

Differential effects of orbital and laminar shear stress on endothelial cells

Alan Dardik, MD, PhD, Leiling Chen, MD, Jared Frattini, MD, Hidenori Asada, MD, Faisal Aziz, MD, Fabio A. Kudo, MD, PhD, and Bauer E. Sumpio, MD, PhD, *New Haven and West Haven, Conn*

Objective: Laminar shear stress is atheroprotective for endothelial cells (ECs), whereas nonlaminar, disturbed, or oscillatory shear stress correlates with development of atherosclerosis and neointimal hyperplasia. The effects of orbital and laminar shear stress on EC morphology, proliferation, and apoptosis were compared.

Methods: ECs were exposed to orbital shear stress with an orbital shaker (210 rpm) or laminar shear stress (14 dyne/cm²) with a parallel plate. Shear stress in the orbital shaker was measured with optical velocimetry. Cell proliferation was assessed with direct counting and proliferating cell nuclear antigen staining; apoptosis was assessed with transferase-mediated deoxyuridine triphosphate nick end labeling staining. Cell surface E-selectin and intercellular adhesion molecule expression were assessed with fluorescence-activated cell sorting. Akt phosphorylation was assessed with Western blotting.

Results: Orbital shear stress increased EC proliferation by 29% and ³[H]thymidine incorporation two-fold compared to 16% and 38% decreases, respectively, in ECs treated with laminar shear stress ($P < .0001$ and $P = .03$, analysis of variance). Cells in the periphery of the culture well aligned to the direction of shear stress similar to the shape change seen with laminar shear stress, whereas ECs in the center of the well appeared unaligned similar to ECs not exposed to shear stress. Shear stress at the bottom surface of the culture well was reduced in the center of the well (5 dyne/cm²) compared to the periphery (11 dyne/cm²); the Reynolds' number was 2066. ECs were seeded differentially in the center and periphery of the wells. ECs in the center of the well had increased proliferation, increased apoptosis, reduced Akt phosphorylation, increased intercellular adhesion molecule expression, and reduced E-selectin down-regulation, compared with ECs in the periphery of the well.

Conclusion: Although the orbital shaker does not apply uniform shear stress throughout the culture well, arterial magnitudes of shear stress are present in the periphery of the well. ECs cultured in the center of the well exposed to low magnitudes of orbital shear stress might be a model of the "activated" EC phenotype. (*J Vasc Surg* 2005;41:869-80.)

Clinical Relevance: The perfect in vitro model to study and assess treatments for atherosclerosis and neointimal hyperplasia does not exist. An extensive body of literature describing effects of laminar shear stress on endothelial cells has contributed to our understanding of the interactions between shear stress and blood vessels. Laminar shear stress is atheroprotective, whereas oscillatory or disturbed shear stress correlates with areas of atherosclerosis and neointimal hyperplasia in vivo. This study describes the orbital shear stress model, its effects on endothelial cell proliferation and apoptosis, and suggests that activation of the intracellular Akt pathway is associated with these differing effects of laminar and orbital shear stress on endothelial cells.

Endothelial cells (ECs) are exposed to the hemodynamic forces of the blood including circumferential stretch and hydrostatic pressure, but they are uniquely exposed to

shear stress at the luminal surface of the blood vessel.¹⁻³ Steady laminar shear stress is thought to be atheroprotective, inhibiting both EC proliferation and apoptosis.⁴⁻⁸ However, nonlaminar or turbulent shear stress produces different effects on ECs than laminar shear stress; areas of the vasculature exposed to nonlaminar or turbulent shear stress are thought to correspond to the localization of atherosclerotic plaque and neointimal hyperplasia.⁹⁻¹²

Although extensive work has characterized the endothelial response to laminar shear stress, less is known about the response to nonlaminar or turbulent shear stress. Cell proliferation and DNA synthesis are increased, and there is loss of cell alignment to the direction of flow.^{13,14} There are different patterns of gene expression in ECs exposed to laminar or nonlaminar flow^{15,16}; for example, transforming growth factor- β_1 is differentially regulated.¹⁷

In vitro studies with orbital shear stress have been used to demonstrate stimulation of EC DNA synthesis, translation, and activation of the mitogen-activated protein kinase (MAPK) and pp70^{s6k} pathways.^{18,19} We have previously demonstrated that orbital shear stress stimulates EC Sp1

From the Section of Vascular Surgery, Yale University School of Medicine, and the VA Connecticut Healthcare Systems.

Competition of interest: none.

Supported by an American College of Surgeons Faculty Research Fellowship (A.D.), the Pacific Vascular Research Foundation, San Francisco (A.D.), the Dennis W. Jahnigen Career Development Scholarship Program, which is administered by the American Geriatrics Society through an initiative funded by The John A. Hartford Foundation of New York City and The Atlantic Philanthropies (A.D.), a National Institutes of Health grant R01-HL47345-05 (B.E.S.), and a VA Merit Award (B.E.S.).

Presented in part at the American College of Surgeons Surgical Forum, New Orleans, La, Oct 10-14, 2004, at the Annual Meeting of the Association for Academic Surgery, Houston, Texas, Nov 11-13, 2004, and at the Annual Meeting of the American Society for Cell Biology, San Francisco, Calif, 2003.

Reprint requests: Alan Dardik, MD, PhD, Yale University School of Medicine, Boyer Center for Molecular Medicine, 295 Congress Ave, Room 436, New Haven, CT 06519 (e-mail: alan.dardik@yale.edu).

0741-5214/\$30.00

Copyright © 2005 by The Society for Vascular Surgery.

doi:10.1016/j.jvs.2005.01.020

phosphorylation and *egr-1* expression, inhibits membrane type 1–matrix metalloproteinase expression, and stimulates platelet-derived growth factor–BB and interleukin-1 α secretion to induce smooth muscle cell chemotaxis.^{20,21} These results suggest that orbital shear stress might not be laminar but might be disturbed or nonlaminar, or even turbulent, shear stress. To determine whether orbital and laminar shear stress have differing effects on ECs, we compared the effects of both orbital and laminar shear stress on EC morphology, proliferation, and apoptosis.

METHODS

Endothelial cell culture. Bovine aortic ECs were obtained by gentle scraping of the intimal surface of bovine aorta obtained from freshly killed calves at a local slaughterhouse as previously described.^{20,22} Human umbilical vein ECs (HUVECs) were isolated via the collagenase method and were obtained from the laboratory of Dr Jordan Pober; HUVECs were used for flow cytometry experiments because bovine ECs do not react with the E-selectin or intercellular adhesion molecule-1 (ICAM-1) antibodies (see below). Cells were maintained in Dulbecco's modified Eagle's medium high glucose–Ham's F-12 (GIBCO BRL, Gaithersburg, Md) medium, supplemented with 10% inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah), 5 $\mu\text{g}/\text{mL}$ deoxycytidine/thymidine (Sigma Chemical, St Louis, Mo), and antibiotics (penicillin 100 U/ml, streptomycin 100 $\mu\text{g}/\text{mL}$, and amphotericin B 250 ng/mL) (GIBCO BRL), and grown to confluence at 37°C in a humidified 5% CO₂ incubator. ECs were identified by their typical cobblestone appearance and indirect immunohistochemistry staining for factor VIII antigen, and lack of reactivity with α -actin. Cells used in this study were between passages 5 and 11, and cells of identical passage were used for parallel orbital and laminar experiments. HUVECs were grown in M199 supplemented with 20% FBS, 10 $\mu\text{g}/\text{mL}$ heparin, and 5 $\mu\text{g}/\text{mL}$ endothelial cell growth supplement (BD Biosciences, Palo Alto, Calif); cells of passages 2 through 3 were used for immunofluorescence studies. ECs were seeded at a subconfluent density of 50,000/cm² on 6-well plates for exposure to orbital shear stress, or on 75 \times 22 mm glass slides (Fisher Scientific, Pittsburgh, Pa) for exposure to laminar shear stress, which were coated with Collagen I (0.013 mg/cm²; Cohesion Technologies, Palo Alto, Calif). ECs were synchronized by incubation for 24 hours in FBS-free medium before shear stress application, with culture in the presence of 10% FBS during shear stress exposure. For cells that were seeded only in the center or only in the periphery of the well, cells were excluded from the unseeded part of the well by covering the unseeded part with a silicone gasket that was removed before shear stress exposure (Fig 1).

Shear stress application. Orbital shear stress (210 rpm) was applied to confluent cell cultures by using an orbital shaker (VWR Signature Model DS-500; VWR International, West Chester, Pa) positioned inside the incubator as previously described.^{20,21,23} The shear stress within the cell culture well is estimated as $\tau_{\text{max}} =$

$a\sqrt{\eta\rho(2\pi f)^3}$ where a is the orbital radius of rotation of the shaker (0.95 cm), ρ is the density of the culture medium (0.9973 g/mL), η is the viscosity of the medium (0.0101 poise measured with a viscometer), and f is the frequency of rotation (rotation/sec).^{19,24} A rotational frequency greater than 200 rpm has been reported to correspond to arterial magnitudes (11.5 dynes/cm²) of shear stress.¹⁹ Laminar shear stress (14 dynes/cm²) was applied to ECs with a parallel-plate chamber as previously described.^{22,25} Control cells were identical passage ECs that were exposed to static conditions (0 dyne/cm²).

Shear stress measurement. Shear stress within the culture well was measured with a MicroS3.v10 probe (Visosense Corporation, Pasadena, Calif). The MicroS3 probe uses optical Doppler velocimetry to measure shear stress within 166 μm of its surface.²⁶ Shear stress was measured in a single well of a 6-well plate, diameter 3.5 cm, wall height 1.8 cm, filled with 2 mL water seeded with TiO₂ particles (particle mean size, 7 μm ; medium density, 0.9973 g/mL; medium viscosity, 0.0101 poise). The probe was mounted, and measurements were taken 1 mm from the center point of the well to sample the shear stress present in the center of the well, as well as 12 mm from the center point to sample the shear stress present in the periphery. These center and periphery sampling areas correspond to the center and periphery of the well that were seeded differentially with cells (see below). The probe was mounted through a hole cut through the bottom of the well such that the tip of the probe was aligned flush with the bottom surface and thus measured shear stress at the level of a seeded cell. Shear stress = $f_{\text{Doppler}} \cdot K \cdot \mu$ where f_{Doppler} is the mean frequency (Hz) of the Doppler shift in the area sampled by the sensor and is calculated by Fast-Fourier Transformation; K is the fringe divergence, a constant characterized for each sensor (0.0594 for the probe used in this study); and μ is the dynamic viscosity and is equal to the product of the kinematic viscosity (ν) and the density (ρ). The Reynolds' number was calculated as $\omega R^2 / \nu$ where ω is the rotational speed of the orbital shaker, R is the radius of rotation of the orbital shaker (0.975 mm), and ν is the kinematic viscosity (1.012 $\times 10^{-6}$ m²/s). The well and attached probe were mounted on the surface of the orbital shaker, which was then adjusted from 60 to 210 rpm, and shear stress was measured at 30-rpm intervals; approximately 100 independent measurements were taken at each point.

Cell density. EC density was assessed by determination of cell number, both before shear stress application and after 1, 3, or 5 days of shear stress exposure, and adjusting for the area in which the cells were seeded. Cells were counted under phase contrast microscopy with a hemocytometer, with some representative samples independently with a Coulter-Counter (Model ZM; Coulter Electronics, Hialeah, Fla), with each value determined by the mean of four counts.

³[H]Thymidine incorporation assay. After exposure to shear stress (24 h), ECs were incubated in culture medium containing ³[H]thymidine (1 $\mu\text{Ci}/\text{mL}$) for 4

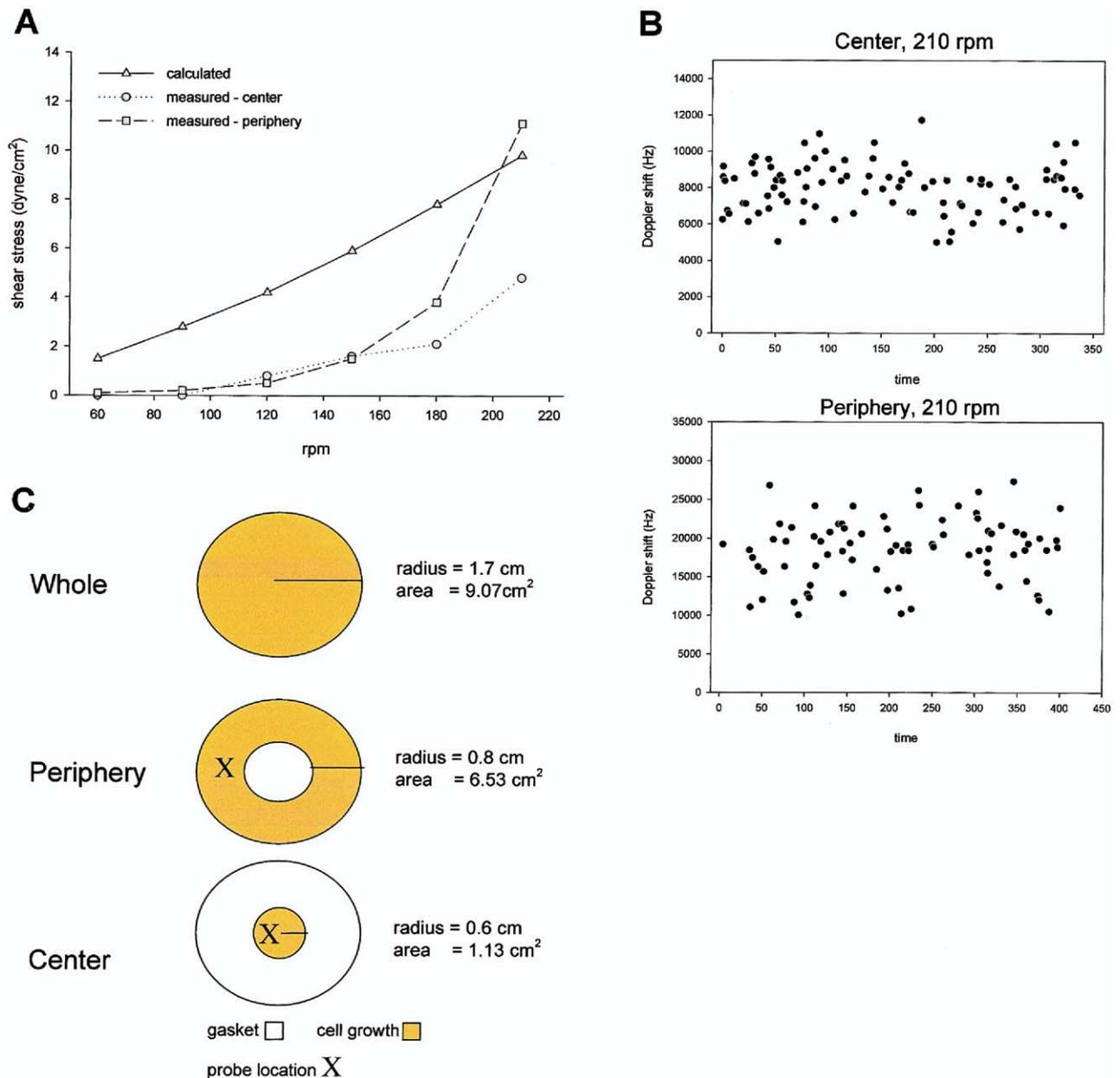


Fig 1. A, Calculated and measured orbital shear stress. Shear stress within the cell culture well is estimated as $\tau_{max} = a\sqrt{\eta\rho(2\pi f)^3}$ where a is the orbital radius of rotation of the shaker (0.95 cm), ρ is the density of the culture medium (0.9973 g/mL), η is the viscosity of the medium (0.0101 poise measured with a viscometer), and f is the frequency of rotation (rotation/sec). Shear stress was measured either in the center or periphery of the culture well with optical Doppler velocimetry; the standard error of mean (SEM) was $\leq 3\%$ in all cases. At 210 rpm, mean $f_{Doppler}$ was 18476 Hz in the periphery and 7945 Hz in the center; the mean flow was 137.18 mm/s in the periphery and 58.99 mm/s in the center; the signal to noise ratio was 58.0 dB. Calculated shear stress was 9.8 dyne/cm²; measured shear stress was 11.1 dyne/cm² in the periphery of the well but 4.8 dyne/cm² in the center of the well, and the Reynolds' number was 2066. **B,** Temporal variation in orbital shear stress. Optical velocimetry was recorded over time; sample recordings in the center (seconds; n = 97) and periphery (seconds \times 50; n = 79) are given for 210 rpm. Each circle represents a separate measurement. Little temporal variation is noted in either recording. **C,** Diagram of differential cell seeding. In some experiments, cells were seeded throughout the entirety ("whole") of the well, or differentially in the periphery or center of the well. For cells that were seeded only in the center or only in the periphery of the well, cells were excluded from the unseeded part of the well by covering the unseeded part of the well with a silicone gasket during the time that cells were seeded and allowed to attach. The silicone gasket was removed after cell attachment but before shear stress exposure. A small transition area between the center and periphery areas was excluded from seeding as well. The "X" marks the location of the probe used in optical velocimetry experiments.

hours under static conditions. Trichloroacetic acid-precipitable proteins were solubilized with 0.2N NaOH, and the incorporated radioactivity was counted with a scintillation counter (Beckmann, Fullerton, Calif).²³

Cell staining. General EC morphology was evaluated with crystal violet staining. After exposure to shear stress, ECs were fixed in 3.7% formaldehyde for 10 minutes, stained with 0.125% crystal violet (Sigma) for 2 minutes, and then observed under phase-contrast microscopy (Olympus IMT2; Olympus Optical, Tokyo, Japan). Staining for F-actin was performed after exposure to shear stress; after fixation, cells were permeabilized with 0.1% Triton X-100 and stained with rhodamine phalloidin (R-415; Molecule Probes, Eugene, Ore) for 30 minutes before observing fluorescence with an epifluorescence microscope under $\times 200$ magnification.

Immunohistochemistry. After exposure to shear stress treatment, ECs were washed with phosphate-buffered saline (PBS), fixed with 2% formaldehyde, and then permeabilized with 75% ethanol. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 minutes before incubation with monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (Clone PC10; Sigma) diluted 100:1 in PBS with 1% bovine serum albumin for 3 hours. Staining was performed by using a secondary antibody conjugated with horseradish peroxidase and 3,3'-diaminobenzidine as a substrate, with counterstaining with Mayer's hematoxylin. The percentage of positively stained nuclei (the number of PCNA-positive nuclei divided by total endothelial nuclei) was determined in five high power microscopic fields. Only definitive nuclear staining was counted.

Apoptosis. The *in situ* death detection kit was used to assess apoptosis (Roche Molecular Biochemicals, Indianapolis, Ind) following the manufacturer's protocol. Briefly, ECs were washed once with PBS and then fixed with freshly prepared 4% paraformaldehyde (pH 7.4) for 1 hour (room temperature). The samples were incubated with 3% H₂O₂ in methanol (10 min) before permeabilization with 0.1% Triton X100 (2 min) and then incubated with the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) reaction mixture. Staining was performed with an antibody conjugated with horseradish peroxidase and 3,3'-diaminobenzidine substrate, followed by counterstaining with Mayer's hematoxylin.

Immunoblotting. After exposure to shear stress in serum-free medium (30 min), cells were washed in ice-cold PBS twice and scraped in lysis buffer containing 50 mmol/L HEPES (pH 7.4), 0.5 mol/L sodium chloride, 1% Triton X-100, 0.1% sodium dodecylsulfate, 1% deoxycholate, 5 mmol/L ethylenediaminetetraacetic acid, 50 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin. Cell extracts were sonicated and centrifuged at 15,000g for 10 minutes, and the supernatant was collected. Equal amounts of protein (30 μ g per lane, BioRad protein assay system; BioRad Laboratories, Inc, Hercules, Calif) were separated by 10% sodium

dodecylsulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Life Science Inc, Arlington Heights, Ill). After blocking for 1 hour with Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk, the membrane was probed with primary antibody, either anti-Akt antibody or anti-phospho-specific (ser 473) Akt antibody (Cell Signaling, Beverly, Mass), and horseradish peroxidase-conjugated anti-rabbit polyclonal secondary antibody (Cell Signaling), before detection of immunoreactivity by enhanced chemiluminescence (Amersham). All blots were quantified with densitometry (BioImage, Ann Arbor, Mich).

Immunofluorescence. EC surface expression of E-selectin or ICAM-1 was measured by indirect immunofluorescence with flow cytometry. HUVECs were seeded on 6-well plates, both on the whole surface and separately in the center or periphery of the wells. Cells were exposed to orbital shear stress or static conditions for 6 hours in the presence or absence of tumor necrosis factor (TNF)- α (10 ng/mL; Calbiochem, San Diego, Calif). After shear stress treatment, cells were washed in ice-cold medium and incubated with anti-E-selectin or ICAM-1 antibody (1:100; R&D Systems, Minneapolis, Minn) for 45 minutes. Excess primary antibody was washed away, and cells were incubated with donkey anti-mouse FITC antibody (1:100; Jackson ImmunoResearch, West Grove, Pa) for 45 minutes. Excess secondary antibody was washed away, and indirect immunofluorescence was measured with flow cytometry (FACSort; Becton Dickinson, Franklin Lakes, NJ). HUVECs were used for these experiments because bovine ECs do not react with the E-selectin or ICAM-1 antibodies (data not shown).

Statistical analysis. Data are represented as the mean \pm standard error of mean, and different groups were compared by using analysis of variance (ANOVA), with post hoc analysis using Fisher protected least significant difference test; paired comparisons were analyzed with a paired *t* test (Statview 5.0; SAS Institute, Inc, Cary, NC). A *P* value $\leq .05$ was considered to be statistically significant.

RESULTS

Optical Doppler velocimetry was used to measure orbital shear stress applied by the orbital shaker. Although the shear stress applied with the orbital shaker has previously been calculated to be uniform across the well,^{19,24} the fluid velocity and shear stress at the center and periphery of the well were found to be different (Fig 1, A). These center and periphery sampling regions correspond to the center and periphery regions of the well that were observed to have different EC morphology and were seeded differentially with cells (see below). Measurements were not accurate below 60 rpm because of the small amount of fluid motion, and measurements could not be made in the center of the well at or above 240 rpm because of centrifugal forces on the liquid, drying out the center. At 210 rpm, the setting used to generate shear stress for experiments, enough medium remained in the center to allow reproducible measurements; shear stress was higher in the periphery (11.1

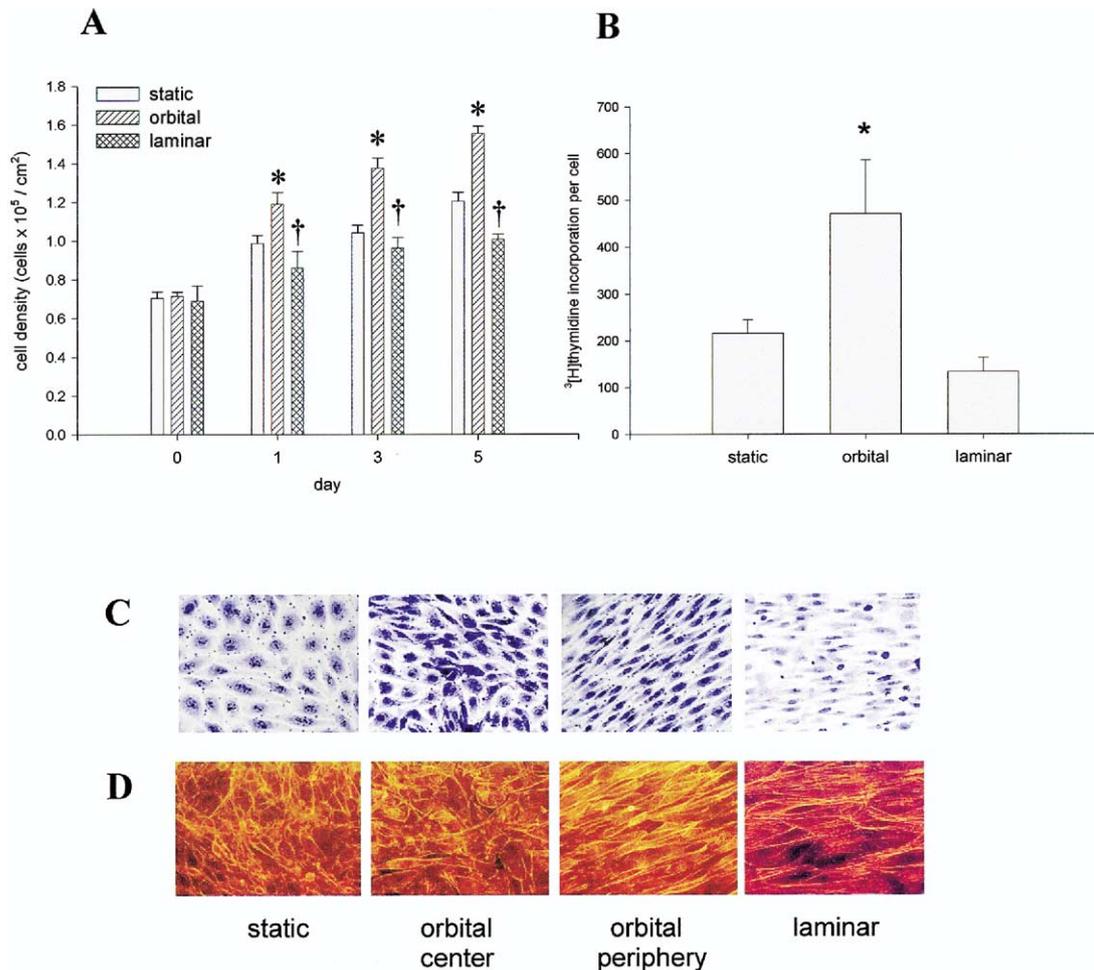


Fig 2. Differential proliferation and morphology of ECs under laminar and orbital shear stress. **A**, Bar graph shows the mean cell density under static ($n = 9$), laminar shear stress ($n = 4$), or orbital shear stress ($n = 5$) at 0, 1, 3, and 5 days. Error bars indicate the SEM. The difference between all groups of cells is significant ($P < .0001$, ANOVA). The difference in cell density between cells exposed to static or orbital shear stress is significant ($*P < .0001$, post hoc) as is the difference between static and laminar shear stress ($\dagger P = .002$). **B**, Bar graph shows the mean cell ^3H thymidine incorporation under static, laminar shear stress, or orbital shear stress for 24 hours before exposure to ^3H thymidine for 4 h ($n = 2-6$). Error bars indicate the SEM. The difference between all groups of cells is significant ($P = .03$, ANOVA); the increase in incorporation in cells exposed to orbital shear stress is significant ($*P = .02$, post hoc). **C**, Morphology of ECs after 5 days of culture. Panels are control ECs exposed to static conditions; ECs exposed to orbital shear stress, center of culture well; ECs exposed to orbital shear stress, periphery of culture well; ECs exposed to laminar shear stress. Flow is from left to right with laminar shear stress; the edge of the culture well is to the right of the panel for orbital shear stress. Original magnification, $\times 400$. Stained with crystal violet. **D**, Corresponding figures, stained for F-actin; original magnification, $\times 200$.

dyne/cm²) than in the center of the well (4.8 dyne/cm²), and the Reynolds' number was 2066 (Fig 1). There was little temporal variation in the shear stress (Fig 1, B).

Bovine aortic ECs were exposed to laminar or orbital shear stress or static conditions, and their cell density was assessed. Although ECs exposed to laminar shear stress exhibited 16% fewer cells/cm² at 5 days compared with control ECs not exposed to shear, ECs exposed to orbital shear stress exhibited 29% increased cell density compared to control ECs at 5 days (Fig 2, A). To determine whether

the increase in cell density with orbital shear stress was associated with an increase in DNA synthesis, the incorporation of ^3H thymidine into protein was determined. There was a two-fold increase of ^3H thymidine incorporation in cells treated with orbital shear stress compared to control cells, whereas there was a 38% decrease in ^3H thymidine incorporation in cells treated with laminar shear stress compared with control cells (Fig 2, B).

The morphology of ECs after exposure to static conditions, orbital shear stress, or laminar shear stress is demon-

strated in Fig 2, C. ECs exposed to static conditions were randomly aligned and uniformly polygonal, whereas ECs grown under laminar shear stress aligned to the direction of flow. ECs in the periphery of the culture well exposed to orbital shear stress were elongated and aligned in the direction of flow similar in appearance to the cells grown under laminar shear stress, although at an angle of approximately 34 ± 6 degrees from the line tangential to the edge of the well. This peripheral area had a radius of 0.8 ± 0.1 cm in a culture well of radius 1.7 cm. ECs in the center of the culture well (radius, 0.6 ± 0.1 cm) appeared similar to cells exposed to static conditions, randomly aligned and polygonal. A small transition zone (radius, 0.3 ± 0.1 cm) was present between the cells of the inside and outside areas that appeared to be composed of cells morphologically similar to both center and periphery cells (Fig 1, C). Similar experiments performed on ECs exposed to shear stress in smaller culture wells demonstrated similar morphologic changes, although with proportionally smaller zones (data not shown). Staining for F-actin demonstrated the effects of shear stress on the organization of the cell cytoskeleton, with alignment of the cytoskeleton to the direction of flow in ECs exposed to laminar shear stress and to ECs in the periphery, but not the center, of the well exposed to orbital shear stress (Fig 2, D). Similar morphologic changes were seen in HUVECs (data not shown).

Because ECs are exposed to different shear stress in the center and periphery of the well, cells were seeded exclusively in the center or periphery zones of the culture well (radius, 0.5 cm) by excluding cells seeded from the transition zone and the periphery or center of the well, respectively, with a removable silicone gasket before exposure to shear stress (Fig 1, C). EC proliferation under orbital shear stress was compared to static conditions for the separate cell zones (Fig 3, A). After 5 days of treatment, cells exposed to orbital shear stress demonstrated a mean increase of 28% in cell number compared to control ECs, similar to our previous data (Fig 2, A). However, ECs in the center of the well demonstrated an increase of 37% in cell number, whereas ECs in the periphery demonstrated a 23% decrease in cell number. To determine whether the increase in proliferation in center cells exposed to orbital shear stress, compared with the lack of increased proliferation in the periphery cells, was associated with an increase in DNA synthesis, the incorporation of ^3H thymidine into protein was determined in both center and periphery cells. Although there was an overall 87% increase of ^3H thymidine incorporation in cells treated with orbital shear stress compared to control cells, center cells demonstrated an increase of 80% and peripheral cells an increase of only 1% in ^3H thymidine incorporation per cell compared to control cells (Fig 3, B).

To confirm that center and periphery ECs exposed to orbital shear stress are different phenotypes, we examined the differential cell surface expression of E-selectin, a marker of EC activation whose translation is diminished by shear stress,^{27,28} in center or periphery cells exposed to orbital shear stress. ECs were exposed to TNF- α in the

presence of orbital shear stress to stimulate E-selectin expression. ECs in the center of the culture well demonstrated no reduction in surface E-selectin expression, similar to TNF- α stimulated cells, whereas ECs in the periphery of the culture well demonstrated reduced E-selectin expression (Fig 3, C). To further confirm the differences between cell surface expression between center and periphery cells exposed to orbital shear stress by using a marker that is induced by shear stress, rather than E-selectin that is down-regulated by shear stress, we examined the differential expression of ICAM-1 in response to shear stress.²⁹ In the absence of TNF- α , expression of ICAM was greater in center ECs compared to periphery ECs exposed to orbital shear stress; expression in center ECs was similar to ECs exposed to TNF- α and without shear stress (Fig 3, C). These results confirm the differences in phenotype between center and periphery ECs exposed to orbital shear stress.

To confirm that the stimulatory effect of orbital shear stress on EC proliferation that was demonstrated in ECs cultured exclusively in the center of the culture well (Fig 3, A) was not an artifact of differential culture, ECs cultured in the whole well were exposed to static or shear stress conditions and then stained for PCNA. ECs exposed to orbital shear stress demonstrated a greater number of cells that stained with PCNA compared to control cells; however, this increase in PCNA staining was confined to the center of the well, with diminished staining in the periphery (Fig 4). The diminished PCNA in the periphery of the well treated with orbital shear stress was similar to that seen in ECs treated with laminar shear stress (Fig 4). Similar results were found after up to 7 days of shear stress (data not shown). These results suggest that the difference in proliferation between center and periphery ECs exposed to orbital shear stress is not an artifact of differential culture.

To determine whether the increase in proliferation in the center of the culture well treated with orbital shear stress was accompanied by an increase in cell turnover, apoptosis was assessed by staining for TUNEL. There was an increase in TUNEL staining in cells in the center of the well, but not the periphery of the well, treated with orbital shear stress compared with static control cells (Fig 4). The diminished apoptosis in the periphery of the well treated with orbital shear stress was similar to the low level of apoptosis in ECs treated with laminar shear stress (Fig 4).

Because there was both increased proliferation and increased apoptosis in the center of the well of cells treated with orbital shear stress compared with cells in the periphery of the well, and activation of Akt is associated with increased cell survival, we determined whether center cells have reduced Akt phosphorylation compared to periphery cells. ECs seeded in the whole well and treated with orbital shear stress demonstrated increased Akt phosphorylation on serine 473 compared with control cells under static conditions (Fig 5). However, this increase in Akt phosphorylation was essentially confined to cells in the periphery of the culture well, with center cells having significantly reduced Akt phosphorylation compared to periphery cells. Periphery cells had increased Akt phosphorylation in re-

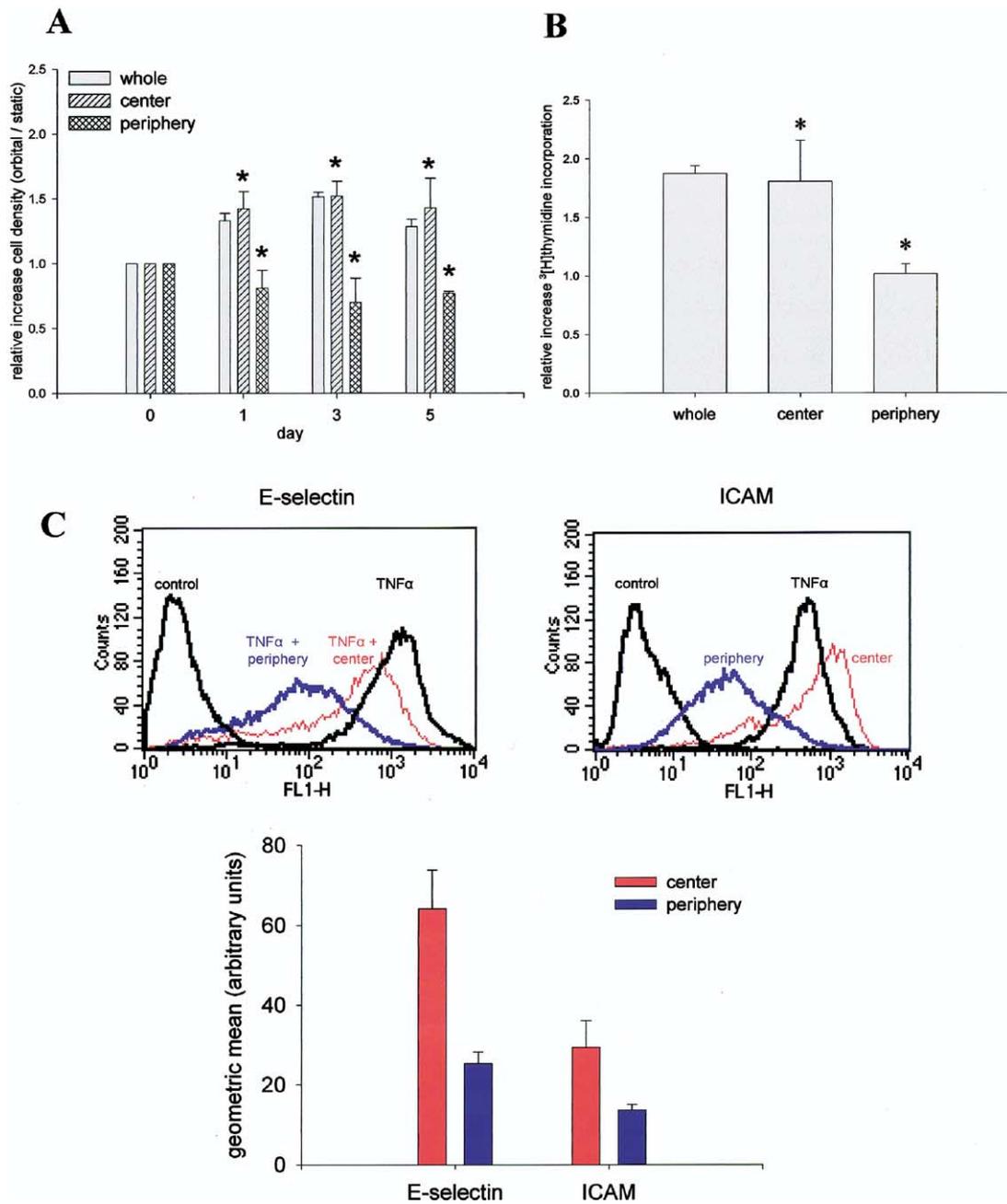


Fig 3. Differential proliferation and cell surface expression of center and periphery ECs with exposure to orbital shear stress. **A,** Bar graph shows the relative increase in mean cell density of matched groups of cells treated with shear stress compared to static control (n = 2-5). Error bars indicate the SEM. The difference between all groups of cells is significant ($P < .0001$, ANOVA). The increased proliferation due to orbital shear stress, compared to static control cells, was significant in cells seeded exclusively in the center, compared to the periphery, of the well ($*P < .0001$, post hoc). **B,** Bar graph shows the relative increase in ^3H thymidine incorporation of matched groups of cells treated with orbital shear stress compared to static control (n = 2-5). The difference between all groups of cells is significant ($P < .0001$, ANOVA). The increased incorporation due to orbital shear stress, compared to static control cells, was significant in cells seeded exclusively in the center, compared to the periphery, of the well ($*P = .03$, post hoc). **C,** Differential expression of E-selectin and ICAM on the surface of ECs exposed to 6 h of orbital shear stress. A representative analysis is shown (E-selectin, n = 5; ICAM, n = 13). Black lines, negative and positive controls (TNF, 0 and 10 ng/mL, respectively); red line, center cells; blue line, periphery cells. The E-selectin experiment is performed in the presence of TNF- α ; the ICAM experiment is performed in the absence of TNF- α . Bar graph reflects the mean of the geometric means of the center and periphery curves for both E-selectin and ICAM; the difference between the center and periphery curves for both E-selectin and ICAM is statistically significant ($P = .03$ and $.02$, respectively; paired t test).

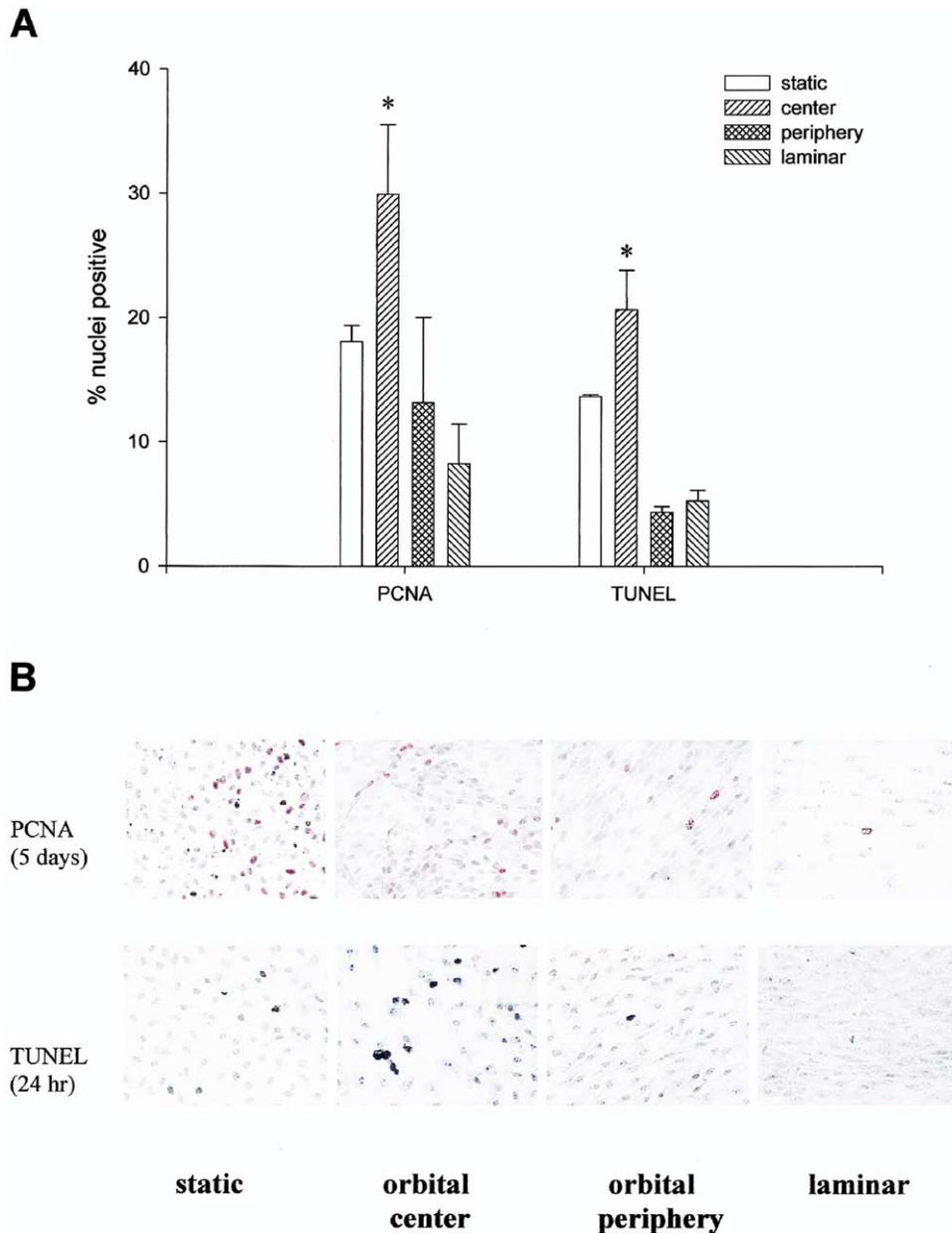


Fig 4. EC proliferation and apoptosis. **A,** Bar graph demonstrates the difference in percentage of cells positive for PCNA or TUNEL staining after 24 h (n = 2-6). For PCNA, the difference between all groups of cells is significant ($P = .04$, ANOVA), including the difference between center and periphery cells exposed to orbital shear stress ($*P = .03$, post hoc), but not between periphery cells exposed to orbital shear stress and cells exposed to laminar shear stress ($P = .43$, post hoc). For TUNEL, the difference between all groups of cells is significant ($P = .003$, ANOVA), including the difference between center and periphery cells exposed to orbital shear stress ($*P = .0005$, post hoc), but not between periphery cells exposed to orbital shear stress and cells exposed to laminar shear stress ($P = 0.72$, post hoc). **B,** Representative samples of PCNA staining after 5 days (*first row*), or TUNEL staining after 24 h (*second row*). Original magnification, $\times 400$. *First panel* is control cells; *second panel* is cells treated with orbital shear stress, center of well; *third panel* is cells treated with orbital shear stress, periphery of well; *last panel* is laminar shear stress.

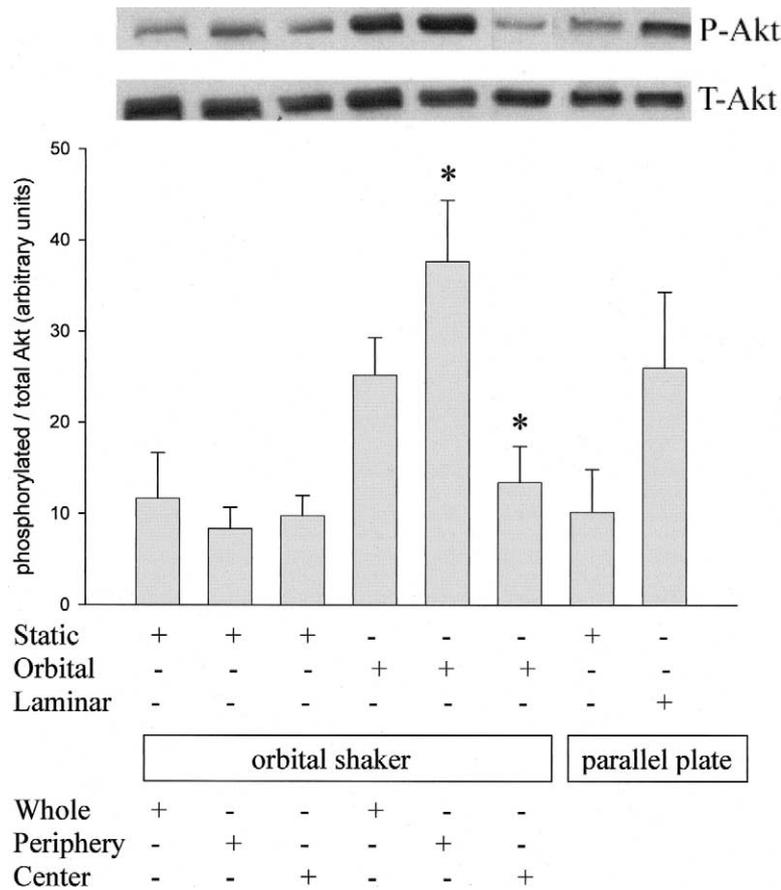


Fig 5. Akt phosphorylation with orbital and laminar shear stress. Bar graph represents the mean relative ratio of phosphorylated to total Akt, arbitrary units, after 30 min of static, orbital shear stress, or laminar shear stress, in serum-free medium (n = 3-5). A representative Western blot is shown. The relative difference in Akt phosphorylation between center and periphery cells exposed to orbital shear stress is significant (*P = .0005, post hoc).

sponse to orbital shear stress, similar to that seen in cells treated with laminar shear stress (Fig 5). These results suggest that the Akt pathway might play a role in the differential response to orbital shear stress between center and periphery ECs.

DISCUSSION

We demonstrate increased EC proliferation and apoptosis with orbital shear stress in the orbital shaker compared to decreased proliferation and apoptosis with exposure to laminar shear stress in the parallel plate. These differences suggest that orbital shear stress is not laminar but is disturbed or turbulent. In the orbital model, cells in the center of the well are exposed to lower magnitudes of disturbed or nonlaminar shear stress compared to cells in the periphery of the well that are exposed to higher magnitudes of shear stress. ECs in the center of the well have increased proliferation, increased apoptosis, reduced Akt phosphorylation, increased ICAM expression, and reduced E-selectin down-regulation, compared with ECs in the periphery of the well or those exposed to laminar shear

stress. These results suggest that ECs in the center of the well exposed to low magnitudes of orbital shear stress correspond to “activated” ECs.

Several commonly used in vitro models of shear stress include the parallel plate, the cone-and-plate, and the roller pump.^{3,30-32} Each of these models has particular drawbacks, including small number of cells and large fluid reservoir, complex and expensive apparatus, and inability to accurately model the in vivo circulation.^{32,33} The orbital shaker has been increasingly used to model complex, disturbed shear stress, with the ability to collect the small amount of conditioned medium, to perform chronic exposure studies, and to add a radioactive tracer to the disposable culture materials.^{18-20,24,34} The major drawback of the orbital shaker model has been the inability to accurately measure the shear stress to which the ECs are exposed, because recirculation currents in the well are complex, only allowing calculation of τ_{max} .

We used an integrated diffractive optics elements probe to measure the shear stress by analyzing the Doppler shift between the transmitted and received light as reflected off

TiO₂ particles suspended within the rotating fluid above it.²⁶ With this optical technology, similar in concept to laser Doppler velocimetry, we were able to measure the shear stress near the bottom surface of the cell culture well, presumably to what the cells are exposed. Although there might be a difference in shear stress because of differences between the TiO₂ solution used for the shear stress measurements and cell culture medium, these differences are likely to be small, because the density and viscosity of each liquid are similar. The TiO₂ solution was used for measurements because of its optical clarity and lack of variability from adsorbed solutes and protein to dissolved particles. Although the shear stress sensor samples a limited number of Doppler shifts within a finite measurement volume, its accuracy in measuring the shear stress magnitude is high (Fig 1). However, this technique cannot measure the intensity of turbulence reliably, and thus inferences regarding the laminar or turbulent quality of the shear stress cannot be made with this technique, but they must be made on the basis of the direction of shear stress and the Reynolds' number.

We verified that the shear stress generated by the orbital shaker in the periphery of the culture well in a 6-well plate at 210 rpm is similar to an arterial magnitude of shear stress (Fig 1). Because we measured reduced shear stress magnitude in the center of the culture well, it is likely that there is a continuous gradient of shear stress that varies along the radius of the well, with the exact center having the minimum shear stress magnitude and with increasing shear stress magnitude with distance to the periphery. We were able to measure the shear stress at only two points in the culture well, however, because of the size of the probe relative to the culture well. Because we also observe less cell alignment in the center cells, it is possible that there is also a gradient of shear stress directionality, with center cells exposed to maximal rotational variation compared to the more uniform shear stress direction in the periphery. Although this suggests that cells in the center of the well are exposed to greater flow disturbance than cells in the periphery, it is possible that the entire difference between center and periphery effects is due to the magnitude variation in shear stress. The significance of the lack of a temporal gradient in either the center or the periphery in this model is unclear.^{35,36} In addition, differences in pressure, inertial force, and complex three-dimensional flow patterns between center and periphery have yet to be defined.

Studies using laminar shear stress have greatly increased our understanding of the response of the EC to its environment,^{6,8} including the importance of shear stress directionality on EC gene expression.³⁷ However, there is less work on the response of the EC to complex flows such as turbulent shear stress. With the cone-and-plate viscometer, Davies et al¹³ demonstrated that ECs have increased DNA synthesis, lack of alignment, and increased turnover, mimicking an *in vivo* model.³⁸ Genomic analysis has confirmed the different patterns of transcriptional activity in response to turbulent and laminar shear stress in this model, including down-regulation of genes involved in structure and

contraction of the cytoskeleton compared with the up-regulation present under laminar conditions.^{15,16}

Previous work with the orbital shaker model has demonstrated decreased monocyte adhesion to ECs, EC release of nitric oxide and arachidonic acid, activation of the MAPK ERK 1/2 and of pp70^{S6k}, increased ³[H]thymidine uptake, increased expression of CDK1, CDK4, and Bcl-3, and inhibited translation of E-selectin.^{18,19,28,34,39} However, these studies have analyzed the overall response of all the ECs in the culture well exposed to orbital shear stress, without regard to subpopulations of cells. This is similar to our finding that cells exposed to orbital shear stress, taken as a whole, appear to have increased proliferation compared to cells exposed to either static conditions or laminar shear stress (Fig 2, A).

We describe two populations of ECs within the culture well under chronic conditions of orbital shear stress, as clearly noted by the difference in cell morphology between ECs in the center and periphery of the well (Fig 2, C). ECs in the periphery of the well are exposed and align to the relatively more unidirectional flow; the cells appear to be at a slight angle to the tangent of the well, likely reflecting the torque due to complex recirculating flow currents. Nevertheless, the cells appear similar in morphology and cytoskeletal alignment as ECs exposed to laminar shear stress conditions applied by using a parallel-plate chamber, which suggests that at least some EC intracellular signal transduction pathways are stimulated similar to the pathways stimulated on exposure to laminar shear stress. However, cells in the center of the well exposed to orbital shear stress are polygonal, with no clear alignment, and similar in morphology to ECs grown under static conditions. The polygonal cell shape of the center cells is similar to the shape of cells exposed to either turbulent shear stress¹³ or purely oscillatory shear stress in a parallel-plate chamber.^{14,17,40} The power of this model is that the periphery cells serve as an internal control for the center cells, because they are clearly seeded from the same original aliquot, but they appear to be exposed to different shear stress conditions. This model clearly demonstrates that ECs respond to different types of shear stress, because all other variables within each culture well are constant. The formation of two cell populations might explain the incomplete reduction of E-selectin expression previously described by using analysis of ECs seeded throughout the whole well.²⁸

Differentially seeding cells exclusively in the center or periphery of the well allows further characterization of these two cell populations. ECs in the periphery of the well appear morphologically similar to ECs exposed to laminar shear stress and have reduced proliferation and apoptosis; ECs in the center of the well appear disorganized like static control ECs and have increased proliferation and apoptosis. Although we excluded ECs within the small transition zone between the center and periphery of the well, it is possible that either population of differentially seeded cells is not completely pure. In addition, after 5 days we noticed some proliferation and migration of the ECs outwards from the edge into the unseeded bare area left after removal of the

silicone gasket; this might account for the somewhat higher baseline proliferation and ³[H]thymidine incorporation noted in the subpopulations, compared to the whole well, under static conditions (data not shown).

We demonstrate that center and periphery cells have differences in rates of proliferation and apoptosis and thus survival, likely reflecting a functional difference between center and periphery cells. Because center cells have an increased rate of proliferation and apoptosis, center cells might be a model of the “activated,” “atherogenic,” or “dysfunctional” EC phenotype.^{8,15,41,42} Additional differences might exist between the center and periphery cells. For example, differences in cytoskeletal components and/or organization might be present (Fig 2, D). Chronic laminar shear stress increases membrane components such as caveolae at the EC luminal surface.⁴³ Because the mechanism of shear stress mechanotransduction is still not well defined, differential localization of structural components, such as caveolae, between center and periphery cells might suggest potential areas of localization of putative mechanotransducing structures.

The increased proliferation and apoptosis in the center cells exposed to orbital shear stress are consistent with previous reports of cell turnover under turbulent shear stress.^{13,38} Increased cell turnover, ie, decreased cell survival, might contribute to the increased proliferation of center cells. It is not surprising that Akt phosphorylation is reduced in center cells, consistent with the role of Akt in cell survival.⁴⁴⁻⁴⁶ Laminar shear stress has been demonstrated to phosphorylate Akt in ECs in vitro; therefore, Akt is presumed to be a pathway by which shear stress inhibits apoptosis and promotes cell survival in vivo.^{22,47,48} Conversely, reduced Akt phosphorylation might be a mechanism by which the balance of intracellular pathways promoting cell death might be stimulated. For example, Akt activation in ECs has been demonstrated to affect caspase-9, nitric oxide synthase, and the MAPK pathways.⁴⁷⁻⁵⁰ We have previously demonstrated that shear stress and cyclic strain both stimulate Akt phosphorylation but might stimulate Bad phosphorylation, a downstream target of Akt, via different intracellular pathways.²² Future work in our laboratory is directed to addressing the downstream targets of Akt differentially expressed in center and periphery cells.

We describe a novel approach to compare EC responses to different types of shear stress by using differential seeding in culture wells and exposure to shear stress by using an orbital shaker. ECs in the center of the well exposed to orbital shear stress have an increased rate of proliferation and apoptosis similar to the “activated phenotype” that is thought to contribute to atherogenesis and neointimal hyperplasia.

We acknowledge the thoughtful comments and suggestions made by Michael Gimbrone, Guillermo García-Cardena, Alex Clowes, Bill Sessa, John Shyy, and Marshall Long. We acknowledge the inspiration and support of the

E.J. Wylie Memorial Traveling Fellowship, Lifeline Foundation (A.D.).

REFERENCES

1. Ballermann B, Dardik A, Eng E, Liu A. Shear stress and the endothelium. *Kidney Int* 1998;54:S100-8.
2. Sumpio B, Riley T, Dardik A. Cells in focus: endothelial cell. *Int J Biochem Cell Biol* 2002;34:1508-12.
3. Paszkowiak J, Dardik A. Arterial wall shear stress: observations from the bench to the bedside. *Vasc Endovasc Surg* 2003;37:47-57.
4. Levesque M, Nerem R, Sprague E. Vascular endothelial cell proliferation in culture and the influence of flow. *Biomaterials* 1990;11:702-7.
5. Dimmeler S, Haendeler J, Rippmann V, Nehls M, Zeiher A. Shear stress inhibits apoptosis of human endothelial cells. *FEBS Lett* 1996;399:71-4.
6. Lehoux S, Tedgui A. Signal transduction of mechanical stresses in the vascular wall. *Hypertension* 1998;32:338-45.
7. Lin K, Hsu P, Chen B, Yuan S, Usami S, Shyy J, et al. Molecular mechanism of endothelial growth arrest by laminar shear stress. *Proc Natl Acad Sci U S A* 2000;97:9385-9.
8. Traub O, Berk B. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol* 1998;18:677-85.
9. Bakker S, Gans R. About the role of shear stress in atherogenesis. *Cardiovasc Res* 2000;45:270-2.
10. Ku D, Giddens D, Zarins C, Glagov S. Pulsatile flow and atherosclerosis in the human carotid bifurcation: positive correlation between plaque location and low and oscillating shear stress. *Arteriosclerosis* 1985;5:293-302.
11. Malek A, Alper S, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. *JAMA* 1999;282:2035-42.
12. Zarins C, Giddens D, Bharadvaj B, Sottirai V, Mabon R, Glagov S. Carotid bifurcation atherosclerosis: quantitative correlation of plaque localization with flow velocity profiles and wall shear stress. *Circ Res* 1983;53:502-14.
13. Davies P, Remuzzi A, Gordon E, Dewey C, Gimbrone M. Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. *Proc Natl Acad Sci U S A* 1986;83:2114-7.
14. Helmlinger G, Geiger R, Schreck S, Nerem R. Effects of pulsatile flow on cultured vascular endothelial cell morphology. *J Biomech Eng* 1991;113:123-31.
15. Garcia-Cardena G, Comander J, Anderson K, Blackman B, Gimbrone M. Biomechanical activation of vascular endothelium as a determinant of its functional phenotype. *Proc Natl Acad Sci U S A* 2001;98:4478-85.
16. Topper J, Cai J, Falb D, Gimbrone M. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci U S A* 1996;93:10417-22.
17. Lum R, Wiley L, Barakat A. Influence of different forms of fluid shear stress on vascular endothelial TGF-beta1 mRNA expression. *Int J Mol Med* 2000;5:635-41.
18. Kraiss L, Ennis T, Alto N. Flow-induced DNA synthesis requires signaling to a translational control pathway. *J Surg Res* 2001;97:20-6.
19. Kraiss L, Weyrich A, Alto N, Dixon D, Ennis T, Modur V, et al. Fluid flow activates a regulator of translation, p70/p85 S6 kinase, in human endothelial cells. *Am J Physiol Heart Circ Physiol* 2000;278:H1537-44.
20. Dardik A, Yamashita A, Aziz F, Asada H, Sumpio B. Shear stress stimulated endothelial cells induce SMC chemotaxis via PDGF-BB and IL-1 alpha. *J Vasc Surg* 2005;41:321-31.
21. Yun S, Dardik A, Haga M, Yamashita A, Yamaguchi S, Koh Y, et al. Transcription factor Sp1 phosphorylation induced by shear stress inhibits membrane type 1-matrix metalloproteinase expression in endothelium. *J Biol Chem* 2002;277:34808-14.
22. Haga M, Chen A, Gortler D, Dardik A, Sumpio B. Shear stress and cyclic strain may suppress apoptosis in endothelial cells by different pathways. *Endothelium* 2003;10:149-57.

23. Haga M, Yamashita A, Paszkowiak J, Sumpio B, Dardik A. Orbital shear stress increases smooth muscle cell proliferation and Akt phosphorylation. *J Vasc Surg* 2003;37:1277-84.
24. Ley K, Lundgren E, Berger E, Arfors K. Shear-dependent inhibition of granulocyte adhesion to cultured endothelium by dextran sulfate. *Blood* 1989;73:1324-30.
25. Azuma N, Duzgun S, Ikeda M, Kito H, Akasaka N, Sasajima T, et al. Endothelial cell response to different mechanical forces. *J Vasc Surg* 2000;32:789-94.
26. Fourguette D, Modarress D, Taugwalder F, Wilson D, Koochesfahani M, Gharib M. Miniature and MOEMS flow sensors. In 31st AIAA Fluid Dynamics Conference and Exhibit. Anaheim, Calif, 2001.
27. Chiu J, Lee P, Chen C, Lee C, Chang S, Chen L, et al. Shear stress increases ICAM-1 and decreases VCAM-1 and E-selectin expressions induced by tumor necrosis factor- α in endothelial cells. *Arterioscler Thromb Vasc Biol* 2004;24:73-9.
28. Kraiss L, Alto N, Dixon D, McIntyre T, Weyrich A, Zimmerman G. Fluid flow regulates E-selectin protein levels in human endothelial cells by inhibiting translation. *J Vasc Surg* 2003;37:161-8.
29. Morigi M, Zoja C, Figliuzzi M, Foppolo M, Micheletti G, Bontempelli M, et al. Fluid shear stress modulates surface expression of adhesion molecules by endothelial cells. *Blood* 1995;85:1696-703.
30. Dardik A, Liu A, Ballermann B. Chronic in vitro shear stress stimulates endothelial cell retention on prosthetic vascular grafts and reduces subsequent in vivo neointimal thickness. *J Vasc Surg* 1999;29:157-67.
31. Dewey C, Bussolari S, Gimbrone M, Davies P. The dynamic response of vascular endothelial cells to fluid shear stress. *J Biomech Eng* 1981;103:177-85.
32. Samet M, Lelkes P. The hemodynamic environment of endothelium in vivo and its simulation in vitro. In: Lelkes P, editor. *Mechanical forces and the endothelium*. Amsterdam: Harwood Academic Publishers, 1999. p. 1-32.
33. Traub O, Yan C, Berk B. In vitro simulation of shear stress and mitogen-activated protein kinase responses to shear stress in endothelial cells. In: Lelkes P, editor. *Mechanical forces and the endothelium*. Amsterdam: Harwood Academic Publishers, 1999. p. 89-109.
34. Pearce M, McIntyre T, Prescott S, Zimmerman G, Whatley R. Shear stress activates cytosolic phospholipase A2 (cPLA2) and MAP kinase in human endothelial cells. *Biochem Biophys Res Commun* 1996;218:500-4.
35. Bao X, Clark C, Frangos J. Temporal gradient in shear-induced signaling pathway: involvement of MAP kinase, c-fos, and connexin43. *Am J Physiol Heart Circ Physiol* 2000;278:H1598-605.
36. Bao X, Lu C, Frangos J. Temporal gradient in shear but not steady shear stress induces PDGF-A and MCP-1 expression in endothelial cells: role of NO, NF kappa B, and egr-1. *Arterioscler Thromb Vasc Biol* 1999;19:996-1003.
37. Passerini A, Milsted A, Rittgers S. Shear stress magnitude and directionality modulate growth factor gene expression in preconditioned vascular endothelial cells. *J Vasc Surg* 2003;37:182-90.
38. Langille B, Reidy M, Kline R. Injury and repair of endothelium at sites of flow disturbances near abdominal aortic coarctations in rabbits. *Arteriosclerosis* 1986;6:146-54.
39. Tsao P, Lewis N, Alpert S, Cooke J. Exposure to shear stress alters endothelial adhesiveness: role of nitric oxide. *Circulation* 1995;92:3513-9.
40. Sorescu G, Sykes M, Weiss D, Platt M, Saha A, Hwang J, et al. Bone morphogenic protein 4-produced in endothelial cells by oscillatory shear stress stimulates an inflammatory response. *J Biol Chem* 2003;278:31128-35.
41. Bonetti P, Lerman L, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol* 2003;23:168-75.
42. Gimbrone M, Nagel T, Topper J. Biomechanical activation: an emerging paradigm in endothelial adhesion biology. *J Clin Invest* 1997;99:1809-13.
43. Boyd N, Park H, Yi H, Boo Y, Sorescu G, Sykes M, et al. Chronic shear induces caveolae formation and alters ERK and Akt responses in endothelial cells. *Am J Physiol Heart Circ Physiol* 2003;285:H1113-22.
44. Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem* 1999;274:16349-54.
45. Romashkova J, Makarov S. NF-KB is a target of AKT in anti-apoptotic PDGF signaling. *Nature* 1999;401:86-90.
46. Zhou H, Li X, Meinkoth J, Pittman R. Akt regulates cell survival and apoptosis at a postmitochondrial level. *J Cell Biol* 2000;151:483-94.
47. Dimmeler S, Fleming I, Fissthaler B, Hermann C, Busse R, Zeiher A. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;399:601-5.
48. Go Y, Boo Y, Park H, Maland M, Patel R, Pritchard K, et al. Protein kinase B/Akt activates c-Jun NH2-terminal kinase by increasing NO production in response to shear stress. *J Appl Physiol* 2001;91:1574-81.
49. Gratton J, Morales-Ruiz M, Kureishi Y, Fulton D, Walsh K, Sessa W. Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. *J Biol Chem* 2001;276:30359-65.
50. Hermann C, Assmus B, Urbich C, Zeiher A, Dimmeler S. Insulin-mediated stimulation of protein kinase Akt: a potent survival signaling cascade for endothelial cells. *Arterioscler Thromb Vasc Biol* 2000;20:402-9.