Smooth Muscle Cells Orchestrate the Endothelial Cell Response to Flow and Injury

Mercedes Balcells, PhD; Jordi Martorell, MS; Carla Olivé, MS; Marina Santacana, MS; Vipul Chitalia, MD, PhD; Angelo A. Cardoso, MD, PhD; Elazer R. Edelman, MD, PhD

Background—Local modulation of vascular mammalian target of rapamycin (mTOR) signaling reduces smooth muscle cell (SMC) proliferation after endovascular interventions but may be associated with endothelial cell (EC) toxicity. The trilaminar vascular architecture juxtaposes ECs and SMCs to enable complex paracrine coregulation but shields SMCs from flow. We hypothesized that flow differentially affects mTOR signaling in ECs and SMCs and that SMCs regulate mTOR in ECs.

Methods and Results—SMCs and/or ECs were exposed to coronary artery flow in a perfusion bioreactor. We demonstrated by flow cytometry, immunofluorescence, and immunoblotting that EC expression of phospho-S6 ribosomal protein (p-S6RP), a downstream target of mTOR, was doubled by flow. Conversely, S6RP in SMCs was growth factor but not flow responsive, and SMCs eliminated the flow sensitivity of ECs. Temsirolimus, a sirolimus analog, eliminated the effect of growth factor on SMCs and of flow on ECs, reducing p-S6RP below basal levels and inhibiting endothelial recovery. EC p-S6RP expression in stented porcine arteries confirmed our in vitro findings: Phosphorylation was greatest in ECs farthest from intact SMCs in metal stented arteries and altogether absent after sirolimus stent elution.

Conclusions—The mTOR pathway is activated in ECs in response to luminal flow. SMCs inhibit this flow-induced stimulation of endothelial mTOR pathway. Thus, we now define a novel external stimulus regulating phosphorylation of S6RP and another level of EC-SMC crosstalk. These interactions may explain the impact of local antiproliferative delivery that targets SMC proliferation and suggest that future stents integrate design influences on flow and drug effects on their molecular targets. (Circulation. 2010;121:2192-2199.)

Key Words: blood flow † endothelium † mammalian target of rapamycin (mTOR) † molecular biology † muscle, smooth † signal transduction † stents

Local delivery of antiproliferative drugs limits the intimal hyperplastic response to vascular intervention but may place vessels at risk of thrombosis even late after initial treatment. The intervention, agents used, and means of administration can synergistically induce endothelial dysfunction and delay recovery,1 leading to impaired vasoreactivity, enhanced platelet aggregation,2 and elevated tissue factor expression.3–5 Sirolimus, for example, inhibits smooth muscle cell (SMC) proliferation and intimal hyperplasia after vascular manipulation presumably through effects on signaling within the mammalian target of rapamycin (mTOR) pathway. mTOR is central to the regulation of protein synthesis, ribosomal protein translation, and cap-dependent translation,6 and its inhibition with sirolimus alters the balance of mTORC1-mTORC2 complexes.7 Prolonged exposure to sirolimus partially inhibits Akt activation and SMC proliferation.8 However, sirolimus also induces tissue factor expression3,4 and dysfunction in endothelial cells (ECs). The impact of flow and drug release on tissue drug distribution and vascular repair has been defined computationally9–12 and in vivo,13–15 but the specific flow effects on individual vascular cells have not been fully defined. ECs are especially flow sensitive,16 and altered hemodynamics may disrupt endothelial health. When ECs become dysfunctional, vascular homeostasis is disrupted, and disease becomes manifest; each element of vascular repair goes awry.17

Clinical Perspective on p 2199

We used vessel-like constructs to examine mTOR pathway signaling in isolated ECs, isolated SMCs, and ECs cultured over SMCs. A model system with sequentially layered SMC/EC vessel-like constructs connected to a perfusion bioreactor allowed fine control of hemodynamic parameters.
recapitulating physiological flow. Importantly, these constructs enabled biologically relevant studies of vascular intervention, including bolus drug administration, balloon deployment, and stent implantation. Our data demonstrate a profound difference in EC biology in the presence and absence of SMCs and suggest a far more sophisticated regulatory interaction between alterations in hemodynamics from above and vessel wall from below than classically appreciated.

**Methods**

**Vascular Cell Culture and Bioengineered Vessel-Like Construct**

Human coronary artery ECs (Promocell, Heidelberg, Germany) were cultured in EB-2 basal medium (Lonza, Basel, Switzerland) supplemented with 5% FBS, 1% penicillin-streptomycin, 0.04% hydrocortisone, 0.4% human fibroblast growth factor 2, 0.1% vascular endothelial growth factor, 0.1% R3-insulin-like growth factor-1, 0.1% ascorbic acid, 0.1% human endothelial growth factor, and 0.1% gentamicin-amphotericin 1000 (EC complete medium). Human aortic SMCs (Cambrex, East Rutherford, NJ) were cultured with SmBM-2 basal medium (Lonza) supplemented with 5% CS, 1% glutamine, and 1% penicillin-streptomycin (SMC complete medium). Cells, passages 4 to 6, were fed every 48 hours and incubated at 37°C.

Before cell seeding, 3.18-mm-ID Silastic tubes (Dow Corning, Midland, Mich.) were washed in 0.2% SDS for 20 minutes, rinsed twice with distilled water for 20 minutes, and steam sterilized. Tube length was 12 cm except for experiments with stents, which used 4-cm-long tubes to reduce background signal from undisturbed cells in unstented parts of the tube. Tubes were coated with 100 μg/mL fibronectin (Sigma, St Louis, Mo) in PBS for 2 hours while rotating at 10 rph at 37°C (Figure 1A) and rinsed to remove loosely adsorbed fibronectin. Homotypic constructs were fabricated with injection of ECs or SMCs into fibronectin-coated tubes (8 × 10^5 cells/mL) and cultured for 24 hours at 37°C under axial rotation. Sequential layering of SMCs, followed by application of ECs, produced SMC/EC vessel-like constructs. SMCs were seeded on fibronectin-coated tubes (8 × 10^5 cells/mL), forming an SMC multilayer (Figure 1B and 1E). After 24 hours of adhesion under axial rotation, constructs were filled with an EC suspension (8 × 10^5 cells/mL) and incubated for 24 hours. Cells within constructs were characterized for constitutive and inducible markers and biosecretory function (Table 1 in the online-only Data Supplement). Appropriate cell assembly within constructs was verified by confocal microscopy, and cell coverage was quantified with MATLAB-based image analysis programs (Video I in the online-only Data Supplement).

The SMC/EC vessel-like constructs were connected to 60-cm-long loops of Silastic tubing containing fresh EC complete medium and rested for 24 hours to ensure complete “endothelialization” (Figure 1C and 1F). Supplemented medium was replaced by EB-2 medium to starve the cells overnight before perfusion. Cell-seeded constructs were placed in a perfusion bioreactor and exposed to controlled coronary artery–like flow: 1-Hz pulsatile flow of 17 dynes/cm² average shear stress. In all experiments, samples without flow served as controls. In experiments with growth factors, EC and SMC complete media were used for EC and SMC homotypic constructs, respectively, whereas SMC/EC vessel-like constructs were perfused with EC complete medium.
Injury Model
EC-only and SMC/EC vessel-like constructs were stented with 7-cell stainless steel NIR stents (3.5×16 mm, Medinol, Tel-Aviv, Israel). To discriminate direct drug-mediated effects from flow-mediated modulation of drug treatment, 1 hour before stent implantation, 10 nmol/L temsirolimus (Wyeth, Andover, Mass) was added to the starvation media. This dose abrogates mTOR signaling in ECs.20 Dimethyl sulfoxide at the doses used has no effect on mTOR activation status.20

Measurement of Phosphorylated S6 Ribosomal Protein Expression by Flow Cytometry
We used our perfusion bioreactor and the vessel-like constructs to examine mTOR signaling in cells exposed to coronary-like flow. mTOR activation status was assessed by measurement of phosphorylation levels of S6 ribosomal protein (S6RP), a downstream substrate of TORC1. Phospho-S6RP (p-S6RP) is a more reliable index in our experiments because total mTOR may be conserved even in the face of shunting to the TORC1 or TORC2 protein complexes. ECs in SMC/EC cocultures were identified with PE-conjugated anti-CD31 antibody (BD PharMingen, San Diego, Calif). Double staining of ECs with this antibody and FITC-conjugated anti–p-S6RP allowed cell and signal colocalization. Levels of p-S6RP were measured at “basal” conditions and after flow exposure, stent placement, and incubation with temsirolimus. Cells were recovered from constructs by 3 minutes of trypsin treatment, washed, and resuspended in fixation/permeabilization buffer solution (BD Biosciences, San Jose, Calif). The gently aspirated supernatant was labeled as digitonin extract.21,22

Radioimmunoprecipitation Assay and Digitonin Extraction
Non-specific cell lysis was performed by rinsing cell-coated constructs twice with ice-cold PBS and then incubating them for 30 minutes on ice in radioimmunoprecipitation assay buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mmol/L EDTA. The supernatant was obtained after centrifugation for 30 minutes at 4°C. Specific cytosolic extraction of surface cells on the constructs was achieved with digitonin. Cells washed with ice-cold PBS were covered with buffer containing 120 mmol/L KCl, 5 mmol/L KH₂PO₄, 10 mmol/L HEPES, pH 7.4, 2 mmol/L EGTA, and 0.15 mg/ml digitonin (Sigma) and rocked gently on ice for 30 minutes. The gently aspirated supernatant was labeled as digitonin extract.21,22

Western Blot Analysis
We used 10% acrylamide gels (Invitrogen, Carlsbad, Calif) for protein separation. Gels were blotted with Invitrogen gel transfer stacks and blotting system. Membranes were blocked with 5% powdered milk and incubated overnight with primary monoclonal anti–p-S6RP (Cell Signaling; 1:1000 dilution) and anti–β-actin (Santa Cruz Biotechnology, Santa Cruz, Calif; 1:15000 dilution) at 4°C while shaking. After 2 washes with PBS-T (PBS, 0.05% Tween20), membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology; 1:3500 dilution) for 2 hours while shaking at room temperature. After two 10-minute washes in PBS-T, Supersignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology Inc, Rockford, Ill) was applied, and luminescence was detected in an Alpha Innotech FluorChem. Densitometry plots were analyzed with ImageJ.

Microscopic Examinations
Endothelial monolayer integrity within constructs was assessed with fluorescence (Perkin-Elmer spinning disk confocal system coupled to a Zeiss Axiovert 200M microscope) and scanning electron microscopy (Figure 1). Constructs, which were embedded in a protective outer tubular layer before removal from the loops to minimize stress and artifactual effects on the cellular lining, were rinsed with PBS and fixed with 4% paraformaldehyde (Mallinkrodt, Hazelwood, Mo) for 20 minutes at room temperature. After 2 consecutive washes with PBS for 10 minutes and 1 hour blotting with 5% goat serum in PBS-BSA (PBS, 1% BSA), ECs and SMCs were labeled for 1 hour with rabbit monoclonal anti-CD31 (Abcam, Cambridge, Mass) and mouse anti–tissue factor (American Diagnos-
Yucatan pigs were implanted with 6-cell, 3×13-mm bare metal (Neo Stent, Co/Cr alloy) or sirolimus-eluting (Neo Stents coated with a 

| Basal mTOR/TORC1 Signaling Is Modulated by Flow in ECs But Not in SMCs |
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| Although ECs and SMCs rendered quiescent by growth factor deprivation exhibited low levels of p-S6RP, they also showed differential responses to flow and growth factor. EC expression of p-S6RP nearly doubled within 20 minutes of exposure to coronary-like flow (1 Hz pulsatile, 17 dynes/cm²) (1.97±0.2-fold increase; P<0.5; Figure 2A). A 1.96±0.4-fold increase in p-S6RP upregulation was confirmed by immunoblotting with Western blotting after whole-cell lysis and with digitonin extraction (Figure 3C and Figure IIB in the online-only Data Supplement). Confocal imaging of adherent ECs revealed 1.91±0.2-fold upregulation of S6RP phosphorylation under flow (Figure 3E and Figure I in the online-only Data Supplement). Growth factor exposure had no significant effect on S6RP phosphorylation in ECs (Figure 2A). In contrast, SMCs were unaffected by flow but exhibited an almost 5-fold increase in p-S6RP expression on growth factor exposure (P<0.05 versus static control; Figure 2B). Thus, mTOR/TORC1 signaling in ECs is most sensitive to alter-

<p>| Results |
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Figure 3. Temsirolimus effectively blocks the mTOR pathway. The flow and temsirolimus response of isolated ECs and SMCs obtained by fluorescence-activated cell sorting (A and B) were confirmed by protein expression by Western blots (C and D) and under confocal immunofluorescence (E and F). n=12 (3 experiments, 4 independent observations of each condition assayed).
ations in shear stress, whereas the local biochemical milieu dominates in SMCs. Homotypic studies were augmented by SMC/EC vessel-like constructs. Interestingly, the presence of SMC reduced flow-induced S6RP phosphorylation in ECs (mean intensity fluorescence flow/static, 1.16±0.04- versus 1.97±0.2-fold in EC-only vessel-like constructs; *P*<0.05; Figure 2D and Figure IIC in the online-only Data Supplement), and ECs eliminated the growth factor responsiveness of SMC p-S6RP expression (Figure 2E).

Differential Effect of Flow and Static Regimens on p-S6RP Status in ECs After Stent-Induced Injury

Under static conditions, p-S6RP expression in ECs lining conduits rose with stent injury (1.32±0.1-fold) but not when SMCs were also present (0.90±0.2-fold; *P*<0.05). Interestingly, the expected flow-mediated increase in p-S6RP expression seen in quiescent, monolayered ECs was lost in ECs injured by stent placement (0.91±0.1-fold; *P*<0.05 versus static) and reduced when ECs were in SMC/EC vessel-like constructs (0.77±0.1-fold; *P*<0.05 versus static).

mTOR Blockade by Temsirolimus Eliminates In Vitro Flow and Growth Factor–Induced mTOR/TORC1 Activation and Delays Poststenting Re-Endothelization

Sirolimus/FKBP12 complexes interact specifically with mTOR/TORC1, blocking downstream targets, S6K1 and its substrate S6RP. Temsirolimus reduced flow- and growth factor–induced p-S6RP levels in ECs and SMCs (Figure 3). mTOR blockade markedly affected re-endothelialization after stenting. The number of CD31-positive cells on the luminal surface of stents struts after 24 hours of flow exposure was 3.5±0.4-fold (*P*<0.05) reduced by temsirolimus in constructs with ECs alone and by 2.2±0.4-fold (*P*<0.05) with SMCs also present (Figure IV of the online-only Data Supplement). These results confirm that temsirolimus is a powerful blocker of the mTOR machinery and suggest that its effects are more profound when expression of S6RP is highest as in ECs without underlying SMCs.

Immunohistochemical analysis of p-S6RP expression was performed in coronary arteries stented with sirolimus-eluting stents or control bare metal stents. Endothelialization was not significantly different in the different stent arteries (3.1±0.2 versus 2.3±0.5). Specific p-S6RP staining was identified in the endothelium and some leukocytes (macrophages and lymphocytes) in the neointima of the 7-day bare metal stents but no cells stained in the 7-day sirolimus-eluting stent (Figure 4A and 4B). Control unstented arteries did not show any cells expressing p-S6RP (Figure 4C). More important, quantitative analysis of the luminal surface of the bare metal–stented vessels revealed the highest percentage of p-S6RP–positive cells at the neointima luminal surface (Figure 4D and 4E). p-S6RP is upregulated in the neointima of bare metal-stented arteries; phosphorylation inversely correlates with the presence of underlying SMCs. These findings provide in vivo evidence that healthy ECs do not express p-S6RP. Conversely, injured ECs do express p-S6RP, and the expression is sensitive to their...
surrounding milieu: fibrin and inflammatory cells in the neointima versus SMCs in the media (Figure III in the online-only Data Supplement). The ability, however, of ECs to restore the balance after intervention is lost in the presence of mTOR inhibitors.

Discussion

The S6/mTOR pathway is critical for vascular reactivity and metabolism. We now show that phosphorylation of S6RP in ECs is flow sensitive and in SMCs is growth factor mediated. We also demonstrate that the response of the intact vessel cannot be appreciated by examination of isolated vascular cells. Using a custom-designed flow system that allowed coating of loop conduits with ECs alone or ECs on layers of SMCs, we demonstrated how EC S6RP signaling is mediated by adjacent SMCs and vice versa. SMCs inhibit flow-mediated mTOR activation in ECs, and reciprocally, ECs regulated the response in SMCs. Cell-seeded perfusion systems allow examination of specific cellular elements under defined flow and shear conditions, with exposure to specific concentrations of vasoregulators and over a short time scale without tissue remodeling. Our vessel-like constructs extend similar systems, by introducing a bilayer coculture of ECs and SMCs within tubular structures that enable microscopic examination, programmed chemical and mechanical intervention, correlation of intracellular signaling events with functional outcome, and under static or coronary artery-like flow. The use of cells and defined media here is superior to excised vessels and whole blood because we can associate particular signals with specific cells under precisely controlled flows. Digitonin extraction enables determination of S6RP phosphorylation in ECs even in culture with SMCs. At low concentrations, digitonin, a gentle detergent, selectively punctures 8- to 10-nm holes in the sterol-rich plasma membrane, allowing leakage of cytosolic proteins of up to 200-kDa mass. Moreover, digitonin exerts effects only in cells it directly contacts. Digitonin-extracted SM/C EC vessel-like constructs revealed S6RP phosphorylation without effects on SMCs because there was no significant release of SM/C-actin (Figure IIA in the online-only Data Supplement).

The trilaminate architecture of the blood vessel provides structural support and a platform from which to launch an array of intricate paracrine regulation, primary among which are the interactions between ECs and SMCs. Each cell can induce phenotypic transformations and different bioregulatory states in the other. ECs spread more slowly and present a less thrombotic phenotype when cultured on SMCs. Similarly, the EC procoagulant and proinflammatory phenotype is amplified in coculture with growth factor–stimulated SMCs and subdued in coculture with growth factor–deprived SMCs. In turn, ECs modulate the contractile and growth properties of vascular SMCs. Our results concerning flow- and growth factor–mediated activation of S6RP provide further evidence of EC-SMC crosstalk. In health, ECs are exposed to flow and SMCs are buffered from blood by the endothelial monolayer. With injury, the endothelium is disturbed, and SMCs are directly exposed to flow. Classic studies cite these events as critical to mediation of vasomotor tone. This view of vascular cell architecture relative to flow perhaps best explains why flow upregulates p-S6RP in ECs and growth factor upregulate this pathway in SMCs. What is fascinating is that this upregulation is observed only for cells in isolation; cocultured cells are far more quiescent. That SMC-EC coregulation extends to p-S6RP is doubly intriguing. The mTOR pathway is a primary metabolic sensor of the cell. SMCs might induce metabolic activity within ECs via a paracrine mechanism or direct cell-cell contact.

Differential expression of proteins within the mTOR signaling pathway has profound implications. Local injury, flow, and chemical inhibition synergize to disrupt the endothelial monolayer, to prevent its restoration, and to impair its function. Interactions between ECs and SMCs are more important in regulating vascular wall hemostasis than previously anticipated; both ECs and SMCs alter the expression of factors in coagulation and fibrinolysis in response to shear stress. Guo and coworkers observed that although laminar shear stress activated AMPK and Akt in ECs, disturbed flow activated only Akt, disrupting the balance of mTOR and S6K and increasing the proportion of ECs in a mitotic state. Our 2-fold upregulation of mTOR in ECs by flow is in direct concert with these studies and has now been expanded to SMCs. Our results indicate that in vessel-like constructs, EC-SMC interaction dominates, altering the dynamics of basal mTOR signaling and overriding growth factor proangiogenic stimulation. Such results take us back to Virchow on the one hand and bring us to the present day of drug-eluting stents and their attendant potential complications on the other. Similar to the triad of Virchow that governs venous thrombosis, we observe that signaling within ECs and SMCs resident in the vessel wall is set by the biochemical milieu established by blood, local flow disruptions, and the state of the wall and its cells. The relative effects of the stent itself on the ECs and SMCs may explain the enhanced sensitivity of the endothelium after local delivery of agents that interfere with mTOR signaling. Endothelial denudation is most prominent between the stent struts at the time of implantation and most rapidly recovers in this region, perhaps because these ECs regenerate over SMCs and can more quickly attain a mature endothelial phenotype. ECs over the stent struts do not readily regain this mature, confluent, and bioregulatory phenotype and retain p-S6RP expression even when physically contiguous because they are distant from SMCs. One might then explain strut-adherent thrombosis in drug-eluting stents with incomplete endothelial healing as evidenced by persistent p-S6RP expression.

The modified triad, inflammation from the foreign materials of the stent, toxic effects of the pooled drug, and flow disruptions from the strut, might contribute to but alone cannot account for the repair in vivo. In vitro stenting results may be masked by surrounding uninjured ECs present in the flow cytometric analysis. Thus, despite the complementary findings that EC-SMC interactions affect EC phosphorylation of S6RP in response to stent injury in vivo and in vitro, the levels of S6RP phosphorylation after stenting in our in vitro system lacked the spatial resolution needed to directly compare them with the in vivo state. Contact-mediated SMC regulation of EC signaling needs to be defined further and
confirmed but may allow better understanding of the vascular response to drug-eluting stents and how drugs that have one putative mode of activity when examined in isolated cultures can behave in unanticipated manners in the intact and injured in vivo system. It is important to remember that stents are placed in diseased arteries with complex bifurcated geometries, blood enriched perhaps in low-density lipoprotein, glucose, and a number of prescription drugs, and plaque. All of those elements will definitely alter the interactions between ECs and SMCs.

The findings that S6RP phosphorylation was flow sensitive in ECs and growth factor sensitive in mural SMCs and that SMCs dominate over ECs have important fundamental and clinical implications. Investigation of the basic signaling should now consider the cells alone and together, and use of drug-eluting stents must integrate stent design influence on flow and drug affect mTOR. The use of flow models, flow-activated cell sorting, intracellular signaling in a coculture of human vascular cells, and modern clinical interventions can provide new insights into fundamental biology. Our flow system can track mTOR and upstream and downstream signaling molecules at rest, under flow, and with exposure to drugs over a span of concentrations and delivery kinetics. Novel endoscopic confocal microscopes allow us to relate flow alterations to cell function and drug localization. Stents are placed in diseased arteries with thickened diseased walls, in the presence of clot and inflammation, with a complex and potentially disarrayed matrix of ECs and SMCs, with complex bifurcated geometries, and with blood enriched with lipoproteins, glucose, and possibly drugs that may be subject to altered flow. All of those elements affect the interactions between ECs and SMCs, and all must be considered in appreciating vascular cell communication and homeostasis, but few of them can be reproduced in a consistent and independent fashion. Flow models with multiple cell systems under defined flow regimens bring us back to Virchow and forward into the era of complex vascular interventions and emerging concepts in vascular biology.

Acknowledgments

We thank Philip Seifert for his support in immunohistochemistry, Sylaja Murikandi and Lathib Rabadi for their support in Western blot analysis, Dr Yoram Richter (Medinol) for the gift of NIR stents, and Wyeth Laboratories for the temsirolimus. We thank Cordis Corp for support to specimens used to obtain preclinical in vivo data.

Sources of Funding

This work was supported by NIH/NIGMS ROI/GM049039 (Dr Edelman) and NIH-NIDDK (1K08DK080946; Dr Chitalia). Dr Balcells is supported by the Barcelona Chamber of Commerce and Fundació Empreses IQS.

Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

Local delivery of antiproliferative drugs limits restenosis but may increase thrombosis even late after intervention. Device implantation and drug administration can synergistically induce endothelial dysfunction and delay recovery, impairing vasoreactivity, enhancing platelet aggregation, and elevating tissue factor expression. As stents are placed in increasingly complex geometries in the face of local and systemic disease, in the presence of clot and inflammation, a complex spectrum of issues must be considered that can no longer be intuited or dissected from in vivo experiments. We developed a model flow system examining signaling in vascular cells under controlled physiological flows. Our data demonstrate a profound difference in endothelial cell biology in the presence and absence of smooth muscle cells and suggest a far more sophisticated regulatory interaction between alterations in hemodynamics from above and vessel wall from below than classically appreciated. Similar to the triad that governs venous thrombosis, we now observe that signaling within vascular endothelial and smooth muscle cells is set by the biochemical milieu established by blood, local flow disruptions, and the state of the wall and its cells. The relative effects of the stent itself on the cells may explain the enhanced sensitivity of the endothelium after local delivery of agents that interfere with mammalian target of rapamycin signaling, placing vessels at risk of thrombosis even late after the initial treatment. Flow models with multiple cell systems bring us back to Virchow and forward into the era of complex vascular interventions and emerging concepts in vascular biology.
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Circulation. 2010;121:2192-2199; originally published online May 10, 2010;
doi: 10.1161/CIRCULATIONAHA.109.877282
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/121/20/2192

Data Supplement (unedited) at:
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Expanded Methods

Phenotypic and functional characterization of endothelial and smooth muscle cells seeded on fibronectin coated tubes

Endothelial (EC) and smooth muscle cell (SMC) coverage was determined using a MATLAB®-based image analysis program. Images were obtained by confocal microscopy of tube sections fixed and stained with specific antibodies (CD31 and smooth muscle cell α-actin, for EC and SMC, respectively). Inducible cell adhesion molecule expression markers (e-Selectin, VCAM-1, ICAM-1) were studied by flow cytometric analysis of cells in suspension. Briefly, cells were washed with PBS, treated with 0.05 % Trypsin / 0.53 mM EDTA for 5 min, and disrupted by gentle shaking. Cell-suspensions were washed and 3x10^5 cells were resuspended in FACS buffer (PBS containing 0.1% bovine serum albumin and 0.1% sodium azide, Sigma Chemicals). EC were incubated with FITC-labeled mouse anti-human ICAM-1 (clone 15.2, Serotec), anti-human E-selectin (clone 1.2B6, Serotec), and mouse anti-human VCAM-1 (clone 1.G11B1; BD Pharmingen) for 30 min at 4°C. Cells were then washed, fixed in 1% paraformaldehyde, and 10^4 cells were analyzed by flow cytometry using a FACScalibur instrument and CellQuest software (Becton Dickinson). Surface tissue factor expression was quantified applying the MATLAB®-based image analysis program mentioned above to samples stained with anti-tissue factor from American Diagnostica. A 6-keto-prostaglandin F1a enzyme-immunoassay system (Amersham) was assayed for prostacyclin in serum-free perfusate collected after 24 h flow exposure to pulsatile arterial shear stress. Total glycosaminoglycan production was quantified by means of the dimethylmethylene blue assay. eNOS activity in cell lysates,
obtained by EDTA (1 mM)/Tris (25 mM) treatment followed by sonication immediately after cessation of 24 h flow exposure, was determined using the [3H]-L-arginine/L-citrulline assay kit (Cayman Chemical). Results were normalized to the total amount of protein determined by the BCA protein assay kit (Pierce).

**Western Blot Analysis**

Cell pellets were washed with cold PBS and immediately treated with 200 µl lysis buffer (20mM tris buffer, 150mM NaCl, 1% triton 100X, 0.1% sodium dodecyl sulfate, 2mM sodium orthovanadate, 2mM PMSF, 50mM NaF, 1 complete protease inhibitor pellet). Lysates were centrifuged at 10,000g for 10min at 4°C and supernatants snap frozen until analysis. Protein was quantified using Biorad protein assay.

**Confocal microscopy – staining, imaging and image quantification**

A Perkin-Elmer spinning disk confocal microscopy system using a Zeiss Axiovert 200M microscope connected to a Hamamatsu Orca-ER camera was used to obtain fluorescence images of cells (EC and SMC) and proteins (p-S6RP, CD31 and Tissue Factor). A DAPI solution (1:500 in PBS/BSA) was used to stain the cells nuclei, while proteins were immunostained with their respective primary antibodies (diluted 1:50 in PBS/BSA) conjugated to Alexa Fluor 448 and/or 647 secondary antibodies. The laser wavelengths used were 405 nm for DAPI, 488 nm for Alexa Fluor 488 and 640 nm for Alexa Fluor 647. The emitted light was discriminated Blue, Green and Red, respectively. The exposure times depended on the experiment, but were constant for each separate experiment. The results of this processing (exposing + filtering) are black and white multi-layer images of the light emitted after exciting the samples. Those images were
artificially coloured red, green and blue (RGB) depending on the wavelength used and combined in order to obtain an RGB stack of every sample. Using a MATLAB-based analysis program, cells were counted and their fluorescence was analyzed. In the case of p-S6RP quantification (cf. Figures 3E, 3F, 1S), the analysis consisted in defining the area of the cell, the artificial colour of the protein of interest (Red, Green or Blue) and finally adding all the values of this colour intensity (0-255) for every pixel of the area. In cases with background fluorescence, this value was estimated and retrieved from the final result. In the case of stent recovery quantification (cf. Figures 1G, 4S), the area of the stent was determined with ImageJ and the number of cells on the stent strut were manually counted.

In vivo studies; animal preparation, surgery details and hystopathological analysis

To prevent or reduce the occurrence of thrombotic events, animals were treated on Day -1 or Day -2 with aspirin (650 mg, per os [PO]) and clopidogrel (300 mg, PO). The animals were then treated with aspirin (81 mg, PO) and clopidogrel (75 mg, PO) daily thereafter. Telazol® (4–6 mg/kg intramuscularly [IM]) was administered as a pre-anesthetic. Pre-operative nifedipine (10 mg, sublingual) was also given. An intravenous (IV) catheter was placed in an ear vein for administration of IV fluids at a rate sufficient to maintain patency of the catheter, or as needed. After induction of anesthesia, an incision was made in the neck to expose the carotid artery. An arterial sheath was introduced and advanced into the artery. Heparin (50–200 U/kg, IV) was administered on Day 0 (after placement of the introducer sheath) to prolong activated clotting time (ACT) to a target range of approximately 300 seconds during device deployment. Arterial blood samples
(<0.5 mL each) were collected within 5–10 minutes of heparin administration, then at intervals of up to 45 minutes to measure ACT. Under fluoroscopic guidance, a guide catheter was advanced through the sheath into the ascending aorta and to the coronary arteries. Angiographic images of the vessels were obtained with contrast media to identify the proper location for the deployment site. Stents were introduced into the coronary arteries by advancing the stent delivery system through the guide catheter and over the guide wire to the deployment site within the coronary artery. The balloon was then inflated at a steady rate to a pressure sufficient to target a visually assessed balloon-artery ratio of 1.1–1.3:1 and held for 30 seconds (from initiation of inflation). Confirmation of this balloon-artery ratio was made when the angiographic images were quantitatively assessed. After the target balloon-artery ratio was achieved, vacuum was applied to the inflation device in order to deflate the balloon and slowly remove the delivery system.

Histopathological scoring was used to grade endothelialization. The scores generally reflected the degree and extent of injury. Scoring was performed using light microscopy. Endothelialization score depends on the extent of the circumference of the artery lumen showing coverage with endothelial cells (0, absent; 1, <25%; 2, 25-75%; 3, >75%; 4 = 100%).
**Supplemental Table 1.** Phenotype and function of EC and SMC seeded on silicone-rubber tubes.

<table>
<thead>
<tr>
<th>Characteristic/function (cell type)</th>
<th>Measured</th>
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<tbody>
<tr>
<td>Coverage (EC*)</td>
<td>Monolayer (267±12cells/mm²)</td>
</tr>
<tr>
<td>Coverage (SMC†)</td>
<td>Multilayer (369±84 cells/mm²)</td>
</tr>
<tr>
<td>CD31⁺ (EC)</td>
<td>&gt;99% positive</td>
</tr>
<tr>
<td>α-actin (SMC)</td>
<td>&gt;99% positive</td>
</tr>
<tr>
<td>Surface Tissue Factor (SMC)</td>
<td>83 ±1.23%</td>
</tr>
<tr>
<td>E-Selectin (EC)</td>
<td>1.2±0.3 % (unstimulated) 58.3±3.4 %(TNFα** stimulation)</td>
</tr>
<tr>
<td>VCAM-1§ (EC)</td>
<td>24.7±5.85 (unstimulated) 89.7±5.4% (TNFα stimulation)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td></td>
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<tr>
<td>Prostacyclin (EC)</td>
<td>1.68<em>10⁻³± 8.05</em>10⁻⁴ pg/cell</td>
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<tr>
<td>eNOS# (EC)</td>
<td>4.40<em>10⁻³± 1.78</em>10⁻⁴ pg/cell</td>
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<tr>
<td>Glycosaminoglycans (EC)</td>
<td>0.56±0.34pg/cell</td>
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<tr>
<td>Soluble tissue factor (SMC)</td>
<td>0.33±0.12ng/cell</td>
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</table>

*Endothelial Cells, †Smooth Muscle Cells, §Platelet Endothelial Cell Adhesion Molecule-1, ¶Vascular Cell Adhesion Molecule-1, ||Inter-Cellular Adhesion Molecule 1, #Endothelial Nitric Oxide Synthase, **Tumor Necrosis Factor Alpha
Supplemental Figure Legends

**Figure 1S.** Expression of phospho-S6 ribosomal protein (p-S6RP) in endothelial cells (EC) depends upon flow exposure (F) and is abrogated by the sirolimus analog, temsirolimus (Tems). Representative immunofluorescent images of phospho-S6RP expression in EC (green) after flow exposure and after treatment with the inhibitor (A-D). Cell nuclei were labelled with DAPI (blue).

**Figure 2S.** A) Digitonin extracts cytosol from EC not from SMC. Digitonin (Dig) and RIPA extracts of EC and SMC grown alone and from EC monolayer co-cultured on top of SMC (SMC/EC co-culture). Cells were treated with digitonin and RIPA for indicated time. Digitonin extracts the cytosol of the cells it comes in direct contact with, while RIPA produces lysate of the entire cell layers. Vascular smooth muscle actin is the marker of SMC. Tubulin is used for loading control. Very little actin is seen in SMC/EC co-culture system indicating that digitonin extracts cytosol from the uppermost EC monolayer with which the detergent comes in direct contact and not the underlying SMC. B) Flow increases abundance of phosphorylated S6RP in EC. Endothelial cells were seeded on fibronectin-coated silastic tubing and exposed to flow and digitonin-extracted fractions were probed for p-S6RP and tubulin. Flow increased the amount of p-S6RP normalized to cell tubulin content. Representative Western blots are displayed from three individual samples. C) SMC inhibit flow-induced increased phosphorylation of S6RP in EC. EC or SMC/EC-vessel like constructs were exposed to flow (+F) or no flow (-F) and
digitonin-extracted fractions were probed for p-S6RP and tubulin. Flow increased abundance of p-S6RP in ECs, while SMC reduced it.

**Figure 3S.** Immunohistochemical images of consecutive slices of the same bare metal stent strut injury. Cells were stained with antibodies against p-S6RP (A), SMC α-actin (B), and CD45RA (C). Photomicrographs suggest that the unstable matrix under the EC in the neointima, poor in SMC and rich in fibrin, is responsible for the upregulation of p-S6RP. The absence of CD45RA positive cells (C) implies that the upregulation of p-S6RP cannot be explained by presence of inflammatory cells in the neointima.

**Figure 4S.** Density of stent coverage on stent struts with and without inhibitor in the presence or absence of flow. After 24 hours flow exposure in the perfusion bioreactor, stents were carefully removed of the vessel-like construct, cut open and imaged using epifluorescence microscopy. EC on stent struts and stent strut surface were quantified using ImageJ®. When EC were cultured alone without underlying SMC, flow increased the response to the injury by promoting rapid re-endothelialization of the stent strut. Static conditions and tensirolimus slowed the repair process. When in co-culture, SMC diminished the ability of EC to re-endothelialize after stent injury.

**Legend Video 1S.** Representative EC and SMC image stack obtained by confocal imaging of multilayered SMC/EC vessel-like constructs. EC and SMC are identified by surface CD31 (green) and surface tissue factor (red), respectively.
Fig 2S

A

<table>
<thead>
<tr>
<th></th>
<th>EC alone</th>
<th>SMC alone</th>
<th>SMC/EC co-culture</th>
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<tr>
<td></td>
<td>Dig 15</td>
<td>RIP 30</td>
<td>Dig 15, RIP 30</td>
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<tr>
<td></td>
<td>30</td>
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<td>30 (min)</td>
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IB: α-tubulin
IB: vascular SMC α-actin

B

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IB: p-S6RP

IB: α-tubulin

C

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<td>+F 1 2</td>
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<thead>
<tr>
<th></th>
<th>SMC/EC vessel-like construct</th>
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<td>-F 1 2 3</td>
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<td></td>
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IB: p-S6RP

IB: α-tubulin