HIGH-RESOLUTION NEAR-WALL FLUORESCENT MICRO-PARTICLE IMAGE VELOCIMETRY REVEALS THE PRESENCE OF A HEMODYNAMICALLY RELEVANT ENDOTHELIAL SURFACE LAYER IN MICROVESSELS IN VIVO

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ABSTRACT

Using high-resolution near-wall fluorescent micro-particle image velocimetry (μ-PIV) in mouse cremaster muscle venules *in vivo*, we show that the glycocalyx endothelial surface layer exerts a significant effect on near-wall microfluidics, and provide the first direct estimates of the effective hemodynamically relevant thickness of the layer *in vivo*. From μ-PIV data of the instantaneous translational speeds and radial positions of FITC-labeled microspheres (diameter, 0.47 μm) in an optical section through the median plane of the vessel, fluid-particle translational speeds were inferred from a detailed three-dimensional analysis of the local fluid dynamics in the vicinity of the vascular endothelium and its glycocalyx surface layer. Regression of a linear velocity distribution based on near-wall fluid-particle speeds consistently revealed a negative intercept when extrapolated to the vessel wall. In venules ~ 25 to 40 μm in diameter, we estimate a mean effective thickness of ~ 0.33 μm for an impermeable layer to ~ 0.44 μm assuming the maximum flow through the layer that can accommodate the no-slip condition on the endothelial-cell surface. For flow conditions typical of the microcirculation, the extent of plasma flow retardation through the layer required to be consistent with our μ-PIV data results in near complete attenuation of fluid shear rate and fluid shear stress on the endothelial-cell surface. These findings have important implications in broad areas of microvascular physiology, and necessitate the revision of previous concepts of leukocyte adhesion, stress transmission to vascular endothelium, and mechanotransduction mechanisms.

INTRODUCTION

The interface between blood and the vascular endothelium is a complex, dynamic, and fundamentally important interface in mammalian physiology. Strategically located at this interface is a glycocalyx surface layer, which is regulated by and expressed on vascular endothelial cells.

Visual evidence for the existence of an endothelial surface layer (ESL) *in vivo* was first provided by the exclusion of plasma-borne FITC-dextran (FITC-dx) in capillaries and small post-capillary venules (<15 μm in diameter)[1]. Using intravital microscopy, these studies showed that 70 kDa dextran molecules were sterically excluded from a region ~0.4 to 0.5 μm in thickness adjacent to the endothelial-cell surface. Indirect evidence that the ESL has an influence on vascular resistance in small vessels <30 μm in diameter has been shown based on decreased flow resistance in microvascular networks[2] after infusion of heparinase, an enzyme that appears to partially degrade the ESL[3,4]. The ability of the ESL to exclude large dextran molecules and limit the cross-sectional area available to red cells passing through capillaries suggests that it might be an important determinant of microfluidics near the vessel wall.

This was first suggested on theoretical grounds by considering the pressure-driven flow of a fluid suspension of rigid spheres in a tube lined with a porous layer[5] and by models of single-file red-cell motion in glycocalyx-lined capillaries[6]. Using an estimate of glycocalyx fixed-charge density, Damiano and Stace[7] were able to estimate the hydraulic resistivity of the ESL from their mechano-electrochemical model of the layer to be between 10^{10} and 10^{11} dyn-s/cm^4. This is consistent with an earlier estimate by Feng and Weinbaum[8] inferred from a fiber matrix model of the glycocalyx.

METHODS

The cremaster muscle of WT C57Bl6 mice was prepared for intravital microscopy and visualized on a Zeiss intravital microscope with a X100 saline immersion objective. Venules 24 to 41 μm diameter with clear focus were selected. A small volume of 470 nm fluorescent microspheres was slowly injected through the carotid cannula until 10 to 20 beads per second passed through the vessel. The microspheres were visualized using stroboscopic double-flash epi-illumination, and recordings were made through a CCD camera. This technique yields, in one picture, two images of the same microsphere displaced a measurable distance over a known time interval. Transillumination was maintained to keep the vessel wall clearly visible.

In some mice, FITC-dx 70 in saline was slowly infused through the carotid cannula until the venular lumen was sufficiently bright without obscuring visualization of fluorescent microspheres. After 10 minutes, the cremaster muscle was continuously epi-illuminated for 5

minutes at 450 to 490 nm with a mercury vapor short-arc lamp and a X20 objective to degrade the glycocalyx surface layer[1]. Microsphere velocity recordings were then made as described above using the X100 objective.

For each vessel, μ-PIV data were used to estimate the thickness, *t*, of the ESL over a range of values of hydraulic resistivity, *K*. Because microspheres influence near-wall microfluidics, the μ-PIV data were used to infer the translational speeds of fluid particles in the plasma-rich layer if no microspheres were present in the flow[9,10]. This was done iteratively in each vessel for each fixed value of *K* that we considered. Beginning with no plasma flow through the ESL ($K \rightarrow \infty$), a linear regression analysis was performed on all of the μ-PIV data in the plasma-rich region of a given vessel. The distance from the vessel wall where the linear regression extrapolated to zero velocity was taken as our initial guess of the ESL thickness. The analysis of Goldman *et al*. was used to infer the translational speed a fluid particle would have if the sphere were not present in the flow, and a linear regression analysis was then performed on the predicted fluid-particle speeds. The distance from the vessel wall where this linear regression extrapolated to zero velocity was then taken as our updated guess of *t*. Iteration continued until convergence on the precise layer thickness, *t*, was achieved.

A similar process was repeated for finite values of *K*. Iteration started with our estimate of *t* from the previous larger value of *K*. Approximating the layer as a Brinkman medium, the analysis of Damiano *et al*. (2002) was used to infer the translational speed a fluid particle would have if the sphere were not present in the flow. Linear regression analysis of the fluid-particle speeds was used to find the slip velocity at the current estimate of the ESL interface. This slip velocity was then compared with the slip velocity predicted in the absence of spheres. Successive updated thickness estimates were made by progressively increasing the current estimate until the value of *t* was obtained that corresponded to a continuous velocity profile across the interface. This procedure was repeated for several values of *K* (10^8, 10^9, and 10^{10} dyn-s/cm^4).

RESULTS

Fluorescent microspheres were visualized flowing in close proximity to the endothelium *in vivo*. Of 79 microspheres in 10 control venules measured within 1.5 μm of the vessel wall, only 2 were measured with their centers within 0.75 μm of the endothelium (the "south pole" of these two microspheres, therefore, were within ~ 0.52 μm of the endothelium). In contrast, 45 microspheres (out of 109 total within 1.5 μm of the vessel wall) were measured in 10 venules with their centers within 0.75 μm of the endothelium after exposure to continuous epi-illumination (light-dye treatment).

Our estimate of the effective, hemodynamically relevant layer thickness was determined for 10 control venules (24 to 41 μm diameter) and 10 venules (18 to 31 μm diameter) after light-dye treatment to degrade the ESL for three finite values of *K* and in the limit as $K \rightarrow \infty$. As expected, estimated mean effective layer thickness, $\bar{t}$, increased slightly with decreasing *K* ranging from a minimum, assuming no flow through the layer, of $\bar{t}$ ~0.33±0.04 μm to a maximum of ~0.44±0.04 μm for $K=10^8$ dyn-s/cm^4 in control vessels and from ~0.07±0.02 μm to ~0.14±0.02 μm in vessels after light-dye treatment to degrade the layer. Thus it would appear that continuous exposure of vessels to epi-illumination results in only a partial degradation of the layer with a total reduction in thickness of ~75%.

DISCUSSION

Using high-resolution near-wall μ-PIV, our results provide the only direct evidence for a hemodynamically relevant ESL in microvessels *in vivo*. Confirmation of the existence and apparent *in vivo* thickness of an ESL, first estimated based on dye and erythrocyte exclusion techniques in capillaries and small post-capillary venules[1,3], and later by an indirect method based on hemodynamic modeling calculations in microvascular networks[2], and now by a third, direct, and completely independent method using intravital μ-PIV in venules, offers increasing confidence in the true extent of this structure *in vivo* over and against earlier estimates inferred from EM studies. An ESL with these properties has significant implications for microvascular physiology. First, any molecule present in the vessel lumen will have to negotiate a very thick, largely unstirred surface layer far beyond the distances conventionally assumed. Second, based on the bounds that we have inferred of the mean effective thickness and hydraulic resistivity of the ESL, retardation of plasma by the layer must be so great that the fluid shear rate (and hence fluid shear stress) on the endothelial-cell surface is effectively completely attenuated. Hence, mechanical stress from the blood flowing in the vessel lumen would be transferred to the macromolecular matrix of the glycocalyx in the form of tension in its proteoglycan/glycosaminoglycan constituents. Finally, while its implications for microvascular rheology are most profound in capillaries, a 0.5-μm-thick ESL, having relatively low hydraulic permeability, would exert a significant influence on microvascular rheology in vessels as large as 50 μm in diameter. Although such a structure would account for only ~2% of the radius of vessels 30-50 μm in diameter, it would occupy nearly one third of the high-shear-rate, low-viscosity plasma-rich region near the vessel wall.

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