INTRODUCTION
Vascular endothelial cells line the lumen of blood vessels, while smooth muscle cells are found within the vessel wall among a matrix of collagen and structural fibers. In healthy vessels, endothelial cells experience laminar flow (shear stresses ranging from 10 to 12 dyne/cm²), whereas turbulent flow (shear stress ranging from 20 to 30 dyne/cm²) can occur in diseases such as atherosclerosis. Atherosclerosis development is described by the response to injury theory: endothelial cells suffer an injury resulting in altered morphology, metabolism, or other cell functions, which stimulates smooth muscle cell proliferation and migration into the lumen, ultimately causing vascular thickening and hardening [1].

Cyclooxygenase (COX)-2, endothelial cell nitric oxide synthase (ecNOS), and platelet-derived growth factor (PDGF-β) are genes with known responses to fluid shear stress and are important in the development of atherosclerosis. Specifically, COX-2 is involved in the production of prostacyclin (PGI₂), an inhibitor of platelet aggregation and smooth muscle cell growth and migration [2]. ecNOS catalyzes the reaction of arginine to citrulline along with nitric oxide (NO), which acts as a vasodilator and mimics PGI₂ in other ways, as a product [3]. Activation of PDGF-β leads to smooth muscle cell proliferation, migration, and differentiation across the lumen and may ultimately lead to vascular wall hardening.

The objective of this study, therefore, was to determine the effects of such soluble and transferable factors (released by endothelial cells in response to fluid flow) on smooth muscle cell function and gene expression. Elucidating this mechanism may lead to a better understanding of atherosclerosis development, and potentially, improved disease treatment.

MATERIALS AND METHODS
Cell Culture
Rat aortic endothelial cells (RAEC) and rat aortic smooth muscle cells (RASMC) (VEC Technologies) were maintained in MCDB-131 Complete Medium (VEC Technologies) or Dulbecco’s Modified Eagle’s Medium (DMEM; Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Hyclone), respectively. Cells were grown under standard cell culture conditions, that is, a humidified, 37°C, 5% CO₂, 95% air environment. All cells were used at population numbers five through twelve without further characterization.

Exposure of RAEC to Laminar Flow
Prior to fluid flow exposure, endothelial cells were seeded (35,242 cells/cm²) onto etched glass cover slides previously coated with fibronectin (10 µg/mL; Sigma). Once confluent (approximately 1.5 days), endothelial cells were exposed to laminar fluid flow in a modified version of the parallel plate flow chamber [4] for 6 or 24 hours. The shear stress generated in this system was 10.4 dyne/cm².

mRNA Expression by RAEC Exposed to Fluid Flow
Following exposure to fluid flow, mRNA from endothelial cells was digested with Triazol (Gibco), extracted with chloroform, and precipitated with isopropanol. Reverse transcriptase-polymerase chain reaction was performed using sample RNA and the Ambio Retroscript Kit with primers (20 pmol/µL; Gibco) specific to either glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cyclooxygenase-2 (COX-2), platelet derived growth factor beta (PDGF-β), or endothelial cell nitric oxide synthase (ecNOS). Electrophoresis of cDNA samples was performed in a gel casting system (Biorad) with 1X TAE buffer for 1.5 hours at 200V. The amount of cDNA in each lane was determined using a phospho imager (Biorad) and densitometry.

RASMC Proliferation and mRNA Expression in Conditioned Medium
RASMC were seeded (3,500 cells/cm²) onto etched borosilicate glass coverslips and incubated under standard cell culture conditions in DMEM for 24 hours. After 24 hours, the standard media was removed and replaced with fresh MCDB-131 medium (control), control-conditioned MCDB-131 medium, or flow-conditioned MCDB-131 medium (experimental). Control-conditioned medium was collected
from a static culture dish (control) of endothelial cells after a 6-hour experiment. Flow-conditioned medium was collected from the laminar flow system after a 6-hour experiment; this medium contained any soluble factors released by endothelial cells upon exposure to laminar flow.

Smooth muscle cells were allowed to proliferate in a static environment under standard cell culture conditions and control, control-conditioned, or flow-conditioned medium for 1, 3, and 5 days. At the end of the prescribed time period, cells were fixed with 4% formalin, stained with Hoechst 33258 (Sigma), and counted using fluorescence microscopy.

In addition, smooth muscle cell mRNA was collected and analyzed as previously described. In smooth muscle cell studies, primers were specific to platelet derived growth factor alpha (PDGF-α), inducible nitric oxide synthase (iNOS), and COX-2; these genes have similar roles to the genes chosen for endothelial cell studies. Specifically, iNOS regulates NO production [5], COX-2 regulates prostacyclin production [6], and PDGF-α is the PDGF isoform expressed in smooth muscle cells and is up regulated in atherosclerotic lesions [7].

RESULTS AND DISCUSSION

This study confirmed previous findings [1,2]; namely, after exposure to a physiological shear stress (10.4 dyne/cm²), vascular endothelial mRNA expressions of COX-2, PDGF-β, and ecNOS were increased.

More importantly, our results provided the first evidence that smooth muscle cell proliferation was increased (compared to that of cells maintained under control media) as a direct result of the soluble and transferable factors released by flow-exposed endothelial cells. Specifically, results demonstrated that after exposure to flow-conditioned medium for 5 days, smooth muscle cell growth was increased by 150%. This effect was due solely to the flow-released mediators, since no significant differences were observed between smooth muscle cells grown under control and control-conditioned medium.

Furthermore, smooth muscle cell mRNA expression was investigated to determine the effect of such chemical mediators on smooth muscle cell gene expression. Results of these studies indicated that these soluble mitogens induced altered iNOS, COX-2, and PDGF-α mRNA expression by smooth muscle cells; these changes occurred in a time-dependent manner. Therefore, this communication pathway between endothelial cells and smooth muscle cells is likely to be involved in the signaling mechanism leading to vascular wall thickening during atherosclerosis development.

CONCLUSIONS

The laminar flow studies reported here provide the foundation for future work, which will focus on understanding the response of vascular endothelial and smooth muscle cells to turbulent fluid flow. These studies will utilize a novel, custom-built turbulent flow system to mimic conditions existing in the vasculature under diseases such as atherosclerosis. Together, results of laminar and turbulent flow studies will provide insight into the cellular mechanism for atherosclerosis development, and could provide information necessary for future drug design to treat such diseases.

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REFERENCES