Shear Stress Regulates Forward and Reverse Planar Cell Polarity of Vascular Endothelium In Vivo and In Vitro

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Abstract—Cultured vascular endothelium displays profound morphological adaptations to shear stress that include planar cell polarity (PCP) that is directed downstream. Endothelial cells in blood vessels are also polarized; however, the direction of polarity is vessel specific, and shear-independent mechanisms have been inferred. The regulation of endothelial PCP is therefore controversial. We report that the direction of PCP in blood vessels is age and vessel specific; nonetheless, it is caused by shear-related regulation of glycogen synthase kinase-3β (GSK-3β), a profound regulator of endothelial microtubule stability. When GSK-3β is inhibited, PCP reverses direction. Endothelium is the only cell type studied to date that can reverse direction of polarity. Tight regulation of GSK-3β, microtubule dynamics, and cell polarity was also required for the striking morphological responses of endothelium to shear stress (cell elongation and orientation with shear). Finally, the cytoskeletal polarity displayed in blood vessels is associated with polarized (shear-directed) cell mitoses that have important effects on endothelial repair. Vascular endothelium therefore displays a novel mode of mechanosensitive PCP that represents the first example of a single cell type that can reverse direction of polarity. (Circ Res. 2006;98:939-946.)

Key Words: shear stress ▪ endothelium ▪ microtubules ▪ planar cell polarity

Vascular endothelial cells display profound morphological adaptations to shear stress that include cell elongation in the shear direction that is directed downstream. Endothelial cells in blood vessels are also polarized; however, the direction of polarity is vessel specific, and shear-independent mechanisms have been inferred. The regulation of endothelial PCP is therefore controversial. We report that the direction of PCP in blood vessels is age and vessel specific; nonetheless, it is caused by shear-related regulation of glycogen synthase kinase-3β (GSK-3β), a profound regulator of endothelial microtubule stability. When GSK-3β is inhibited, PCP reverses direction. Endothelium is the only cell type studied to date that can reverse direction of polarity. Tight regulation of GSK-3β, microtubule dynamics, and cell polarity was also required for the striking morphological responses of endothelium to shear stress (cell elongation and orientation with shear). Finally, the cytoskeletal polarity displayed in blood vessels is associated with polarized (shear-directed) cell mitoses that have important effects on endothelial repair. Vascular endothelium therefore displays a novel mode of mechanosensitive PCP that represents the first example of a single cell type that can reverse direction of polarity.
and align with shear when GSK-3β activity or microtubule dynamics were suppressed with exogenous inhibitors or mutant forms of the kinase. GSK-3β–related regulation of microtubules is therefore critical to morphological adaptations of endothelium to shear stress.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Imposition of Physiological Levels of Shear Stress
Porcine aortic endothelial cells were grown to 2 days postconfluence on glass slides or cover slips and then subjected to shear stress of 15 dyne/cm² in parallel plate flow chambers. When indicated, GSK-3β activity was inhibited by treatment with LiCl or SB415286. Nocodazole (100 mmol/L) or taxol (5 ng/mL) were used to disrupt or hyperstabilize microtubules, respectively.

Immunofluorescence
Immunofluorescence staining of cells was viewed by laser scanning confocal microscope. For studies of γ-tubulin localization, nuclei were counterstained with propidium iodide or Sytox green.

Analysis of Endothelial Cell Alignment With Shear Stress
Endothelial cell elongation in the shear direction was assessed by determining cell length:width ratios using a novel, simple, and accurate methodology that precludes inaccuracies inherent in “shape factor” determinations that rely on cell perimeter–area relations (see online data supplement).

Microtubule-Organizing Center Localization
MTOCs were grouped (see supplemental Figure I) as upstream of nucleus (scored 1), upstream of midpoint of nucleus (scored 2; includes cells scored 1), and downstream of the midpoint of the nucleus (scored 3), which included cells downstream of the nucleus (scored 4).

Polarization of Acetylated Microtubules
Cultures were immunostained against acetylated tubulin and α-catenin, and nuclei were counterstained with propidium iodide. An observer, blinded to whether shear direction was left-to-right or right-to-left, assessed the position of the majority of stable microtubules in each cell relative to the nucleus. Decoded data were presented as a percentage of cells with stable microtubules predominantly upstream versus downstream of the nucleus.

Immunoblotting
Ten micrograms of protein from cell lysates were subjected to polyacrylamide gel electrophoresis, and blots were probed with antibodies as indicated. Labeled secondary antibodies were visualized using enhanced chemiluminescence.

Microinjections
In 3 experiments, nuclei of at least 30 endothelial cells in postconfluent monolayers were microinjected with constitutively active GSK-3β (GSK3S9A). Coinjection of rhodamine-labeled dextran was used to localize microinjected cells. After 3 hours, flow of culture medium was initiated and cells were then examined after 24 hours of shear stress.

In Vivo Assessment of Cell Polarity
Blood vessels from adult and 5-week-old rabbits were immunostained en face. Some rabbits were treated with 7 mmol/L lithium carbonate in drinking water to inhibit GSK-3 for 48 hours before euthanasia.

Figure 1. Microtubule dynamics is required for shear-induced changes in endothelial cell shape. A through D, Immunofluorescence images of microtubules in endothelial cells. Microtubules projected throughout the cytoplasm except that the periphery of the cell was frequently spared (arrows in A and B). Without shear, cells display a polygonal, nondirectional morphology (A), whereas cells elongated in the direction of shear stress within 24 hours (B). Direction of shear stress in all micrographs is left to right. Both microtubules and dynamic instability were required for cell elongation, because treatment with nocodazole (C) or taxol (D), respectively, prevented cell elongation. E, Quadratification of cell length:width ratios indicated that the cells became 2.5 to 3 times as long as they are wide after 24 hours of shear and that the toxins that targeted microtubules fully prevented this response. *P<0.05; N=3/group.

To examine the effects of reversing flow/shear direction in vivo, rabbits were anesthetized and the midabdominal aorta was narrowed by 50% with 5-0 suture. These stenoses produce a downstream vortex that reverses flow near the endothelial surface for up to 10 vessel diameters downstream of the stenosis (Figure 6A). One week later, the rabbits were killed by barbiturate overdose and vessels segments in the vortex region were stained en face.

To test for polarized mitosis, 3- and 5-week-old rabbits were given intramuscular injections of bromodeoxyuridine (BrdUrd). En face BrdUrd immunostaining was performed after 24 hours. At this time, BrdUrd-labeled cells had completed mitosis, so that doublets of daughter cells were detected and the relative positions of the labeled nuclei indicated the direction of mitosis. Alternatively, cultured cells were pulse labeled with BrdUrd immediately before shear. These experiments were approved by the Animal Care Committee of the University Health Network and were conducted in accord with the standards of the Canadian Council on Animal Care.

Statistics
Sample sizes are presented in figure legends. Effects of drugs (nocodazole, taxol, SB415286) on cell length-width ratios were assessed using a Dunnett’s test. Differences in MTOC position after shear, and position relative to the nucleus of acetylated microtubules, were determined using Student’s t tests. P<0.05 was taken as statistically significant.

Results
Microtubule Dynamics Is Required for Shear-Induced Changes in Endothelial Cell Shape
Postconfluent endothelial cells elongated in the direction of shear stress within 24 hours, with mean cell length in the shear direction becoming 2.5 to 3 times cell width (Figure 1A, 1B, and 1E). Microtubules in these cells emanated from the MTOC and projected throughout the cytoplasm, except that the perijunctional region was frequently spared (Figure 1A and 1B). These filaments became highly oriented in the direction of shear stress. Microtubules are required for cell elongation, because treatment with nocodazole or taxol, respectively, prevented cell elongation.
Microtubules can be stabilized by posttranslational modifications to tubulin. Acetylation of tubulin coincides with stabilization, and it can be detected with an acetylation-specific antibody. Immunoblots detected low levels of acetylated tubulin in postconfluent monolayers of endothelium that were not exposed to shear stress; however, the acetylated form was dramatically upregulated by 1 hour of shear stress, and further increases were seen by 24 hours (Figure 3A). When the same antibody was used for immunofluorescence studies, only a sparse population of perinuclear microtubules was labeled in static cultures (Figure 3B), whereas extensive labeling of much of the microtubule population was detected after 24 hours of shear (Figure 3C). Stabilized microtubules were found predominantly downstream of the cell nucleus in sheared cells (Figure 3D). These findings indicate major modifications to microtubules under the influence of shear stress. Importantly, the cells have completed shape change by 24 hours, so polarity and microtubule stability are not associated with directional motility at this time.

Figure 3. Shear stress induces profound stabilization of microtubules. A, Western blot probed with an acetylated tubulin (ac-tubulin)-specific antibody demonstrates that shear stress dramatically upregulates this manifestation of microtubule stability. When the antibody was used for immunofluorescence staining of endothelial cells, only a very sparse, perinuclear distribution of stable microtubules (green) was observed in static cultures (B). Cell junctions were immunostained for α-catenin (red) and nuclei were counterstained with propidium (also red). Shear stress induced the formation of an elaborate network of stable microtubules (C) and assessment by an observer blinded to flow direction confirmed that the stable microtubules were predominantly downstream of the nucleus (D). *P<0.05; N=3 for static cultures and N=4 for sheared cultures.

GSK-3β Regulates Stabilization and Polarization of Microtubules by Shear Stress

GSK-3β regulates planar cell polarity in migrating cells; furthermore, this kinase suppresses microtubule stability by phosphorylating multiple microtubule stabilizing proteins. We therefore assessed the role of GSK-3β in endothelial cell responses to shear stress. Western blots failed to detect the inactive (phosphorylated) form of GSK-3β (p-GSK-3β) in static cultures (Figure 4A). In contrast, p-GSK-3β was readily detected in sheared cells over a time course that matched that of tubulin acetylation, except that p-GSK-3β waned at 24 hours, when tubulin acetylation was maximal (Figures 3A and 4A). Total GSK levels were insensitive to shear stress in these experiments (not shown).
To test whether GSK-3β/H9252 regulates morphological responses to shear stress, we exposed cells to shear with and without treatment with the GSK-3β/H9252 inhibitors LiCl and SB415286. Cell elongation in response to shear stress was totally blocked by GSK-3β/H9252 inhibition (Figure 4B through 4D). Our findings that GSK-3β/H9252 is suppressed by shear stress, whereas total inhibition using exogenous inhibitors of GSK-3β blocks shear-induced changes in cell morphology, indicate that tight control over this kinase is critical to endothelial responses to shear. Accordingly, cells failed to 

elongate after nuclear microinjection of vectors expressing constitutively active GSK3S9A (Figure 4E and 4F). Polarity of MTOCs also was compromised, ie, 57% of MTOCs were downstream of the nuclear midpoint of microinjected cells, compared with more than 90% of uninjected cells. As expected, we also observed a sparser distribution of acetylated microtubules in cells expressing GSK3S9A. Microinjection of a kinase dead construct (GSK3K85A) was without effect, consistent with reports that this mutation does not yield dominant-negative function (J.A. Woodgett, personal communication, 2005).

Given the importance of microtubule dynamics in control of cell shape and the capacity of GSK-3β to regulate microtubule stabilization in other cells, we examined the capacity of GSK-3β to influence microtubule stability in unsheared endothelium. Treatment with LiCl (or SB415286; supplemental Figure II) to inhibit GSK-3β caused the distribution of stable microtubules to convert from a sparse perinuclear expression (Figure 3B) to an elaborate network (Figure 5A). This stabilization resulted in extensive elongation of microtubules, so that the total microtubule population circled the cell periphery in a toroid pattern (Figure 5B).

We asked whether microtubule polarity, as indicated by MTOC position, was affected by inhibition of GSK-3β. Based on studies of migrating cells, we anticipated randomization of MTOC position after shearing cells in the presence of an exogenous inhibitor.20 Intriguingly, polarity was reversed by this treatment (Figure 2C and 2D), ie, the MTOCs redistributed to the upstream side of the nucleus after initiation of shear. These findings suggest that shear stress induces competing, GSK-3β-dependent and GSK-3β-independent, modes of polarity control in endothelium.

### Endothelial Cell Polarity is Age and Vessel Specific In Vivo

MTOCs in the thoracic aorta and vena cavae of rats and pigs localize to the heart side of the nucleus (upstream in arteries, downstream in veins).11,21 The same is true for carotid arteries and vena cavae of rabbits, although polarity declines with age in the latter vessel, and it is not evident in thoracic aortas in this species.11,22 These findings led to the inference that endothelial polarity in vivo is cardiocentric and independent of blood flow. Although we confirmed that MTOCs were downstream of nuclei in the vena cava of the adult rabbit,

![Figure 4. Tightly regulated inhibitory phosphorylation of GSK-3β is required for endothelial cell shear-induced shape change. Western blots with a phosphorylation-specific antibody failed to detect the inactive form of GSK-3β in static cultures (A), whereas phosphorylation was readily detected in sheared cells (B) over a time course that resembled that of tubulin acetylation (see text and Figure 3A). An exogenous inhibitor of GSK-3β, SB415286, prevented elongation of sheared endothelial cells (C and D). *P<0.05; N=3/group. E and F, Sheared cells failed to elongate after nuclear microinjection of vectors expressing constitutively active GSK3S9A. Images display immunofluorescence staining of tubulin (E) or fluorescence detection of Rhodamine dextran (F), which was co-injected with the construct to identify microinjected cells (arrows). Failure to elongate of cells contiguous with microinjected cells (lower left and right) was common. N=3 per experiment.](image-url)

![Figure 5. GSK-3β regulates microtubule stability in endothelium. A, When endothelial cells not exposed to shear stress were treated with lithium to inhibit GSK-3β, the population of stabilized microtubules was dramatically upregulated (A) (compare with Figure 3B); furthermore, inhibition of GSK-3β caused hyper-elongation of the total population of microtubules, so that this network of filaments circled the cell periphery in a toroid pattern (B). N=10/group.](image-url)
upstream in the adult carotid artery and unpolarized in the adult thoracic aorta (data not shown), we also found that endothelium of the abdominal aorta reversed polarity with age; MTOCs were downstream of nuclei in 5-week-old rabbits and upstream in abdominal aortas of mature rabbits (Figure 6A and 6B, solid bars). MTOCs were distributed predominantly downstream of the nucleus in the vena cavae of young (Figure 6D) and mature (not shown) rabbits. These findings indicate that polarity of endothelium in vivo is vessel and age specific, and it is not exclusively cardiocentric.

Endothelial Cell Mitoses Also Polarize in the Shear Direction

Polarization of cell morphology and the microtubule system may prime cells for polarized mitosis; therefore, we also tested whether spontaneous mitoses of endothelial cells in immature rabbits were consistently unidirectional. When S-phase cells were labeled in vivo with BrdUrd and endothelial surfaces of arteries were prepared for imaging 24 hours later, the relative positions of the 2 daughter cells was consistently along the vessel axis. Figure 7A illustrates this feature in an in vivo preparation of the carotid artery of a 3-week-old rabbit; however, identical results were obtained in aortas and carotid arteries of 5-week-old rabbits. This response was genuinely flow derived because nonaxial mitoses were observed in the blind-ended stumps of carotid arteries that had been ligated (Figure 7B); furthermore, mitosis were flow aligned in vitro as well as in vivo. Indeed, even though in vitro flow was initiated (immediately) after exposure to BrdUrd, cell division was aligned with shear (Figure 7C through 7E). This alignment included cells that, after 24 hours of shear stress, were in telophase (Figure 7C), cytokinesis (Figure 7D), or were postmitotic (Figure 7E).

Endothelial Cell Polarity Is Shear Dependent In Vivo

Our finding that, under different conditions, shear stress can induce either upstream or downstream polarization of MTOCs in cell cultures led us to examine its influence on MTOC polarity in intact blood vessels. We surgically produced 50% narrowing (stenosis) of the midabdominal aorta of both 5-week-old and mature rabbits. This procedure causes a vortex to form downstream of the stenosis, and we confirmed previously that blood flow near the vessel wall, and therefore wall shear stress, was directed toward the heart for many vessel diameters distal to the stenosis (Figure 8A). Flow reversal at the vortex site eliminated the downstream polarity of the MTOCs in immature abdominal aortas and the upstream polarity in mature vessels (Figure 8B and 8C). A slight reversal of these polarities was not statistically significant. These findings indicate that polarization of endothelial MTOCs in vivo is shear dependent.

Figure 6. MTOC polarity in vivo is vessel specific, age dependent, and GSK-3β dependent. A, En face, whole-mount immunofluorescence staining for γ-tubulin (MTOC) in endothelium of abdominal (abd.) aorta of 5-week-old rabbit (Sytox green nuclear counter-stain). B through D, Graphs of quantification of cell polarity for endothelial cells in blood vessels (see Materials and Methods and legend of Figure 2). Solid bars represent data from untreated rabbits and open bars are data from lithium-treated animals. Lithium treatment for 48 hours reversed the upstream polarization of endothelium in the abdominal aorta of adult animals (B), and it suppressed the downstream polarity of endothelium of the abdominal aortas (C) and vena cavae (v.c.) (D) of 5-week-old rabbits. *P<0.05, control vs lithium treated. N=3 per experiment.

Figure 7. Shear stress induces polarized mitosis of endothelial cells. BrdUrd immunohistochemistry illustrating nuclei of daughter cells that were labeled in S phase of the cell cycle and fixed 24 hours later (arrows). Polarization of the axis of mitosis was seen in the carotid arteries of a control 3-week-old rabbit (A), and in 5 week old rabbits (not shown), but not in a rabbit 1 week after carotid ligation at 5 weeks of age (B). Polarized mitoses were also seen when cultured cells were labeled with BrdUrd and then sheared for 24 hours. Cells that were in telophase (C), cytokinesis (D), or were postmitotic (E) at the time of fixation are shown. N=3/group.
Effects of GSK-3
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This study has shown that shear stress induces tight control
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The flow dependence of endothelial cell polarity both in vivo
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(supplemental Figure III). These findings confirmed that
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Discussion
This study has shown that shear stress induces tight control
over microtubule organization, stability, and function and that
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We confirmed that MTOCs displayed downstream polarity
when endothelial cells were subjected to shear stress,4 and we
showed that shear induces marked stabilization of microtu-
bules as evidenced by tubulin acetylation. Although the
effects were profound, stabilization must be tightly regulated
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bule stabilizing agent, taxol, blocked cell morphological
responses to shear stress. These findings demonstrate that
shear stress induces primary manifestations of PCP.

Because shear had major effects on microtubules that
proved important to cell morphological responses, we exam-
ined regulation of microtubule modifications. When cells
migrate at wound edges, integrins are activated at the cell
anterior, as new cell–substrate interactions occur, and down-
stream activation of Cdc42 leads to the anterior assembly of
a complex that includes Cdc42 and the scaffold protein, Par6,
atypical protein kinase C-ζ (aPKCζ), and GSK-3β. An
outcome of this assembly is inhibitory phosphorylation of
GSK-3β, which is pivotal to establishment of PCP.20 GSK-3β
is multifunctional, but its roles in PCP appear related to its
capacity to dissociate APC from microtubule + ends.20 How
inhibition of GSK-3β, and the resulting association of APC
with the peripheral tips of microtubules, effects microtubule
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bule binding to proteins/structures at the cell anterior that
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Based on the pivotal role of GSK-3β in PCP of migrating
cells, we examined its role in shear-induced responses. Shear-
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link. At late time points, GSK-3β phosphorylation waned,
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periphery in a toroid pattern. Again, tight regulation, rather
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cell morphological responses because the cells failed to
e-longate when the activity of the kinase was blocked using
exogenous inhibitors. Regulation of intracellular gradients,
as well as absolute activity, of GSK-3β is probably required.20

Surprisingly, exogenous inhibitors of GSK-3β activity did
not suppress MTOC polarity, as occurs in migrating cells,21
they reversed it so that cells polarized in the upstream
direction. Recent work indicates that at least some epithelial

Figure 8. Upstream and downstream polarities of endothelial
microtubules in blood vessels are flow dependent. A, Constriction
(stenosis) of the abdominal aorta was used to create a vortex
and, thereby, establish retrograde flow near the downstream
vessel wall as previously described.14 Graphs indicate that both
the polarization of MTOCs away from the heart in the abdominal
aortas of 5-week-old rabbits (B) and the polarization toward the
heart in the abdominal aortas of adult rabbits (C) are sup-
pressed when flow is reversed in the vortex downstream from
stenoses. Solid bars indicate unmanipulated animals, open bars
indicate animals with stenoses. *P<0.05, control vs stenosis.
N=5/group.

Endothelial Cell Polarity Is Regulated by GSK-3β
In Vivo
The flow dependence of endothelial cell polarity both in vivo
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direction. Recent work indicates that at least some epithelial
cells display posterior PCP when migrating; however, this is the first example of a single cell type that can reverse direction of polarity. Endothelium may therefore provide an informative model for studying directional polarity control. We infer from this observation that endothelial cells can undergo GSK-3β-independent, shear-induced reverse PCP that is unmasked by the inhibitors.

Reversal of shear-induced microtubule polarity by inhibition of GSK-3β was particularly interesting given that, in some species, endothelial MTOCs are upstream of nuclei in arteries and downstream in veins.10,11,21 This previous observation was interpreted as indicating flow independence of endothelial cell polarity and novel alternatives were proposed, including sheet-like migration of peripheral vascular endothelial cell polarity and novel alternatives were proposed—posed, including sheet-like migration of peripheral vascular endothelium toward the heart.25 We found, however, that cardiocentric polarity was not ubiquitous in vivo; furthermore, polarity in the abdominal aorta of rabbits reversed with age. This finding, plus our observation that shear can induce upstream or downstream polarity in vitro, depending on p-GSK-3β levels, prompted us to directly assess the influence of shear on endothelial polarity in vivo. Experimental reversal of blood flow in the vortex that forms downstream from stenoses in vivo eliminated endothelial polarity, regardless of whether polarity was directed upstream or downstream in the unmanipulated artery. A modest reversal of polarity was not statistically significant, which may reflect either the less pronounced polarity that is observed in vivo or the precise nature of shear stress and its temporal variations downstream from stenoses. Nonetheless, these findings demonstrate that endothelial cell polarity in blood vessels is shear dependent.

Endothelial cell polarity was GSK-3β dependent in blood vessels, as it was in cell culture. This hypothesis was testable because lithium administration, to inhibit GSK-3β activity, is frequently used to treat bipolar disorder in humans.26 Lithium given in drinking water to rabbits suppressed or reversed polarity in both arteries and veins. Failure to always observe reversal of downstream polarity, as was seen in cell cultures, may indicate that circulating lithium levels fluctuated substantially when the rabbits were given lithium-treated water ad libidum, so that GSK-3β may have transiently but repeatedly escaped inhibition.

How microtubules influence morphological adaptations to shear stress is unclear. While the cell is changing shape, polarized microtubules might deliver cell membrane, protein and other cargoes to the cell poles (downstream pole in vitro) as well as regulate disassembly of substrate adhesion complexes. However, this simple interpretation is not readily reconciled with our previous finding that the actin assembly that drives shear-induced cell elongation proceeds with equal frequency in the upstream and downstream directions.1 Furthermore, polarization and stabilization of microtubules persisted at 24 hours of shear, after shape change had reached completion.1 The latter finding suggests that microtubule functions in planar cell polarity go beyond contributing to motility. Additional functions are unknown, although we have argued that the highly oriented distribution of the cytoskeleton and adhesion complexes in cells elongated by shear represents a low entropy state and that the organization of these dynamic structures must undergo constant renovation to prevent drift toward random orientation. The persistent interactions of highly stabilized and oriented microtubules with adhesion complexes might contribute to continuous maintenance of these structures, as might the steady state actin assembly/turndover that we described previously.1

The high degree of PCP displayed by endothelial cell morphology and cytoskeleton led to the hypothesis that mitosis would exhibit directional bias as well. Accordingly, spontaneous mitoses in the arterial endothelium of young, rapidly growing rabbits ubiquitously displayed mitosis along the shear vector. Shear dependence was confirmed when polarized mitoses were observed in sheared endothelium in vitro and when disruption of blood flow by carotid ligation caused orientation of mitoses to randomize in vivo. These findings indicate that mechanical forces may be potent regulators of directional tissue growth and repair. Possibly, mitotic spindle formation during polarized mitosis is associated with the biased distribution of microtubules that is induced by shear stress; alternatively, there may be an interplay between forces generated by mitotic microtubules and associated motor proteins at the cell cortex that align mitosis27 and externally applied forces that are transmitted to these structures.

These adaptations of endothelium to shear may have implications for important vascular pathologies. For example, endothelial denudation when arteries are traumatized by angioplasty or stent implantation contributes to restenosis; consequently, rapid repair of endothelium is important to arterial integrity. Shear-induced endothelial cell polarity directs cell elongation and proliferation preferentially in the long axis of the vessel, the effects of which are vividly demonstrated by examining outgrowth of endothelium from branches of denuded arteries, which proceeds much more rapidly along the vessel axis than circumferentially.28 If substantial lengths of vessel are denuded, a bias in cell motility and mitosis in the lengthwise direction will accelerate repair.

Furthermore, continual microtubule-dependent remodeling of the endothelial monolayer in response to changing blood flow conditions, and associated disruption of cell–cell junctions,3 may contribute to high endothelial permeability in regions of complex blood flow at arterial branch sites and thereby contribute to the predilection of these sites for atherogenesis.29 At these locations, magnitude and direction of shear stress changes are caused by exercise, circadian rhythms in cardiac output, or longer-term changes in cardiovascular function that are associated with exercise regimens, reproductive cycles, pregnancy, and disease states.30

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References


Shear Stress Regulates Forward and Reverse Planar Cell Polarity of Vascular Endothelium In Vivo and In Vitro
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ONLINE SUPPLEMENT

DETAILED METHODS

Antibodies and reagents

Primary antibodies used in these studies were raised against α-catenin (SC1497, Santa Cruz Biotechnologies; Santa Cruz, CA); α-tubulin and γ-tubulin (T9026 and T6557, respectively, Sigma (Oakville, Canada)); GSK-3β (Upstate, Charlottesville, VA) and phospho-GSK-3β (ser 9) (Cell Signaling, from NEB, Pickering, Canada), and acetylated tubulin (Sigma). Nuclear stains included Sytox green (Molecular Probes, from Invitrogen, Burlington, Canada) and propidium iodide (Calbiochem, La Jolla). Secondary antibodies included Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes), which was used to detect total and acetylated tubulins, and CY3-conjugated donkey-anti-mouse IgG (Jackson Immunoresearch, from BioCan, Mississauga, Canada), which was used to detect α-catenin and γ-tubulin. Nocodazole and taxol were purchased from Sigma and the GSK-3 inhibitor, SB415286, was purchased from BioMol (Plymouth Meeting, PA). Other chemicals were from Sigma unless otherwise specified.

Imposition of physiological levels of shear stress

Endothelial cells were grown to confluence on glass slides (Corning, Corning, NY) in Medium 199 containing antibiotics and 10% fetal bovine serum (Invitrogen). At 2 days post-confluence, cells were subjected to laminar fluid shear stress of 15 dynes/cm² in a parallel plate flow chamber that was perfused by gravity feed from a glass reservoir. Cells maintained in static culture served as controls. When indicated, GSK-3β activity
was inhibited by pre-incubating cultures 2 hours with 30 mM LiCl or 50 mM SB415286 and cells were then sheared for 24 hours in the presence of inhibitor.\textsuperscript{2,3} To disrupt microtubules or microtubule dynamics, cultures were pre-incubated for 1h with either nocodazole (100 mM) or taxol (5 ng/mL), respectively, and then sheared 24 hours in the presence of these reagents.

**Immunofluorescence**

Endothelial cells were fixed with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; 2 mM NaH\textsubscript{2}PO\textsubscript{4}, 8 mM Na\textsubscript{2}HPO\textsubscript{4}, 150 mM NaCl, 0.1 mM CaCl\textsubscript{2}, 0.1 mM MgCl\textsubscript{2} [pH 7.4]) for 20 minutes at room temperature, washed and then permeabilized with 0.2% Triton X-100. Alternatively, cultures were fixed with methanol for 2 minutes at -20°C. Cells were incubated with primary antibodies for one hour, washed with PBS, and incubated with fluorophore-conjugated secondary antibodies for 30 minutes. Samples were examined using a Bio-Rad 1024 laser scanning confocal microscope (Nikon X60 oil-immersion objective, NA = 1.4 oil). For studies of γ-tubulin localization, nuclei were counter-stained with propidium iodide (cell cultures) or Sytox green (\textit{in situ}).

**Analysis of endothelial cell alignment with shear stress**

Cultures of endothelial cells exposed or not exposed to shear stress were immunostained against α-catenin to label cell-cell junctions and images were collected using laser scanning confocal microscopy. A rectangular grid, with lines parallel and perpendicular to shear stress (parallel and perpendicular to the long axis of the slide for unsheared cultures), was placed over each field and the number of cells/mm that were intersected by
lines parallel to shear was counted (cells/mm parallel to shear stress). The inverse of this number (mm/cell) provided the mean cell dimension in the direction of shear stress. Because cells elongate in the direction of shear stress, this dimension was referred to as mean cell length. Mean cell dimension in the direction perpendicular to shear stress (mean cell width) was determined in a similar manner. The mean length:width ratio of the cells was then determined from fifteen fields for each experiment. This simple method for assessing directional elongation of cells in monolayers avoids limitations of "shape factors" that are based on cell area/perimeter relations. These limitations are related to indeterminacy of cell perimeter due to its fractal nature.

**Microtubule-organizing centre localization**

Static cultures were immunostained against γ-tubulin to define the location of the MTOC and the nuclei were counterstained with Sytox Green. Alternatively, cultures were sheared 24 hours with or without treatment with the GSK-3 inhibitor, SB415286 (50μM). The MTOC were grouped as upstream of nucleus (position 1), upstream of midpoint of nucleus (position 2), downstream of the midpoint of the nucleus (position 3) or downstream of the nucleus (position 4)(see Online Figure 2).

**Polarization of acetylated microtubules**

Static or sheared (24h) cultures sheared were immunostained against acetylated tubulin and α-catenin, and nuclei were counterstained with propidium iodide. An observer, blinded to whether shear direction was left-to-right or right-to-left, assessed the position of the majority of stable microtubules in each cell relative to the nucleus. Decoded data
was presented as % of cells with stable microtubules predominantly upstream versus downstream of the nucleus.

**Immunoblotting**

Cell lysates were collected from static and sheared cultures. Cells were washed twice with ice-cold PBS and lysed at 4°C in lysis buffer (50mM tris base, 1 protease inhibitor tablet (Roche, Laval, QC) per10 mL buffer, 0.3M NaCl, 1mM EDTA, 50mM sodium fluoride, 1mM sodium orthovanadate, 1mM PMSF, diluted in PBS: 2mM NaH₂PO₄, 8mM Na₂HPO₄, 150mM NaCl, 0.1mM CaCl₂, 0.1mM MgCl₂ [pH 7.4] (Sigma, Oakville, On)). Ten µg of protein was electrophoresed on a 10% polyacrylamide gel and transferred to PVDF membranes (Bio-Rad, Mississauga, Canada) that were probed with primary antibodies raised against phospho-GSK-3β (ser 9), or acetylated tubulin (Sigma), followed by incubation with the appropriate secondary antibody (IgG conjugated to horseradish peroxidase). Membranes were visualized using ECL+ (Amersham Life Science, Baie D'Urfe, Canada) according to manufacturer's instructions.

**Plasmids and Microinjections**

Endothelial cells were grown on glass slides until 2 days post-confluence. GSK⁵⁹A or GSK⁸⁵A, cloned into pcDNA3 (gift from J.R Woodgett), were injected into the nuclei of cells (10 µg/mL) using an Eppendorf transjector 5246. Texas Red-conjugated dextran (70,000 MW, Molecular Probes) was co-injected with the plasmid at a concentration of 10 mg/ml, as a marker of microinjection. Three hours later, cells were subjected to shear stress for 24h.
In vivo assessment of cell polarity

Adult and 6-week-old male New Zealand white rabbits (bw = 3.0 kg and approx. 900 g) were euthanized by barbiturate (Pentobarbital Sodium, Bimeda-MTC Animal Health Inc.) overdose. The vessels were excised and immersion fixed in -20°C 100% methanol for 4 minutes and immunostained \textit{en face} as described under Immunofluorescence. Some rabbits were treated with lithium (7mM lithium carbonate in drinking water) to inhibit GSK-3 for 48h before euthanasia.\textsuperscript{2,4}

We also examined the effects of reversing flow/shear direction in the abdominal aorta. Full surgical anaesthesia of 6-week-old and adult rabbits was induced, as previously described,\textsuperscript{5} by intramuscular xylazine (0.8 mL/kg, 2 mg/mL; Bayer, Toronto) and ketamine (90 mg/mL; Bioniche Animal Health Canada, Belleville, Canada) and then anaesthesia was maintained by intravenous infusion of this anaesthetic (0.02-0.03 ml/min). Pendure Neat (Langford, Guelph, Canada; 150,000 I.U./mL benzathine penicillin G, 150,000 I.U./mL procaine penicillin G, 0.9 mg methylparaben and 0.1 mL propyl paraben) was injected intra-muscularly prior to surgery and 0.1 mL of the analgesic, Temgesic (Schering-Plough, UK; 0.3 mg/mL buprenorphine HCl), was injected subcutaneously 1h after surgery. Under sterile conditions, the abdominal aorta was exposed by a midline incision and the vessel diameter was narrowed by approximately 50% by partial ligation with 5-0 suture.\textsuperscript{6} The suture was tied, figure-of-eight fashion around the artery and the shaft of a 16G hypodermic needle so that the vessel was occluded. The needle shaft was then removed so that the artery opened at the suture point to an internal diameter that equaled the external diameter of the needle.
Incisions were closed in layers and the animals were monitored daily. We previously showed that these stenoses produce a downstream vortex that reverses flow near the endothelial surface for up to 10 vessel diameters downstream of the stenosis (shown in Figure 6A). One week later, the rabbits were killed by barbiturate overdose (Pentobarbital Sodium) and vessels were fixed by immersion in methanol. Vessels segments extending 4-14 mm downstream of the stenosis were stained en face for γ-tubulin and cell nuclei were counter-stained with Sytox green. Over this segment of vessel, reverse flow is detected at the vessel wall throughout systole.

Finally, we tested whether polarized morphology and cytoskeleton of endothelium in vivo translated into polarized mitosis. To do so, 3-week-old and 6-week-old rabbits were given intramuscular injections of bromodeoxyuridine (BrdU, Sigma). Young animals were examined because endothelial cell replication rates fall to below 0.1%/day in adults. BrdU is cleared from the circulation and taken up by S-phase cells within 60 min, so that fixation and immunostaining at this time is often used to assess cell proliferation rates. However, for the purposes of the current experiments, we euthanized the rabbits and perfusion-fixed the abdominal aorta at 100 mmHg using 3% paraformaldehyde after 24h. At this time, BrdU labeled cells had completed mitosis so that doublets of daughter cells were detected and the relative positions of the labeled nuclei indicated the direction of mitosis. BrdU was detected in en face histological sections by immunostaining of whole mount preparation (mouse anti-BrdU antibody, Abcam, Cambridge, MA; goat anti-mouse antibody, Sigma-Aldrich, Oakville, ON).

Statistics
Sample sizes, presented in Results, were $\geq 3$ replicates/experiment. Effects of drugs (nocodazole, taxol, SB415286) on cell length-width ratios after 24h of shear stress were assessed using a Dunnett's test. Differences in MTOC position after shear stress, and position relative to the nucleus of acetylated microtubules, were determined using Student's t-tests. P<0.05 was taken as statistically significant.
**Online Figure 1.** Grading of polarity of MTOC. Cells were scored 1 if the MTOC was upstream of the nucleus, 2 if the MTOC was upstream of the center of the nucleus (includes MTOC scoring 1), 3 if the MTOC is downstream of the nucleus and 4 if the MTOC is downstream of the nucleus.
Online Figure 2: Figure 5A of the accompanying journal article demonstrates that treatment of endothelium with LiCl to inhibit GSK-3 induces profound stabilization of microtubules as evidenced by dramatic upregulation of immunofluorescence staining for acetylated microtubules. Online Figure 2 confirms that similar staining is observed when cells were treated with the alternate GSK-3 inhibitor, SB415286.
Online Figure 3. Acetylated tubulin in unmanipulated ((A & C) or ligated (B&D) carotid arteries of adult rabbits without (A & B) or with (C & D) administration of LiCO$_3$ in drinking water to inhibit GSK-3β (see text).
Reference List


