Temporal Gradients in Shear, but Not Spatial Gradients, Stimulate Endothelial Cell Proliferation

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Background**—**The effect of temporal and spatial gradients in shear on primary human endothelial cell (HUVEC) proliferation was investigated. The sudden-expansion flow chamber (SEFC) model was used to differentiate the effect of temporal gradients in shear from that of spatial gradients. With a sudden onset of flow, cells are exposed to both temporal and spatial gradients of shear. The temporal gradients can be eliminated by slowly ramping up the flow.

Methods and Results**—**HUVEC proliferation in the SEFC remained unstimulated when the onset of flow was slowly ramped. Sudden onset of flow stimulated a 105% increase of HUVEC proliferation (relative to ramped onset) within the region of flow reattachment. To further separate temporal and spatial gradients, a conventional parallel-plate flow chamber was used. A single 0.5-second impulse of 10 dyne/cm^2 increased HUVEC proliferation 54$\pm$3% relative to control. When flow was slowly ramped over 30 seconds, HUVEC proliferation was not significantly different from controls. Steady laminar shear over 20 minutes inhibited HUVEC proliferation relative to controls regardless of step (36$\pm$8%) or ramp (21$\pm$5%) onsets of flow.

Conclusions**—**The results indicate that temporal gradients in shear stress stimulate endothelial cell proliferation, whereas spatial gradients affect endothelial proliferation no differently than steady uniform shear stress. **(*Circulation*. 2001;103: 2508-2513.)**

Key Words: hemodynamics ■ endothelium ■ blood flow ■ atherosclerosis

Hemodynamic forces have long been implicated in the initiation and localization of atherosclerosis. Given the focal nature of plaque formation within regions of arterial curvatures, branches, and bifurcations, it has been suggested that certain characteristics of fluid shear stress unique to these regions may potentiate atherogenesis.[1] Detailed analyses of fluid mechanics in atherosclerosis-susceptible regions of the vasculature have identified unique patterns of disturbed flow characterized by regions of flow separation, recirculation, reattachment, and perhaps most importantly, significant temporal and spatial gradients of shear stress.[1,2] Temporal shear stress gradients are defined as the increase or decrease of shear stress over a small period of time at the same location, whereas spatial shear stress gradients are defined as the difference of shear stress between 2 close points of a cell at the same point in time. To date, the role of temporal and spatial gradients of shear stress in the pathogenesis of atherosclerosis remains unclear. Some studies link atherogenesis to the large temporal gradients in shear due to the change of shear direction,[2,3] whereas others relate this to different spatial distributions of mean wall shear stress.[4]

To specifically investigate the proatherosclerotic effect of flow recirculation on endothelial cells, DePaola et al[5] and Truskey et al[6] have developed 2 similar models that simulate in vivo spatial patterns of flow separation, recirculation, and reattachment. By creating a sudden asymmetrical expansion in the flow path of perfusing media, these models generate a large spatial gradient in shear stress over a relatively small region of a cultured endothelial monolayer. This high gradient is caused by flow separation: near to the expansion step, flow recirculates in an eddy, whereas further downstream, the flow reforms to the regular parabolic profile. In between, there is a point of flow reattachment where shear stress is zero (stagnation point). Utilizing these in vitro models of recirculating flow, a number of studies have suggested that localized spatial gradients in shear stress can induce a proatherosclerotic endothelial cell proliferation-migration-loss cycle at the point of flow reattachment.[5,7,8]

Although it is true that these model systems generate large spatial gradients when flow is fully established, recirculating flow undergoes a distinct developmental phase of at least several hundred milliseconds, even if the onset of flow is instantaneous.[9] As such, large temporal gradients can also be produced over the same spatial region if the onset of flow is sudden, or if flow is pulsatile. Given that temporal gradients have also been shown to induce atherogenic phenotypes,[10,11] either type of gradient could account for these observations.

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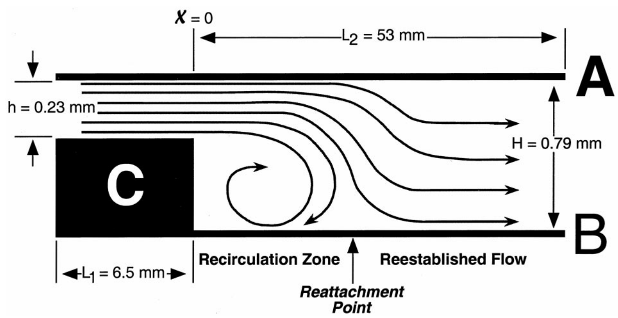

Figure 1. Cross section of SEFC. Flow channel (21 mm wide) is formed between polycarbonate body (A) and glass slide (B), where cell monolayer is grown. Two silicone gaskets with different-size openings provide inflow channel and sudden-expansion step (C). Inflow channel is sufficiently long (6.5 mm) to ensure parabolic velocity profile before expansion point, and after >15 mm, flow assumed parabolic profile that can be observed in equivalent PPFC. All locations are given relative to sudden-expansion point (x=0). Fluid flows from left to right in direction of arrows.

To resolve this controversy, the present studies examine the effect of temporal and spatial gradients on endothelial proliferation in the recirculating flow model system. To separate the effects of temporal and spatial gradients in this model system, we used a modification of the asymmetrical sudden-expansion flow chamber (SEFC) described by others.[6] Detailed numerical analyses of the SEFC showed that a negligible temporal change can be achieved in this model if the onset of flow is slowly ramped up over time. We also isolated and examined temporal gradients using a conventional parallel-plate flow chamber (PPFC) and well-defined flow profiles.

Methods

Cell Culture

Primary human umbilical vein endothelial cell (HUVEC) isolation was performed as described previously.[12] Cells were seeded onto glass microscope slides and grown to confluence within 3 days in M199 media (Irvine Scientific). All cell cultures were maintained in a humidified 5% CO_2-95% air incubator at 37°C.

Flow Experiments

DMEM (Irvine Scientific) supplemented with 2% FBS (Hyclone) and 0.5 U/mL penicillin, as well as 0.05 mg/mL streptomycin, was used as the perfusing medium for all experimental procedures. All flow chambers and accompanying apparatus were maintained at 37°C throughout the experiment. Time-matched sham controls (slides mounted on flow chambers without flow) and static controls (undisturbed slides in Petri dishes) were performed for all experimental groups.

The SEFC (Figure 1) was a modification of the chamber described by Truskey et al.[6] HUVEC monolayers were subjected to 4 hours of flow. One of 2 methods for the onset of flow was used: (1) ramped onset (a smooth ramped increase from 0 to 3.5 mL/s within 15 seconds) or (2) sudden onset (the initiation of fully established flow at 3.5 mL/s within 300 ms). The flow rate of 3.5 mL/s was calculated to produce a shear stress of 10 dyne/cm^2 in the region of reestablished flow downstream from the reattachment point. The continuous flow of media through the SEFC was maintained with a constant hydrostatic pressure head flow loop apparatus.[12] Ramped flow was manually controlled through a screw-type pinch valve (Flow-Rite, PV-9). Immediately after the completion of each specific flow profile, slides were removed from the SEFC to be assayed for HUVEC proliferation (see below).

In a conventional PPFC, confluent HUVEC monolayers on glass slides were subjected to 1 of the following 5 laminar flow profiles (Figure 4A): (1) impulse (0.5-second impulse of 10 dyne/cm^2 followed by 20 minutes of no flow); (2) ramped transient (a smooth 15-second ramped increase from 0 to 10 dyne/cm^2, sustained for 0.5 seconds, followed by a 15-second ramped decrease and 20 minutes of no flow); (3) step flow (a sudden shear stress increase from 0 to 10 dyne/cm^2, and then steady shear sustained for 20 minutes); (4) ramped flow (a smooth 15-second ramped increase from 0 to 10 dyne/cm^2, and then steady shear sustained for 20 minutes); (5) 1-Hz pulsatile (a repeated sequence of 0.5-second impulses of 10 dyne/cm^2 at a frequency of 1 Hz sustained for 20 minutes). Perfusing medium was driven by a computer-controlled syringe pump (pump 22, Harvard Apparatus with a controlling PC). Immediately after the completion of each specific flow profile, slides were removed from the PPFC to be assayed for HUVEC proliferation (see below).

Immunofluorescent Staining

Proliferating HUVECs were identified by use of a commercially available in situ monoclonal antibody kit for the detection of bromodeoxyuridine (BrdU) incorporation into cellular DNA during DNA synthesis (Boehringer Mannheim). Immediately after exposure to flow in either the SEFC or PPFC, slides were quickly removed from the chamber and incubated at 37°C in M199-BrdU (10 fmol/L BrdU) for 22 hours. Slides were fixed in 70% ethanol (in 50 mmol/L glycine buffer, pH 2.0), and immunostained for BrdU incorporation. BrdU-positive cells were visualized under a fluorescence microscope (Nikon, Diaphot TMD). Proliferating cells were counted by eye within adjacent 40× high-power fields of view (HPF) along the centerline of each slide. In the SEFC, each HPF was divided into 1.1-mm sections extending 20.2 mm downstream from the expansion point. In the PPFC, at least 20 HPFs were counted for each slide.

Numerical Simulations of Flow

The simulation of the fluid flow and the computation of the time-dependent shear stress was performed with a procedure described elsewhere.[9] A 2D model was used because the flow is homogeneous for ≈80% of the width of the flow chamber.[6] The model size of the flow chamber, 15×0.79 mm, was resolved by a grid of 600×100 nodes. The Reynolds number (Re) of 243 was computed from the average inflow channel velocity and geometry. For all simulations, the steady-state flow at Re=10 was used as the initial condition. To verify the numerical results, and also to obtain the time for flow development under sudden-onset conditions, the flow chamber was videotaped with ink-stained flow medium. As determined by the video visualization, a ramp time of 200 ms was used for the simulation of the sudden-onset flow and 15 seconds for the ramped flow. During the ramp time, Re was increased linearly from 10 to 243 and held constant thereafter.

Similarly, the PPFC was simulated by use of a model size of 20×0.23 mm and a grid of 200×50 nodes. A ramp time of 56 ms for the ramp from Re=0.07 to 8.8 was programmed, based on values obtained by independent video visualization. The maximum temporal gradient of shear stress was determined from this simulation.

Statistical Analysis

All experimental values are given as mean and SEM. All reported values of n refer to the number of separate and independent experiments from multiple primary HUVEC cultures. Significant differences between means were calculated with a Student's *t* test. The Wilcoxon test was used to test for a significant departure of the median from sham control. Statistical significance was taken at the $P<0.05$ level.

Results

Mathematical Modeling of Flow Recirculation in the SEFC

The calculated and visualized reattachment points differed by 7%. Video visualization also revealed that downstream flow developed fully within 200 ms (data not shown). For fully established flow at 3.5 mL/s, the calculated reattachment point was found 4.8 mm downstream from the sudden-expansion point. The computer simulations revealed that the location of the highest temporal gradient (5200 dyne · cm^{-2} · s^{-1}) for sudden onset was 4.1 mm downstream from the sudden-expansion point. The highest temporal gradient for the ramped flow was 16 dyne · cm^{-2} · s^{-1} and occurred at the same location. As calculated, during the dynamic-onset phase, the reattachment point moved from 0.6 mm (at Re=10) to its final position at 4.8 mm. This movement is the primary source of the temporal gradient in shear stress (Figure 2).

Effect of Flow Onset in SEFC on HUVEC Proliferation

The region extending 9.0 mm downstream from the point of expansion was taken to fully contain the spatial pattern of flow separation and reattachment. The region of reestablished unidirectional flow between 9.0 and 20.2 mm downstream from the expansion point was used as an internal control for HUVEC proliferation. Proliferation within the region of reestablished flow (in BrdU-positive nuclei/HPF) was not significantly different between ramped onset (35±2, n=5) and sudden onset (38±1, n=7) (Figure 3). For both profiles, the overall fraction of nuclei positive for BrdU within this region was ≈3%. When the onset of flow in the SEFC was ramped, no significant change in proliferation (31±1 BrdU nuclei/HPF) was observed within the region of recirculating flow (relative to internal control). Sudden onset of flow stimulated a peak 105% increase in proliferation (relative to the corresponding region of ramped onset) within the region of recirculating flow. Peak proliferation was observed within a region 4.5 to 5.6 mm from expansion. This region closely correlates with the calculated and visualized location of flow reattachment.

Effect of Flow Onset in PPFC on HUVEC Proliferation

HUVEC proliferation was expressed as percent change relative to sham controls (Figure 4B). No significant differences were observed in the level of proliferation between sham controls and static controls (data not shown). The overall fraction of nuclei positive for BrdU in sham control was ≈2%. When HUVECs were exposed to a single 0.5-second impulse of flow, proliferation increased 54±3% (n=6). The temporal gradient generated by the single impulse was calculated as 304 dyne · cm^{-2} · s^{-1}. A single ramped transient of flow reduced proliferation by 24±13% (n=8). Steady laminar shear for 20 minutes significantly inhibited proliferation regardless of step (36±8%, n=8) or ramped (21±5%, n=7) onsets of flow ($P<0.05$). Levels of proliferation were not significantly different between step flow and ramped flow. When HUVECs were exposed to continuous oscillations in flow at a frequency of 1 Hz sustained for 20 minutes, proliferation increased by 49±7% (n=7). No significant differences were observed in the level of proliferation between a single impulse and 20 minutes of 1-Hz oscillation. The maximum temporal gradient generated with the sudden onset of flow in the PPFC was calculated to be 300 dyne · cm^{-2} · s^{-1}.

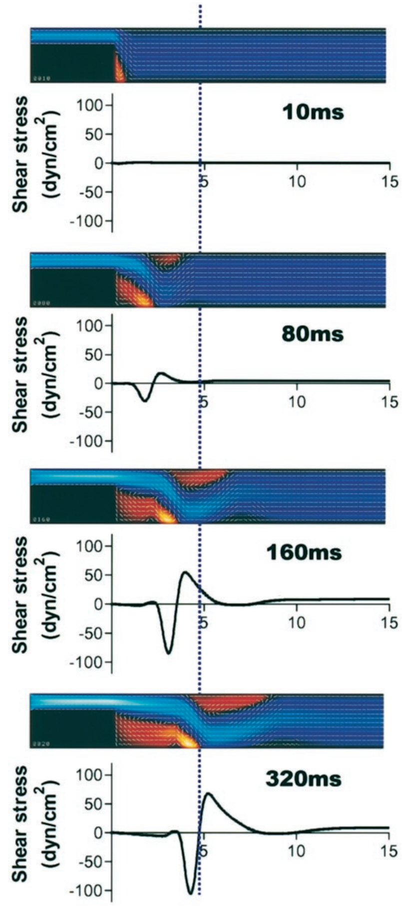

Figure 2. Development of recirculation eddy during onset phase of flow. Top frame shows flow in channel after 10 ms, where small eddy has already developed. Blue colors indicate forward flow, whereas red tint represents flow to left (backward). White bars indicate approximate direction of flow. Graphs show corresponding wall shear stress. Frames that follow show developing primary eddy after 80 and 160 ms. Secondary eddy at top becomes visible. Bottom frame shows fully established steady flow after 320 ms. Final steady-state reattachment point is indicated by dotted line.

Discussion

The key point of this study is the separation of temporal and spatial gradients of shear stress to determine their individual role in endothelial proliferation. Two flow systems were used to expose the cells to different flow characteristics. In the SEFC, spatial gradients were generated near the region of flow separation and reattachment. In a PPFC, flow was uniform, and therefore steady shear stress without spatial gradients was generated throughout the chamber. All cells in the PPFC were exposed to the same flow profile and temporal gradient. In both chamber systems, a sudden onset of flow led to the production of a significant temporal gradient. A detailed numerical analysis of both chambers predicted that the temporal gradients were negligible with the ramped onset of flow (bottom panel in Figure 3). In the SEFC, the peak temporal gradient was shown to be localized close to the

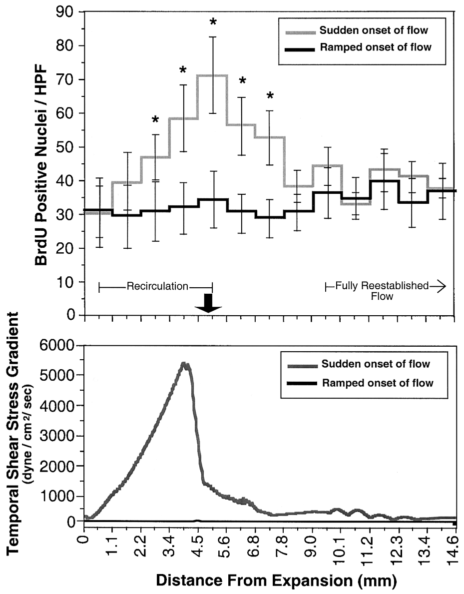

Figure 3. Effect of ramped and sudden onset of flow in SEFC on HUVEC proliferation. Top, Proliferation rate of HUVECs exposed to 4 hours of recirculating flow. Initial onset of flow was either 15-second ramped onset (n=5) or sudden onset (n=7). Arrow indicates calculated point of flow reattachment. Asterisks indicate significant difference between corresponding regions of ramped onset and sudden onset ($P<0.05$). Bottom, Profile of maximum temporal shear stress gradient downstream from expansion point for sudden onset of flow (maximum gradient 5200 dyne $\cdot$ cm^{-2} $\cdot$ s^{-1}) and for slowly ramped flow (maximum gradient 16 dyne $\cdot$ cm^{-2} $\cdot$ s^{-1}). Note that cells were subjected to range of temporal and spatial gradients. Significant stimulation of proliferation occurred in regions (segment 6.7 to 7.8 mm) corresponding to temporal gradients (355 dyne $\cdot$ cm^{-2} $\cdot$ s^{-1}) observed in vivo.[2] In contrast, a large range of spatial gradients (0 to 4000 dyne/cm^3, calculation not shown), which include physiologically relevant gradients,[21] were unable to stimulate proliferation in absence of temporal gradients in ramp flow.

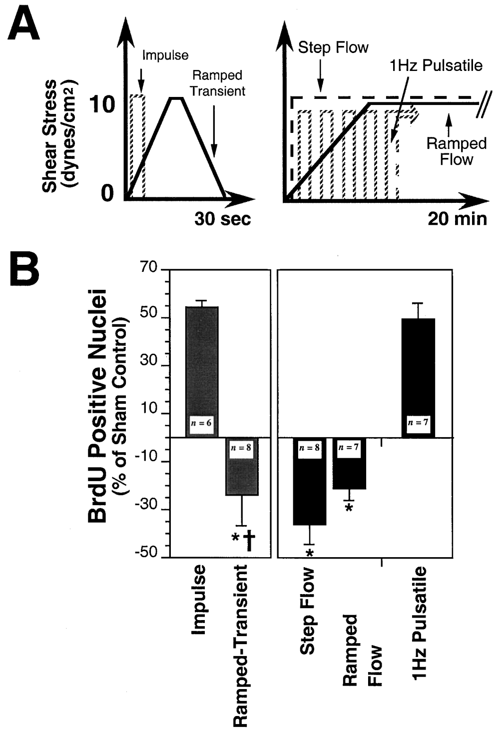

Figure 4. Effect of impulse, ramped transient, step flow, ramped flow, and 1-Hz pulsatile on HUVEC proliferation. A, Laminar flow profiles: impulse (0.5 second), ramped transient (left illustration), step flow, ramped flow, and 1-Hz pulsatile (right illustration). Maximal shear stress in each profile is 10 dyne/cm^2. B, Confluent HUVECs were exposed to single transient spike of flow (left) or flow sustained for 20 minutes (right). Values are expressed as percent change relative to sham controls (mean±SEM). † indicates proliferation was not significantly different from sham control. *Significant difference from impulse flow ($P<0.05$).

point of flow reattachment and the point of highest spatial gradient (Figures 2 and 3). The Table summarizes the combinations of steady shear, temporal, and spatial gradients the cells were exposed to in this study. Through the use of 2 different flow systems, cells were exposed individually and specifically to flow with and without temporal and spatial gradients as well as to combinations of the 2.

In the present study, the sudden onset of flow in the SEFC was found to stimulate HUVEC proliferation at the site of flow reattachment. When the effects of the temporal gradient were eliminated from the SEFC by ramping the flow onset, it was found that spatial gradients alone could not stimulate proliferation. However, when temporal gradients were individually isolated in a conventional PPFC with well-defined flow profiles, they were found to be potently mitogenic. Moreover, sustained steady laminar shear stress was found to completely inhibit the mitogenic effects of temporal gradients on proliferation.

Endothelial proliferation was specifically chosen as a marker for the effect of temporal and spatial gradients in the recirculating flow model system. Increased endothelial turnover in regions of recirculating flow has long been implicated

Shear Stress Gradients Occurring in Flow Chambers as a Function of Flow Onset

Chamber Type	Onset of Flow	Duration of Flow	Shear Profile Generated in Chamber	Profile Name
SEFC	Ramped	4 h	Spatial gradient	...
	Sudden	4 h	Temporal gradient and spatial gradient	...
PPFC	Sudden	0.5 s	Temporal gradient	Impulse
	Ramped	30 s	Negligible temporal gradient	Ramped transient
	Sudden	20 min	Temporal gradient and steady shear stress	Step flow
	Ramped	20 min	Steady shear stress and negligible temporal gradient	Ramped flow
	Sudden	1 Hz for 20 min	Repeated temporal gradient	1 Hz Pulsatile

in the process of atherogenesis.[5,8] A number of studies have demonstrated enhanced macromolecular permeability of aortic endothelial cells during mitosis.[13,14] Because the vascular endothelium serves as a dynamic interface between circulating blood elements and the interstitial tissues, disruption of its permeability characteristics may permit the localized influx of circulating LDL and other proinflammatory macromolecules into the artery wall.[15] Consistent with previous studies,[5–7] when the onset of flow in the SEFC was sudden, endothelial proliferation was significantly stimulated at the site of flow reattachment (Figure 3). In sharp contrast to previous studies,[5–7] when the onset effects of the temporal gradients were eliminated with a ramped onset of flow, endothelial proliferation remained unstimulated within the same spatial region. Both onset flow profiles generate the same spatial gradient in shear stress, which is maximal at the site of flow reattachment. Given the highly transient nature of the temporal gradient, and given that both maximum temporal and spatial gradients overlap, these results suggest that the induction of atherogenic phenotypes in the sudden asymmetrical expansion model seen in previous studies[5–7] may have been due to temporal rather than spatial gradients of shear stress. However, because the dynamics of flow initiation were not expressly specified in the previous studies[5–7] and longer exposures to recirculating flow and different chamber geometries were used, it is difficult to make direct comparisons.

In good agreement with patterns of proliferation seen in the SEFC, sudden onset of flow in the PPFC significantly stimulated HUVEC proliferation relative to the ramped onset in flow (Figure 4). Patterns of endothelial proliferation in the PPFC were significantly altered when the temporal gradient generated during the onset of flow was followed by sustained steady shear stress. Consistent with previous findings,[16,17] sustained steady shear stress was found to completely inhibit endothelial proliferation (relative to sham controls) regardless of the flow onset profile. The ability of sustained steady shear stress to inhibit the mitogenic effects of a temporal gradient can also be seen in the suppression of proliferation within the region of reestablished unidirectional flow in both the ramped and sudden-onset flow profiles used in the SEFC.

The ultimate response of an endothelial cell to any flow pattern is a balance between the magnitudes of the atherogenic/mitogenic signal (temporal gradient) and the anti-atherogenic/antimitogenic signal (steady shear).[1,2,10,11] In the SEFC, the relative contribution of steady versus dynamic components varies with the location. Although the peak temporal gradient occurs between 3.4 and 4.5 mm (Figure 3), a significant steady shear stress component is still present that partially suppresses the proliferative response to the temporal component. At the reattachment point, the mean wall shear stress within that region ranges from zero to very low. Without steady flow, the effect of the temporal gradient generated during the onset of flow is preserved.

A strong positive correlation between plaque location and low mean shear stress has long been recognized within arterial bifurcations.[1,3] Marked oscillations in the direction of wall shear where mean shear stress is low have been suggested to further enhance atherogenesis.[1,2,18] Given the pulsatile nature of blood flow, the enhancement of plaque formation may result from the repeated generation of strong temporal gradients at the point of flow reattachment where mean shear stress is low. Therefore, the finding that 20 minutes of sustained 1-Hz pulsatile flow in the PPFC equaled but did not further enhance endothelial proliferation relative to a single impulse was of interest (Figure 4). It is possible that the maximum attainable proliferation in our system was achieved with a single impulse. Given the geometry of the PPFC, a single 300 dyne $\cdot$ cm^{-2} $\cdot$ s^{-1} temporal impulse in the absence of steady flow was a potent mitogenic event, possibly reaching saturation levels of stimulation. Continued repetition of this stimulatory event likely could not further increase the proliferation rate.

The biophysical mechanism by which large temporal gradients in shear stress stimulate a mitogenic response in cells remains to be determined. Rapid mechanochemical signal transduction during the sudden onset of flow similar to that observed in the present study has been reported previously,[19] where specific mitogenic G-protein activation occurs within 1 second. Congruously, it has been shown that G-protein activation in cardiac fibroblasts by strain is strongly dependent on the rate of application of strain.[20] Enhanced transport of mitogenic factors to the surface of cells is unlikely to mediate the mitogenic stimulus of temporal gradients, because ramped flow provided comparable transport yet led to antimitogenic effects.

In summary, we have shown that temporal gradients in shear stress lead to enhanced endothelial proliferation, whereas spatial gradients in shear stress affect endothelial proliferation no differently than steady uniform shear stress. Additionally, the promitogenic stimulus of the temporal gradient was dependent on the absence or presence of steady shear stress. When one considers these findings, it is important to bear in mind that atherosclerosis is a protracted and multifactorial disease that involves many circulating blood elements, hemodynamic forces, and a complex cascade of molecular events within the endothelium and the arterial wall. The present study was designed to emphasize a potent yet overlooked mechanical stimulus that may link recirculating flow to localized atherogenesis.

Acknowledgments

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