]; and ◇ vs [a(SMC);a(EC)]). All experiments were n = 6.

10FB-MEM cultures	2.5FB-MEM cultures
[a(EC)]	[a(EC)]
[a(SMC);a(EC)]	[a(SMC);a(EC)]
[b(SMC);a(EC)]	[b(SMC);a(EC)]
[c(SMC);a(EC)]	[c(SMC);a(EC)]

A.

B.

Figure 5.

(A) Representative images of VE-cadherin immunostaining of EC monocultures and cocultures supplied with either 10FB-MEM or 2.5FB-MEM. x10 objective. Note that all monolayers are highly confluent. The direct coculture case [a(SMC); a(EC)] is characterized by a highly rounded morphology as quantified in Table 1. Scale bar 120 μ m. (B) The sample mean shape factor for random samples of 10 BAEC cells from each culture configuration compared to EC alone format in 10FB-MEM.

Figure 6. Confocal images of cell tracker green localization in BAEC and BASMC cocultures (green) grown in 10FB-MEM on PET membrane (blue). En face view of the (A) BAECs and (B) BASMCs. (C) (*top*) is a cross sectional view in the Z-plane of each panel; co-culture of BAECs on the apical **side** and BASMCs on the basal side (green), demonstrating no connecting processes across the PET membrane (blue). Scale bar=20μm.

Figure 7. Confocal images of cell tracker green localization in BAEC and BASMC direct cocultures (green) grown in 10FB-MEM on PET membrane (blue). En face view of the (A) BAECs and (B) BASMCs. (C) (*top*) is a cross sectional view in the Z-plane of each panel; showing intimate contact between the direct co-culture of BAECs and BASMCs on the apical side (green) of the PET membrane (blue). Scale bar=20μm.