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Endothelial Cell Layer Subjected to Impinging Flow Mimicking the Apex of an Arterial Bifurcation

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Abstract

Little is known about endothelial responses to the impinging flow hemodynamics that occur at arterial bifurcation apices, where intracranial aneurysms usually form. Such hemodynamic environments are characterized by high wall shear stress (WSS >40 dynes/cm²) and high wall shear stress gradients (WSSG >300 dynes/cm³). In this study, confluent bovine aortic endothelial cells were exposed to impinging flow in a T-shaped chamber designed to mimic a bifurcation. After 24–72 h under flow, cells around the stagnation point maintained polygonal shapes but cell density was reduced, whereas cells in adjacent downstream regions exposed to very high WSS and WSSG were elongated, aligned parallel to flow, and at higher density. Such behavior was not blocked by inhibiting proliferation, indicating that cells migrated downstream from the stagnation point in response to impinging flow. Furthermore, although the area of highest cell density moved downstream and away from the impingement point over time, it never moved beyond the WSS maximum. The accumulation of cells upstream of maximal WSS and downstream of maximal WSSG suggests that positive WSSG is responsible for the observed migration. These results demonstrate a unique endothelial response to aneurysm-promoting flow environments at bifurcation apices.

Keywords

Wall shear stress; Spatial wall shear stress gradient; Migration; Aneurysm

INTRODUCTION

Endothelial cells (ECs), which form the inner lining of blood vessels, are known sensors of wall shear stress (WSS)^{9,12} and play critical roles in vascular homeostasis. As flow conditions differ at different vascular locations, ECs respond accordingly. In straight arterial segments, where the WSS is normally maintained at the physiological baseline of 15–20 dynes/cm², ECs are quiescent, nonproliferating, and constitute a selective permeability barrier.^{3,18}

To this date, most flow-endothelium research has focused on EC exposure to shear stresses of low or normal values (up to 15–20 dynes/cm²). These values are relevant to studies of

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atherogenesis, which preferentially occurs in low flow regions in sinuses along the arterial tree characterized by disturbed flow or flow recirculation and reattachment, high oscillating shear index (OSI), and high WSS gradients (WSSGs).^{6,16,35} To produce such disturbed flow regions *in vitro*, flow devices incorporating a backward-facing step were used¹⁴ to generate recirculation zones with combined low WSS and high WSSG. ECs subjected to this condition exhibited increased motility, proliferation, permeability, turnover, surface adhesion, and altered protein expression,^{10,11,13,14,18,24,30} responses that *in vivo* would make an artery prone to atherogenesis.

On the other hand, there is little information on the behavior of ECs in the vicinity of the apex of an arterial bifurcation, a location prone to saccular aneurysm formation in the cerebral vasculature.^{15,17,22,29} The hemodynamics at the apex of a bifurcation is quite different. With the blood flow impinging at the apex and accelerating downstream, the apex environment constitutes an area exposed to flow stagnation and low WSS but high WSSG, and an adjacent area experiencing both high WSS and high WSSG.^{25,26} In contrast to the well-studied EC function under low WSS and disturbed flow at arterial sinuses, there have been few studies on the effect of impinging flow on EC function. The current study describes a culture chamber in which ECs can be exposed to a well-defined impinging flow. Using this device, we examine how the morphology, alignment, and movement of ECs are affected by impinging flow and show those responses differ in the different hemodynamic regions surrounding the impingement.

METHODS

Impingement Chamber Design

In order to study the effect of flow on endothelial cells at arterial bifurcations, an *in vitro* flow chamber was designed and constructed to match the conditions found *in vivo* at a surgically created bifurcation previously studied in our laboratory.²⁵ In our previous *in vivo* study, we defined three distinct flow regions that lead to distinct types of vascular remodeling.²⁵ They are:

1. Region I, a stagnation region characterized by a stagnation point and low to normal WSS relative to the baseline level in straight vessels.
2. Region II, an accelerating flow region characterized by high positive WSSG and high WSS.
3. Region III, a recovery region characterized by negative to zero WSSG and high WSS.

Because we were interested in quantitatively investigating the effect of flow on the ECs, the design of the chamber also had to be such that each distinct flow region could contain a large enough number of cells for analysis. To accommodate a maximum area of cells when they are cultured on a standard 22 mm × 50 mm microscope coverslip, we extended each flow region in a direction normal to the flow and as close to the edge of a 22 mm × 50 mm rectangle as possible.

As a starting point for determining appropriate dimensions to obtain the desired hemodynamics, we simulated the impinging flow environment as a simple T-shaped lumen with a two-dimensional flow jet impinging at the center of a cover slip of ECs and then splitting in opposite directions along this cover slip. Average cross-sectional areas of the vessels in our *in vivo* system were used as the cross-sections of the chamber inlet and outlets. Rectangular cross-sections were used in the flow chamber to facilitate the use of standard cell coverslips and the inlets, and outlets to the “T” were extended over 10 cross-sectional diameters to allow for flow development. Two-dimensional (2D) geometries were extracted from this model. Using this initial geometry, we performed computational fluid dynamic (CFD) simulations

(see Fig. 1) and examined the resulting flow environment near the cells. Specifically, the WSS and WSSG fields on the cell coverslip were examined to determine if they were similar in both magnitude and spatial distribution to Regions I, II, and III of the *in vivo* model described previously. We iterated between modifications of flow rate (by increasing or decreasing the inlet flow rate) and geometry (by increasing or decreasing the height or width of the inlet and/or outlet channels) of this T-junction to fully capture the flow environment. If, for example, Region I was not sufficiently long, the height of the outlet chambers was increased and 2D CFD was run again. Finally, three-dimensional (3D) CFD was run to verify that the side walls of the chamber do not substantially affect the WSS distribution on the cells. Figure 2 illustrates a typical WSS distribution that a layer of cells would be subjected to.

For this study, inflow rates of either 250 mL/min (corresponding to a Reynolds number $Re = 125$ based on inlet diameter) or 500 mL/min ($Re = 250$) were used. ECs were exposed to a flow environment characterized by a stagnation region adjacent to an accelerating flow region (high positive WSSG, high WSS), followed by a recovery region (negative to zero WSSG, high WSS). The higher flow rate ($Re = 250$) is based on calculations from our *in vivo* created canine bifurcation exposed to elevated flow using methods described previously.²⁵ The lower flow rate was chosen because it resulted in peak WSS and WSSG of similar magnitudes to those found through calculations on a normal canine carotid bifurcation. Such flow rates were considered as representative of “normal” and “high flow” conditions *in vivo*. All experiments were run under normal physiological pressure (95 ± 5 mmHg).

The final flow chamber (Fig. 3) was constructed of autoclavable materials so that the chamber could be assembled with the remaining components of the flow loop and then autoclaved together, thus minimizing the risk of contamination through handling. The main body of the chamber was manufactured by machining the geometry from polycarbonate blocks, which were then permanently bolted together using stainless steel hardware. The inflow and outflow holes of the main body were tapped with NPT pipe threads and then fitted with appropriate nylon tube fittings. These holes were drilled with a tapered bit to create a relatively smooth transition from circular tube fitting to rectangular chamber flow channel. The coverslip holder was also machined from a polycarbonate block by cutting a groove so that a microscope coverslip of ECs, when placed in the groove, sits flush with the top of the holder.

CFD Simulations

Input geometrical boundary conditions for the CFD were created using ProEngineer (PTC, MA) 3D parametric design software. For the fluid simulations we assumed Newtonian fluid ($\mu = .0034$ Pa·s) and incompressible, laminar flow (density = 1020 kg/m^3) in a rigid-wall model, with traction-free boundary conditions at both outlets. The fluid properties were obtained from measurements on the experimental working fluid. Tetrahedral and prism grids were generated with ICEM-CFD software (Ansys) and CFD solutions of steady-state flow conditions were obtained through commercial software Star-CD (CD-Adapco). Validation of the CFD has been described previously.²⁰ In this study, particle image velocimetry (PIV) measurements were obtained from an experimental aneurysm model and compared with CFD results computed using the same model geometry. It was found that CFD simulation results closely match the PIV measurements.

In Vitro Flow Loop

Flow was driven by a peristaltic pump that drew flow media from a reservoir, which was open to the atmosphere via a filter. The fluid was then passed through a fluid capacitor (to dampen the pulsatility of the pump) and then through the impingement chamber. A pressure tap was located just before the impingement chamber to enable systemic (static) pressure measurement near the device. After the impingement chamber, and just before the reservoir, we placed a

tubing clamp to adjust loop resistance (and, therefore, the static pressure of the system). Silicone tubing (.25") was used to connect the components of the loop. An ultrasonic flow probe (Transonic Systems) calibrated for both the silicone tubing and flow media was used for flow rate measurement. This probe could be placed at multiple locations along the flow loop to measure inlet and outlet flow rates at any point during the experiment.

Cell Culture and Flow Media

Bovine Aortic Endothelial Cells (BAECs) between passages 14 and 20 were seeded on sterile 22 mm × 50 mm glass coverslips and grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). The cell cultures were maintained in a humidified incubator at 37 °C and 5% CO₂ and fed every 3–4 days until they reached confluence. Once confluent, they were placed in flow media for 24 h to allow the cells to adapt to the new media. Flow media consisted of DMEM, 5% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 80 mg/mL Dextran 70 (GE Healthcare). Dextran was added to increase the viscosity to 0.0034 Pa·s at 37 °C; its use has been documented previously by others.³²

Cell Proliferation Inhibition

To study the response of an endothelial layer with inhibited proliferation, we ran two experiments with cells that were incubated with Mitomycin-C (MMC), an irreversible inhibitor of cellular proliferation,⁴ just prior to their placement in the flow loop. Cells were incubated at a concentration of 15 µg/mL for 2 h and then washed three times with PBS to remove trace amounts of the inhibitor. This concentration completely inhibited EC proliferation in control experiments.

Experimental Protocol

Prior to each experiment, all of the components of the flow loop were wrapped and sterilized by steam autoclave. Once cooled, the loop was placed in a clean tissue culture hood. Using sterile methods, the entire flow loop was assembled in the hood and flow media was added to the reservoir. A coverslip of cells was then placed in the groove of the impingement chamber coverslip holder. The main body of the chamber was placed on top of the coverslip holder and secured together using stainless steel bolts (see Fig. 3).

The flow pump was immediately started to prevent the cell monolayer from drying out, and silicone caulk was used to further seal chamber-to-loop joints. Using the flow probe, total chamber outflow was measured and pump speed was adjusted accordingly. Chamber inlet flow rates from the CFD simulations, as described previously, were used as target flow rates for the experiments (±1% to account for pump variability). Once the desired flow rate was established, the pressure was increased to a physiological level (100 mmHg) by constricting flow with a pressure clamp located just after the impingement chamber. The entire flow loop was placed in an incubator at 37 °C and 5% CO₂ to ensure stable temperature and pH for the duration of the experiment. Flow rates were monitored periodically. Experiments were run for 12, 24, 48, and 72 h time periods. A monolayer of cells cultured under the same conditions was placed in the incubator in a Petri dish with flow media (no flow) for the duration of the experiment and was used as a control.

Morphological Examination

Microscopic examination and characterization of the morphology (shape, size, and orientation) of the EC monolayer was achieved by observing the cells with an inverted phase contrast microscope (Zeiss Axiovert 40). Digital images were taken using an inverted microscope (Zeiss Axiovert 135) with a mounted CCD camera (Hamamatsu Orca-ER) and IPLab software.

Immunostaining

Further examination and quantification of cell morphology and population (density) was achieved by immunofluorescent staining of actin stress fibers and cell nuclei. After each experiment, the impingement chamber was disassembled and the cell coverslip was removed and (with controls) immediately placed in fixative. Fixation of the cells was achieved by incubation in 10% formalin for 45 min at room temperature. Cells were permeabilized with 0.2% TritonX-100 for 5 min. Actin fibers were stained with Oregon Green 488 phalloidin (1:500, Molecular Probes) in BSA/PBS. Cells were mounted on microscope glass slides using Vectashield (Vector Laboratories) mounting media with DAPI (for nuclei labeling).

Cell Density Quantification

In order to spatially map cell densities with WSS distributions, a composite image of the entire length of the EC monolayer was created by stitching together sequential images of the cells taken at 20× magnification. Images were acquired and assembled using a Zeiss Axioimager motorized fluorescence microscope and accompanying Axiovision software (Zeiss). Quantification of DAPI-positive cells was achieved using macros in NIH ImageJ software.¹ The macros divided composite images into 200 μm sections, or regions of interest (ROI), along the length of the slide. For each ROI, the total number of DAPI-positive cells was obtained and used to calculate cell density in each region.

RESULTS

Flow Data

Running flow experiments in the final impingement chamber at $Re = 250$ and 125 resulted in two distinct flow patterns, each corresponding to specific WSS and WSSG distributions along the EC layer as shown in Fig. 4. Following the definitions of Meng *et al.*,²⁵ the hemodynamic environment experienced by the endothelial layer was divided into three regions. Region I was characterized by a stagnation point followed by a fairly rapid acceleration from low to normal shear stress. This acceleration results in high WSSG values. Region II was characterized by continued high WSSG, as the WSS accelerated to its peak value. In Region III, WSS decelerated from its peak value (WSSG was negative during deceleration) and leveled off at a constant downstream value (0 WSSG). Both flow conditions reached peak WSS values (206 dynes/cm² and 80 dynes/cm²) at the same distance from the stagnation point.

Intuitively, one expects that pressure at the stagnation point would be significantly higher than elsewhere due to the conversion of kinetic energy into pressure energy at the impingement site. However, our CFD results show that the total pressure at the impingement was merely 2 mmHg above the static pressure of 100 mmHg. In other words, impingement generated only a 2% local pressure increase.

Cell Morphology and Alignment

In all flow regions, cells remained confluent and did not show any signs of damage; there were no gaps, no signs of cytoplasmic retraction or rounding up of cells. The morphology, shape, and alignment of the ECs were highly dependant on the local flow dynamics. As shown in Fig. 5, the location of flow impingement (Region I) was quite obvious from cell morphology and alignment in the phase-contrast microscopic image taken at completion of the flow experiment. In Region I ECs remained confluent but were polygonal, enlarged, and nonpolarized. In Regions II and III, cells maintained cell-cell contacts and were highly elongated, having a more spindle-like rather than “pancake” shape, and were aligned parallel to the direction of flow, as in the rest of Region III extending to the end of the flow channel. The cell shape change

and reorientation had already begun by 12 h and was completed after 24 h of flow exposure under both flow conditions.

Cell Distribution

Endothelial cells subjected to impinging flow exhibited a spatially varying density. At the impingement, cells were sparse and large, whereas they were crowded downstream in both branches of the impingement chamber. Cell quantitation verified this impression, as seen in Fig. 6, where cell density (number of cells/mm²) after 48 h of flow exposure to low flow ($Re = 125$) or high flow ($Re = 250$) is plotted as a function of distance from the stagnation point. Three independent experiments under each condition produced very similar plots. A consistent pattern was observed in which both high- and low-flow experiments showed the same trend: decreased cell density (compared to the static control) forming a trough at the stagnation point in Region I, and increased cell density forming a peak in Region II. In Region III, density returned to a relatively constant level that was lower than the peak but higher than the trough. However, since culture age and passage number were different among experiments and can affect the cell density, there was some variability among six experiments that were performed in terms of absolute density and exact peak location. Nonetheless, the density distribution pattern was always the same.

Cell Density Distribution was Unaffected by Cell Proliferation

To determine if the cell density peak in Region II was a result of localized cell proliferation, cells were treated with MMC to inhibit proliferation. Inhibited cells were then subjected to high flow ($Re = 250$) experiments for 48 h. In two separate experiments, the MMC-treated ECs showed remarkably similar cell density distributions compared to untreated cells (Fig. 7), with low densities in Region I and a density peak of similar size and location in Region II. Therefore, the formation of the cell density peak was not due to cell proliferation.

Cells Accumulate in the High WSSG Region

If cell density peaks were not due to cell proliferation, an alternative explanation is that cells migrated into the peak region from a neighboring area. Because it has been shown by Hsu *et al.*²¹ that WSS can cause ECs to migrate in the direction of shear, we expected cells to move in the direction of flow and accumulate *downstream* of the zone of highest WSS. In other words, we postulated that a cell density peak would form in Region III. However, our experiments consistently show that the cell density peak was located in Region II instead.

We ran three additional experiments longer than 48 h to see if the EC density peak would move downstream to Region III over time. The density peak did in fact move downstream (Figs. 7b, c) while becoming wider (Fig. 7b) with time. While at the earlier time points the peak appeared sharper and closer to the stagnation region, at 72 h it had moved downstream and had flattened out. However, even though the density peak moved downstream, it still remained in Region II at all tested durations (12, 24, 48, and 72 h). At 72 h (i.e., after three days of flow exposure), the peak flattened out, giving no evidence that longer durations would cause the cells to accumulate downstream of the highest WSS.

DISCUSSION

Previous *in vitro* research on EC responses to hemodynamics has focused on low to normal WSS environments, driven by the relationship between these environments and pathogenesis of atherosclerosis.^{7,8,10,14} Little is known about endothelial behavior in impinging flow environments seen at the apices of bifurcations, where intracranial aneurysms usually form. We designed an *in vitro* EC device to create impinging flow such as occurs at bifurcations *in vivo*.^{25,26} This system allows us to gain knowledge of EC behavior in an impinging flow

environment. Such knowledge could lead to new insight into aneurysm-initiating vascular remodeling.

The finding that stagnation pressure at the impingement point is not significantly different from the static pressure is consistent with previous *in vivo* calculations of stagnation pressure at arterial bifurcations.^{19,25,28} Specifically, local pressure increase due to impingement in this *in vitro* study accounts for only 3% of the peak total pressure and is much smaller than temporal pressure changes *in vivo* (pulse pressure). Thus, we do not believe that impingement pressure has a major effect on EC behavior at the bifurcation apex. Rather, the high and spatially changing WSS (thus WSSG as well) resulting from flow impingement may be the stimuli for EC responses in bifurcations.

Besides EC morphology and alignment, our experiments show distinct cell redistribution following exposure to impinging flow, and that such redistribution is unaffected by inhibiting cell proliferation. White *et al.* have also shown that high WSSG does not stimulate cell proliferation.³³ Therefore, we can deduce that ECs migrate in the flow direction in response to the impinging flow. Previous studies have shown that high WSS can drive EC migration; ECs migrate under laminar shear stress *in vitro*^{5,21} and there is evidence that ECs migrate downstream when exposed to high flow (WSS in excess of 80 dynes/cm²) *in vivo*.²⁷ Other *in vitro* studies, focusing on the effect of disturbed flow on EC migration, have shown that ECs migrate away from the high WSSG region in a low flow environment.^{14,30} Hence, both WSS and WSSG have been reported to affect EC migration. However, based on our observations, cells accumulated very close to the stagnation point, just downstream of the low WSS and high WSSG region and upstream of maximal WSS. Therefore it seems that WSSG in this particular environment drives EC movement more strongly than WSS. If migration were stimulated mostly by WSS magnitudes, one would expect that cells exposed to the highest WSS magnitudes in our system would exhibit the strongest migration. If this were the case, more cells would leave the highest WSS area than would enter it, thereby forming a density trough at the highest WSS area, followed by a density peak just downstream. This clearly does not occur. Furthermore, the greatest depletion of cells, and therefore the most cell migration, occurred in Region I, where WSS is the lowest but WSSG is high. This finding suggests that WSSG rather than WSS is the stronger driving force for downstream migration.

A possible mechanism for WSSG driving EC migration is by creating differences in the shear forces that are experienced by adjacent cells. This force differential can act at the intercellular junctions and activate the junctional proteins such as PECAM-1 and connexin-43.^{13,23,31} A positive WSSG (Regions I and II) would act to stretch the cells apart (in the direction of flow), while a negative WSSG (Region III) would compress the cells together. The trough and peak in EC density distribution in our experiments are confined to regions of high, positive WSSG, while cell distribution is essentially uniform in Region III, despite the fact that the absolute magnitudes of the (negative) WSSG at the beginning of this region are quite appreciable. Therefore, the present study shows that the accelerating flow stimulates cell migration away from the high positive WSSG region, while the decelerating flow does not stimulate migration.

Since the flow environment of the *in vitro* chamber was based on that seen previously *in vivo*, we expected a similar EC response, namely that such high shear stresses (WSSG >300 dynes/cm³) would lead to EC loss or damage.^{25,26} On the contrary, cells in our chamber not only remained confluent and intact, but they demonstrated significant cell motility and migration. Therefore, high WSS and high WSSG do not directly cause EC damage.

However, the fact that the ECs have a higher motility under impinging flow compared to that under laminar flow suggests that cells behave differently at the apex of bifurcation than in the straight arterial segments. Yet, it is not known if such difference could predispose an apex to

pathologies such as aneurysm formation. While the increase in cell migration away from the high WSSG did not disrupt the EC layer after two days of increased flow *in vitro*, it is possible that a chronic flow increase at an arterial bifurcation would lead to a chronic EC migration away from the impingement, and the continuity of the endothelium would depend on an equally increased proliferation at the stagnation point to serve as cell source. Furthermore, during migration, cell–cell contacts are destabilized, resulting in increased permeability that could potentially compromise the barrier function after a long exposure to increased accelerating flow. Additionally, increased migration is usually associated with increased matrix metalloproteinase (MMP) activity,² which could degrade the basement membrane and make the region exposed to impingement flow more susceptible to destructive remodeling that leads to localized aneurysm pathogenesis.^{25,26} Recently we have shown that the combination of high WSS and high, positive WSSG stimulates MMP expression in intimal and medial cells of a canine carotid artery and leads to an aneurysm formation (unpublished data). However, *in vivo* it is difficult to delineate which of these changes are directly attributable to ECs responding to the local hemodynamic environment, and which events are secondary to the initial aneurysm-inducing stimulus. The current *in vitro* system shows that high WSS and high positive WSSG directly affect the EC behavior by increasing their motility and potentially their MMP production.

In addition, the migration of ECs away from the apex requires the remaining ECs to spread over a larger surface area, creating additional tension within the endothelium at the apex. This tension could be relieved by local proliferation of the apical ECs to provide additional cells to spread over the apex; this would also “feed” more downstream migration. Tardy *et al.*, studying the atherosclerotic effect of disturbed flow (high WSSG, low WSS) on ECs, have shown that cells continuously proliferate in a high shear stress gradient region and then migrate downstream away from the high WSSG region.³⁰ Because arterial bifurcations exist in numerous locations throughout the body without pathology, we expect that, normally, there exists an equilibrium of cell production at the impingement and migration downstream. Wright³⁴ found that mitotic ECs along the aortic tree are most prevalent at branch points, consistent with bifurcation apices being foci of EC proliferation. Thus bifurcation apices may be a continuous source of ECs for other parts of the vessel, with WSSG driving the endothelial flux. Any disruption of the EC source or the subsequent downstream cell migration would then lead to areas of abnormally decreased cell density and diminished cell–cell contacts, leading to altered EC function, which would make such regions of the vasculature prone to pathology.

In conclusion, although the ECs remain confluent at the impingement and align with flow under high WSS in the adjacent regions, they do migrate away from the impingement region, possibly driven by the local positive WSSG, which imposes an additional stretching. This added physiological “stress” may contribute to the special biology around branch points *in vivo*.

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FIGURE 1. CFD simulation for 2D geometry with $Re = 250$. This image illustrates velocity magnitude, direction in the impingement flow field, and the three flow zones (I, II, III).

FIGURE 3.

Final 3D geometry of impingement chamber: (a) exploded ProEngineer rendering (main body, coverslip holder, coverslip), (b) assembled rendering highlighting the lumen of the device, and (c) photograph of manufactured device.

FIGURE 4.

WSS (top) and WSSG (bottom) vs. distance from impingement point for two flow conditions: $Re = 250$ (closed squares) and $Re = 125$ (open diamonds). Regions I, II, and III are labeled accordingly.

FIGURE 5.

Phase-contrast image of endothelial cell monolayer at the impingement after 24-h exposure to high flow ($Re = 250$).

FIGURE 6.

Endothelial cell density as a function of distance from impingement after 48-h exposure to low flow ($Re = 125$, open squares) and high flow ($Re = 250$, closed diamonds). Static control for each experiment is represented at far left with a matching shape.

FIGURE 7.

(a) Cell density vs. distance for endothelial cells treated with MMC (open squares) and without MMC (closed squares), after exposure to high flow ($Re = 250$) for 48 h. (b) Representative cell density peak for untreated cells exposed to high flow ($Re = 250$) for 72 h, showing a shift downstream compared to 48 h. (c) Peak location measured from the impingement at 48 and 72 h experiments under high flow; each bar represents the average of five experiments $\pm$ SEM.