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Effect of Intraluminal Pillars on Particle Motion in Bifurcated Microchannels

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Abstract

A central feature of intussusceptive angiogenesis is the development of an intravascular pillar that bridges the opposing sides of the microvessel lumen. In this report, we created polydimethyl siloxane (PDMS) microchannels with geometric proportions based on corrosion casts of the colon microcirculation. The structure of the PDMS microchannels was a bifurcated channel with an intraluminal pillar in the geometric center of the bifurcation. The effect of the intraluminal pillar on particle flow paths was investigated using an in vitro perfusion system. The microchannels were perfused with fluorescent particles and the particle movements were recorded using fluorescence videomicroscopy. We found that the presence of an intravascular pillar significantly decreased particle velocity in the bifurcation system ($p < .05$). In addition, the pillar altered the trajectory of particles in the center line of the flow stream. The particle trajectory resulted in prolonged pillar contact as well as increased residence time within the bifurcation system ($p < .001$). Our results suggest that the intravascular pillar not only provides a mechanism of increasing resistance to blood flow, but may also participate in spatial redistribution of cells within the flow stream.

Introduction

A fundamental mechanism of morphogenesis, intussusceptive (nonsprouting) angiogenesis is a process of intravascular replication (Burri and Tarek 1990; Caduff et al. 1986; Patan et al. 1993). Intussusceptive angiogenesis is a dynamic process that can produce two lumens from a single vessel (Djonov et al. 2003; Patan et al. 1996). The central feature of intussusceptive angiogenesis is the development of a pillar that bridges the opposing sides of the microvessel lumen. Physical expansion of the pillar eventually leads to division of the vessel into two lumens. Recently, the structural and functional changes in the inflamed mouse colon (Tsuda et al. 2008; Turhan et al. 2007) suggest the process of intussusceptive angiogenesis in the chronically inflamed mouse colon (Konerding et al. 2007). The discovery of inflammation-induced intussusceptive angiogenesis implies a potential functional interaction between inflammatory blood cells and the intussusceptive pillar.

Attempts to define the flow fields in the process of intussusceptive angiogenesis have focused on the fluid phase of the blood. Djonov et al. (Djonov et al. 2000) have shown that

pillars develop predominantly in regions of enhanced flow. In the chick chorioallantoic membrane model, clamping an upstream arterial side-branch results in rapid pillar formation with subsequent structural remodelling in the region of increased flow (Djonov et al. 2002). Similarly, Szczerba and Szekely (Szczerba and Szekely 2005) have used computational flow dynamic analysis to simulate the emergence of blood vessel patterns as a consequence of shear stress-induced remodelling. Although these studies have explored the fluid dynamics associated with intussusceptive angiogenesis, the implications of the intussusceptive pillar on particle dynamics are largely unknown.

In this report, we created polydimethyl siloxane (PDMS) microchannels with geometric proportions based on corrosion casts of colonic microvessels. The effect of an intraluminal pillar on particle flow paths was investigated with an in vitro perfusion system and fluorescence videomicroscopy. These studies demonstrated that the presence of an intravascular pillar significantly decreased particle velocity in the bifurcation system as well as altered the trajectory of particles within the centerline flow stream.

Methods

Morphometry and image analysis

Corrosion casts of the mucosal plexus of the mouse colon have been previously described in detail (Ravnic et al. 2005; Ravnic et al. 2007a). Images of the corrosion casts were processed with the MetaMorph Imaging System 7.1 software (Molecular Devices, Brandywine, PA). The 14-bit grayscale images were thresholded and standard distance calibration was performed. The MetaMorph's Region Measurement and Caliper applications were used to measure the vessel dimensions. The data was logged into Microsoft Excel 2003 (Redmond WA) by dynamic data exchange.

Spatial statistics

Selected bifurcations in the mucosal plexus of the mouse colon were identified using corrosion casts and distance calibrated images were obtained. Selected images of the corrosion casts were oriented on the geometric center of the bifurcations and superimposed. The 14-bit grayscale images were converted to a numerical matrix and arithmetically combined. The 2- dimensional contour limits reflected the 95% confidence limits of the combined data were plotted in Origin 7.5 (OriginLab, Northampton, MA).

PDMS microchannel fabrication

The polydimethyl siloxane (PDMS) microchannels were produced using a soft photolithographic method (McDonald et al. 2000)(Figure 1). Computer-aided design (AutoCAD, Autodesk, San Rafael, CA) of the microchannels was transferred to a SU-8 photoresist on a silicon wafer and exposed to ultraviolet light to create the microchannel "master" mould. A PDMS cast was produced from the master and plasma bonded to a standard microscope slide for imaging experiments. The microchannels were 100µm in diameter with a 30µm radius of curvature and a 10µm channel depth. The bifurcation angles were determined from SEM analysis on corrosion casts. The pillar diameters were 10µm, 15µm, 20µm or 25µm. To perfuse the microchannels, the channels were directly accessed through the PDMS using perfusion tubing connected to a height-adjustable reservoir containing fluorescent microspheres. Multiple replicates of the prototype design were fabricated by the Stanford Microfluidics Foundry (Stanford, CA).

Scanning electron microscopy

The PDMS microchannels were imaged after coating with gold in an argon atmosphere with a Philips ESEM XL30 scanning electron microscope (Eindhoven, Netherlands)(Ravnic et al.

2005). The SEM images were used to confirm microchannel dimensions and assess surface rugosity.

Microchannel imaging system

The microchannel perfusion was imaged using a Nikon Eclipse TE2000 inverted epifluorescence microscope using Nikon Fluor 10x and 20x objectives. An X-Cite (Exfo; Vanier, Canada) 120 watt metal halide light source and a liquid light guide was used to illuminate the samples. Excitation and emission filters (Chroma, Rockingham, VT) in separate LEP motorized filter wheels were controlled by a MAC5000 controller (Ludl, Hawthorne, NY) and MetaMorph software 7.10 (Molecular Devices, Brandywine, PA). The flow chamber 14-bit fluorescent images, with 1000×1000 pixel resolution, were digitally recorded on a C9100-02 EMCCD camera (Hamamatsu, Japan). Images were transferred to a Dell Xeon workstation running MetaMorph 7.10.

Preparation of tracer particles

The production of the polystyrene spheres and their superior fluorescence properties have been previously described (Ravnic et al. 2006b; Ravnic et al. 2007b). The green (ex 488; em 510) particles were labeled with derivatives of the BODIPY fluorochromes using organic solvents (Invitrogen; Eugene OR). The custom manufactured particles were 500nm in diameter.

Speckle displacement velocimetry

After routine distance calibration, the typical image stack was obtained at 20msec acquisition intervals. The recorded image stack was processed using a custom MatLab (MathWorks, Natick, MA) algorithm. The speckles were identified as high contrast regions with speckle position determined at subpixel accuracy. The speckle displacement was identified using a cross correlation centroid-finding algorithm to determine the best match of the speckle displacement in successive images. Instantaneous velocity was calculated and plotted based on the 100mhz system bus clock of the Xeon processor (Intel, Santa Clara, CA). Average velocity calculations were based on velocity calculations across the channel width.

Particle tracking velocimetry

Particle tracking was performed on digitally recorded and distance calibrated multi-image “stacks.” The image stacks produced a sequential time history of velocity and direction as the acquired images were time stamped based on the 100mhz system bus clock of the Xeon processor (Intel, Santa Clara, CA). The movement of individual particles was tracked using the MetaMorph (Molecular Devices) object tracking application. The intensity centroids of the particles were identified and their displacements tracked through planes in the source image stack. With routine distance calibration, the overlay of the image stack provided a quantitative assessment of the particle/cell path.

Time-series flow visualization

The stream acquired images were stacked to create a time-series of 500 or 1000 consecutive frames. The stacks were systematically analyzed to ensure the absence of motion artifact. The stack “maximum” operation selected the highest intensity value for each pixel location throughout the time-series. The resultant image produced a time-series reconstruction of particle locations during the time interval of the image stack.

Statistical analysis

The statistical analysis was based on replicate measurements. The unpaired Student's t test for samples of unequal variances was used to calculate statistical significance. The data was expressed as mean $\pm$ one standard deviation. The significance level for the sample distribution was defined as $P < .05$.

Results

Microchannel geometry

The 2-dimensional spatial geometry of the PDMS microchannels was based on corrosion casting and SEM of the murine colon. The colonic mucosal plexus is a quasi- polygonal network associated with inflammation-induced intussusceptive angiogenesis (Figure 2A). The bifurcation angles used in this study represented the mean of six similar planar bifurcation angles observed in a single mouse mucosal plexus (Figure 2B). Based on these geometric proportions, scaled-up PDMS microchannels were constructed with or without an intravascular pillar and fused to a standard microscope slide (Figure 2C). The proportions of the pillar and microchannel diameter were consistent with in vivo observations (Konerding et al. 2007); however, the initial dimensions of an in vivo pillar can be as small as 1–3 μ m whereas the structural properties of the PDMS limited the pillar to a 10 μ m diameter. SEM of the microchannels demonstrated random rugosity consistent with the known limitations of the soft lithography technology (McDonald et al. 2000)(data not shown).

Pillar effects on the flow field

The height of the perfusion reservoir containing *aqueous suspension* of fluorescently labeled particles was systematically varied to produce 3 different pressure heads (10, 20, and 40cmH₂O) which produced a steady physiologically relevant low Reynolds number flow ($Re < 1$) within the bifurcated microchannels. The downstream ends of the identical daughter channels were open to the atmosphere. The flow fields within the microchannels were documented using high speed (supravideo frame-rate) videomicroscopy and analyzed using both speckle displacement and particle tracking velocimetry. At a constant perfusion pressure, mean flow velocities in the bifurcation system—the measured averaged across the upstream channel —were significantly reduced in the bifurcations with a pillar ($P < .05$) (Figure 3). A complementary assessment of particle flow paths was provided by time- series reconstructions of the flow paths (Figure 4C,D). Both the speckle displacement velocimetry and the time-series reconstructions demonstrated diminished flow velocity and altered flow fields in the region of the pillar extending to the apex of the bifurcation (Figure 4D, ring).

Pillar effects on particle flow paths

The pillar had an effect, not only on the overall channel flow resistance, but also on the path of individual particles. The fluorescent particles were tracked from the upstream segment through the bifurcation. In the control microchannel (no pillar), the centerline particles appeared to be randomly distributed between the downstream segments. In contrast, the presence of the pillar resulted in markedly different flow paths (Figure 5A). When a particle approached the pillar, its velocity significantly reduced compared to that in the neighboring flow stream (Figure 5B). Particles in the near-centerline of the flow stream contacted the pillar and maintained contact through varying degrees of the pillar circumference (Figure 5C). After passing beyond the pillar, the particles contacted the bifurcation apex and were distributed between the downstream segments.

Microchannel residence time

As a result of significantly decreased flow velocity in the vicinity of the pillar, residence time of particles around the pillar was more than two-fold longer (857 ± 374 msec versus 379 ± 148 msec) than that of particles away from the pillar (Figure 6A). In addition, flow velocity prior to pillar contact also had a significant influence on the length of the contact. When the average velocity of particles contacting the pillar was less than 600 μ m/sec, the particles maintained contact for more than 100 degrees of the pillar circumference (Figure 6B).

Discussion

In this report, we used a photolithography fabrication technique to produce microchannels in the elastomer PDMS. The technique permitted the fabrication of microchannels with 2D geometric proportions similar to those observed in vessel bifurcations of the colonic mucosal plexus. This approach allowed us to study the effect of an intravascular pillar – a structure similar to those observed in intussusceptive angiogenesis – on flow patterns around the pillar. We found that the presence of an intravascular pillar significantly decreased flow in the bifurcation system. In addition, the pillar altered the trajectory of particles in the center line of the flow stream. Assuming the relevance of particle distribution to cell motion, our results suggest that the intravascular pillar not only provides a mechanism of increasing resistance to blood flow, but may also participate in spatial redistribution of cells within the flow stream.

A contribution of this study is the use of PDMS microchannels to model complex microvessel anatomy. The application of microfluidic fabrication technology to experimental biology has several advantages. Foremost, PDMS fabrication techniques allow the rapid and economical development of a variety of microchannel geometries. In our experimental system, this flexibility is useful because of the complex vessel geometries associated with intussusceptive angiogenesis. Also, the inert and translucent PDMS permits the prolonged observation of perfused microchannels using standard optical videomicroscopy.

As noted previously, our PDMS bifurcation model has a statistically acceptable degree of geometric similarity. The force due to flow unsteadiness in the microcirculation was negligible compared to the forces due to flow inertia and fluid viscosity (i.e., the Womersley parameter is less than unity). Hence, it was the latter two forces that determined the flow patterns and balance the driving force (pressure gradient). The ratio of inertial to viscous forces is known as the Reynolds number (Re). Therefore, by matching Re in our model to those (typically $Re < 1$) measured in the *in vivo* mucosal plexus by intravital microscopy (Ravnic et al. 2007a; Turhan et al. 2007), dynamic similarity was also achieved. In this study, we have assumed that a) particle motion reflects the fluid flow pattern, and b) particle motion provides a reasonable approximation of cell motion within these flow fields.

The disadvantage of PDMS is that the photolithography technique limits the biological models to 2D geometry. Although more complex 3D geometries are possible, the construction is painstaking and time-consuming. Based on our experience, current PDMS fabrication technology is unlikely to adequately simulate naturally occurring cylindrical geometry. Further, the structural proportions of the PDMS limit the depth-to-width aspect ratio to approximately 5:1. As a result, the pillar width in our experiments was limited to 10 μ m. More important to matching the physical size of the *in vivo* pillar, however, PDMS allowed us to construct a model with geometric, dynamic and kinematic similarities to *in vivo* intussusceptive angiogenesis. As noted previously, matching Re in the PDMS system and the *in vivo* mucosal plexus is an essential feature of our model.

Another disadvantage of PDMS is its hydrophobic surface. It should be noted, however, that there are many approaches to modifying the PDMS for use in biological applications (Fuji 2002; Shevkoplyas et al. 2005; Simpson et al. 1998; Soldani et al. 2004; Squires and Quake 2005; Stone et al. 2004). Finally, the photolithography technique produces random irregularities (rugosities) that must be considered in developing appropriate sampling techniques and rigorous experimental design (Fuji 2002; Roberts et al. 1997).

The PDMS microchannels illustrate a potential physiological role of the intussusceptive pillar; namely, the adaptive regulation of blood flow. In our experiments, the presence of the pillar increased resistance in the bifurcation system. Such a structural adaptation may be necessary in vascular beds that are not protected by arteriolar resistance mechanisms in the bifurcation system. Vessels in the chick chorioallantoic membrane do not have muscular arterioles (Patan et al. 1996). Also, the developing lung does not have arteriolar resistance vessels comparable to those in the systemic circulation (Burri and Tarek 1990). Similarly, we have not observed precapillary sphincter-like regulation of blood flow in the murine mucosal plexus (Ravnic et al. 2007a; Turhan et al. 2007). In each case, the intussusceptive pillar appears to be a structural mechanism that is capable of 1) reducing blood flow by increasing local resistance, and 2) increasing the functional capillary density by intussusceptive angiogenesis. This appears to be an adaptive mechanism that exists in both development and inflammation.

The analysis of flow fields within the PDMS microchannels provided a useful illustration of the relative value of both eulerian and lagrangian experimental approaches (Ravnic et al. 2006a). The eulerian approach describes the velocity field at a given point in space and moment in time. This approach provided a highly resolved velocity profile in the region of the pillar; however, it did not predict the movement of individual particles. Tracking the movement of individual particles—a so-called lagrangian approach—provided more information regarding the fate of individual particles.

Particle tracking demonstrated an unexpectedly long contact time between the particles and the pillar. Particles below a threshold velocity contacted the pillar and maintained contact through nearly half of the pillar circumference. An intriguing but speculative possibility is that the pillar provides a mechanism for the delivery of endothelial precursor cells (EPCs) to the expanding pillar (Schattelman et al. 2007). The blood flow delivery of EPCs could provide a mechanism for the rapid increase in pillar surface area. Another possibility is that the decreased flow velocity and spatial redistribution of particles (cells) may contribute to the perivascular accumulation of inflammatory cells in chronic inflammation. The distribution of particles at the apex and medial wall of the bifurcation is consistent with the spatial accumulation of inflammatory cells in murine colitis (Ravnic et al. 2007a). It is possible that the presence of a pillar could contribute to mononuclear cell contact, adherence, and transmigration in murine colitis.

In summary, our studies using PDMS microchannels demonstrated that the presence of an intraluminal pillar has a substantial effect on 2D flow fields as well as on the fate of individual particles within the flow stream. We suspect that the effect of an intraluminal pillar has adaptive significance for the distribution of cells in the effected flow stream. The present study was limited to cylindrical pillar geometry and a central pillar location. We suspect that variations of the shape and location of the intraluminal pillar may provide further insight into the biological and structural significance of the pillar in intussusceptive angiogenesis.

Acknowledgments

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Abbreviations

2D	2-dimensional
3D	3-dimensional
PDMS	poly-dimethyl siloxane
EPCs	endothelial precursor cells
SEM	scanning electron microscopy

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Figure 1.

Microchannel fabrication using soft photolithography. The computer designed microchannel was transferred to the SU-8 photoresist to create the “master” for subsequent PDMS moulding. The PDMS was removed from the silicon wafer and plasma bonded to a standard microscope slide. The fabricated microchannels were accessed with the perfusion tubing for subsequent perfusion and imaging.

Figure 2.

Geometry of microchannel design. A) Corrosion casts and SEM were used to select six similar bifurcations. B) The spatial dimensions of the bifurcations were converted to numerical matrices and digitally recombined with the contour lines reflecting 95% confidence limits. These geometric parameters were combined with the established geometry of the intraluminal pillar for the fabrication of the PDMS microchannels. C) The computer-aided design of the PDMS microchannels on the outline of a standard (25×76mm) microscope slide.

Figure 3.

Particle velocity in the bifurcation system measured in the upstream segment of the microchannels with ('+';gray) and without ('-';white) an intraluminal pillar. Particle velocities were calculated by both speckle displacement and particle tracking velocimetry. The aggregated data is shown. The data was plotted with the box defining the 25th and 75th velocity percentiles with the whiskers defining the 5th and 95th percentile. The median value was plotted as a square. Corresponding Reynolds number was calculated based on the microchannel width, the mean particle velocity, and the viscosity of the working fluid.

Figure 4.

Flow fields of the vessel bifurcation with and without a pillar in the geometric center of the bifurcation. A,B) Vector flow fields demonstrating by speckle displacement velocity profiles; pillar and channel walls are schematically represented. C,D) Flow field visualization produced by digital recombination of 500 consecutive images processed by standard MetaMorph (Molecular Devices) filters. The highlight (ring) demonstrates the area of significant variation in flow paths (pillar=20um).

Figure 5.

Particle paths around a pillar in the geometric center of the bifurcation. A) Time-position plots of two consecutive near-centerline particles. The particles contacted the pillar (20um) at its leading edge and maintained contact through 120–160 degrees of the pillar circumference. Both particles contacted the bifurcation apex before being distributed to the downstream segments. B) Flow velocity of 100 consecutive particles in three segments: the upstream (a) segment, the pillar (b), and the post-pillar (c) segment. The data was plotted with the box defining the 25th and 75th velocity percentiles with the whiskers defining the 5th and 95th percentile. The median value was plotted as a square. C) Serial videomicroscopy images of sequential centerline particles (yellow and green arrows) impacting the pillar; elapsed time is 270msec.

Figure 6.

Residence times and pillar contact of centerline particles within the bifurcation. A) Residence time of particles within the bifurcation. Cumulative residence time is shown as a function of particle traveling distance. Slope reflects an inverse of particle velocity. The flow paths of centerline particles (a) from entrance to the exit of the system were longer than particles in a juxta-centerline position (b); residence times of centerline particles was also significantly longer ($p < .001$). B) The relationship of particle velocity moving around the pillar (peri-pillar velocity) and arc length, expressed as degrees, is shown. Particles at two perfusion heights (20cm, open circles and 10cm, closed circles) are plotted. Linear curve fit was defined using a linear regression model that estimated the linear parameters using the method of least squares with the bands representing the 95% confidence limits of the plotted data.