Chapter 13

VAScular MIMetic Microfluidic Systems For the Study of Endothelial Activation and Leukocyte Recruitment in Models of Atherogenesis

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Monocyte recruitment to inflamed endothelium is an early event in atherogenesis and occurs at focal regions where the average shear stress is on the order of one dyne/cm². Applying soft lithography to fabricate microfluidic channels that assemble above a living endothelial monolayer grown in culture, we have produced a vascular mimic system that enables detailed studies of the influence of inflammation, hydrodynamics, and dietary lipid on the expression and function of vascular adhesion molecules.

1. Shear Stress Modulates Endothelial Adhesion Molecule Expression

The focal nature of inflammation can be observed by histological examination of atherosclerotic lesions in aorta and arteries.¹ Plaques and atheromata develop preferentially within characteristic geometries, such as curvatures and bifurcations that exhibit disturbed flow characteristics.²,³ A hallmark of atherogenesis is the upregulation of endothelial cellular adhesion molecules (CAMs) and concomitant recruitment of monocytes, which rapidly emigrate across inflamed EC and over time differentiate into foam cells within the vessel intima.

Shear stress imparted by the viscous flow of blood plays a significant role in the homeostasis of vascular structure and function in part through the action of the mechanically-responsive endothelium. The presence of low fluid shear (i.e. ~1 dyne/cm²), high gradients of stress, and flow disturbances
all correlate with atherogenesis, whereas steady laminar shear is required to maintain vessel homeostasis. \(^4\) Thus, the magnitude of fluid shear stress and the level of cytokine stimulation tightly regulate where and when in arteries monocyte recruitment occurs. Elevated levels of shear stress as observed in arteries of healthy human subjects (i.e. \(\sim 15 \text{ dyne/cm}^2\)) are atheroprotective. This is in part attributed to the mechanical influence of shear force acting on endothelium that results in attenuation of VCAM-1 expression, despite the fact that ICAM-1 expression is upregulated at high shear stress. Conversely, preconditioning EC at low shear stress (i.e. \(\sim 2 \text{ dyne/cm}^2\)) as associated with regions of flow disturbance at bifurcations and the inner curvature of the aorta, results in pro-atherogenic conditions. For instance, arterial regions of low shear stress and large spatial gradients exhibit amplified upregulation of VCAM-1 and E-selectin, which in turn increase the efficiency of monocyte capture even at very low concentrations of cytokine stimulation (i.e. TNF-\(\alpha\) 0.1 ng/ml).\(^5-8\) Thus, differential regulation of CAM transcription could result in changes that are both profound and anti-inflammatory in the context of leukocyte recruitment.\(^9-12\) Since atherosclerosis is a focal disease that typically develops at vascular sites of bifurcations and curved arteries that harbor steep shear stress gradients, a detailed study of how CAM expression and monocyte recruitment maps on inflamed endothelium exposed to shear is of keen interest for thorough understanding of disease.

2. Monocyte Recruitment During Atherosclerosis

Monocyte capture, rolling, activation, and arrest to nascent atherosclerotic plaques is a coordinated effort by selectins, integrins, and their respective ligands. Capture from the free stream of blood is supported by P-selectin glycoprotein-1 (PSGL-1) and other glycosylated ligands on the monocyte which bind P-selectin, and E-selectin on inflamed endothelium.\(^13,14\) Monocyte capture is an important first step of atherosclerosis. Hypercholesterolemic mice lacking these molecules are atheroprotected.\(^15,16\) Selectins enable leukocytes to be recruited and roll along the endothelium due to their exceptionally high on and off rates, which govern how quickly bonds are formed and broken.\(^17\) P- and E-selectin require a threshold level of shear stress on the order of 1 dyne/cm\(^2\) to initiate tethering through receptor tension; interruption of the shear field causes rolling leukocytes to detach.\(^18\) This is due to their catch bond nature,\(^19\) the
force imparted by the fluid flow strengthens the bond. This phenomenon in conjunction with presentation of additional ligand to selectins as the monocyte rolls allows new bonds to form before old ones are broken.29

Integrins are responsible for decelerating the rolling leukocyte and mediating firm adhesion through binding Ig superfamily members expressed on the endothelial membrane. The β1 integrin, very late antigen-4 (VLA-4 or α4β1), is activated on the monocyte through inside-out signaling following ligation of chemokines such as MCP-1 and subsequently binds vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 expression is detected at early plaques on hypercholesterolemic rabbits and its expression precedes macrophage accumulation.21 In addition, hypercholesterolemic mice lacking a VLA-4 binding domain in VCAM-1 have decreased atherosclerosis.22 It is controversial whether VCAM-1 expressed at atherosusceptible sites is an initiating event in monocyte recruitment and plaque formation.

The β2 (CD18) integrins also play a role in monocyte trafficking to atherosclerotic lesions as CD18−/− mice have decreased atherosclerosis (Nageh et al., 1997). The β2 integrin, lymphocyte functional antigen-1 (LFA-1 or α1β2) binds ICAM-1 on the endothelium and their interactions are important for atherogenesis (Kitagawa et al., 2002). Recently, CD11c/CD18 has been reported to bind VCAM-1 during monocyte firm arrest on inflamed endothelium.23 Furthermore, CD11c−/− hypercholesterolemic mice have decreased atherosclerosis and their monocytes exhibit a defect in recruiting to recombinant VCAM-1 and E-selectin in shear flow.24 A cooperative process appears to underlie recruitment of monocytes in response to activation and VCAM-1 ligation of VLA-4 and CD11c. In both humans and mouse models, a high fat diet can induce upregulation of CD11c and enhanced efficiency of monocyte recruitment to VCAM-1 in shear flow (Gower et al., Unpublished data).

During inflammation the endothelium drastically alters its adhesive phenotype increasing the expression of E-selectin, ICAM-1, and VCAM-1 allowing monocytes that come in contact with the vessel wall to capture, roll, firm arrest, and emigrate into the tissue. These dynamic endothelial-leukocyte interactions are crucial for immune surveillance, however, when this process becomes dysregulated, chronic inflammatory disease ensues. To examine how hydrodynamic shear superposes with inflammatory insult to promote monocyte recruitment, we have developed microfluidic networks that allow us to study these interactions in real-time using small sample
volumes that enable of the study of limited and expensive biological specimens and reagents.

3. Design and Fabrication of Vascular Mimetic Microfluidic Chambers

In vitro models that mimic physiological parameters of blood vessels such as shear stress and geometry are powerful tools to study the molecular and cellular events associated with inflammation occurring at the membrane of the endothelium. Conventional parallel-plate flow chambers (PPFC) have been implemented to assay the endothelial or leukocyte response to defined magnitudes of shear stress. The shear stress at the endothelial membrane in a PPFC may be approximately by:

\[
\tau_w = \frac{6\mu Q}{wh^2}.
\]

(3.1)

Where \(\mu\) is the fluid viscosity, \(Q\) is the volumetric flow rate, \(w\) is the channel width, and \(h\) is the channel height. Owing to their macro-scale dimensions, PPFC require large volumes to infuse cell media or buffer solution at constant flow rates. Assuming the viscosity of water (1 cP), a typical PPFC with a height of 300 \(\mu m\) and a width of 2 cm requires flow rates on the order of 40 ml/min to maintain a shear stress of 20 dyn/cm\(^2\); the stress present at the endothelial membrane at regions of the coronary artery that are athero-protected. Thus, these devices have limited utility in assays requiring limited reagents such as small molecule inhibitors, antibodies, or sparse leukocyte populations such as monocytes.

To address the need for smaller working volumes in assays studying leukocyte or endothelial mechanobiology in shear flow, we designed novel microfluidic devices capable of delivering fluid with precisely controlled chemical composition and flow rate. The general design of the flow device consists of two major components: (I) microfluidic flow channels and (II) a vacuum channel network. As shown in Fig. 1, the vacuum channel network is a spider web-like pattern that serves to seal the device to a glass coverslip in the absence of adhesives or chemistries that are incompatible with cells or proteins. These microchannels have heights between 50 and 100 \(\mu m\) and a variety of widths ranging from 200 \(\mu m\) to 2 mm. We have coined these devices vascular mimetic microfluidic chambers (VMMC) in contrast to their generic macro-scale counter parts, PPFC.
Fig. 1. Schematic of the vascular mimetic microfluidic device. Four independent chambers containing microchannels are recessed into a PDMS disc and assemble above an endothelial monolayer (here grown in a 6-well tissue culture plate). The network of vacuum channels resembles a spider-web. When connected to house vacuum through access ports punched into the PDMS, the device is sealed to the biological substrate. A blunt 20-gauge needle hub serves as a reservoir for monocyte suspensions, chemokines, or antibodies. A syringe pump applying negative pressure at the outlet drives flow. From Schaff UY et al. (2007) Lab Chip (4): 448-456. Reproduced by permission of the Royal Society of Chemistry.

Standard methods of soft lithography are applied to create the VMMC. The network of microchannels are designed in CAD (Autodesk, San Rafael, CA) and printed at 5000 dpi on a transparency. Negative photoresist (SU8, Micro Chem, Newton, MA) is spun onto a silicon wafer at a user-defined thickness between 50 to 100 μm. The transparency is then overlayed on the coated wafer and exposed to UV light. During development photoresist that is not polymerized by the incident UV light is removed and a positive replica (master mold) of the design remains on the wafer. Polydimethylsiloxane (PDMS) (Sylgard 184) prepolymer is poured over the master mold and cured. A disc containing the design is excised and access ports are punched with a hollow blunt needle into the PDMS (Fig. 1). The VMMC's small profile allows for incorporation of multiple microfluidic designs into a single master mold. This yields a practical advantage of batch production and because PDMS is inexpensive, the flow chamber can be discarded if soiled by chemicals or deteriorated.
Microchannels recessed in the PDMS disc form individual micro-scale parallel plate flow chambers above a live endothelial monolayer when vacuum-sealed to the glass coverslip (Fig. 1). Unique properties of our VMMC include precise microfluidic delivery of reagents and versatility with which flow can be driven. In acute experiments involving the addition of leukocytes, antibody, or inflammatory stimuli a syringe pump applying negative pressure to the outlet rather than positive pressure to the inlet drives flow. A blunt 20-gauge needle with a 100 μL capacity is press-fit directly into the PDMS disc and serves as a sample reservoir. The inlet dead volume of this configuration is less than 2 μL, a value three orders of magnitude smaller than a PPFC. Furthermore, the open “on-chip” reservoir facilitates rapid addition of leukocytes, stimuli such as cytokine, or antagonists such as monoclonal antibodies, with a standard micropipette. Thus a number of important questions relating to the earliest events of acute inflammation may be addressed by controlling the cellular and chemical composition entering the VMMC in real time.28

Depending on the design, two to eight independent and vacuum-isolated VMMC are integrated into each PDMS device and are addressed by sequential connection to a syringe pump and perfused with separate suspensions of leukocytes or soluble inflammatory agonists. This makes it possible to apply multiple experimental conditions to a single endothelial monolayer, allowing for higher experimental throughput and less variability. In contrast, a conventional PPFC requires an individual glass slide coated with cells for every experimental condition due its much larger footprint.

In experiments that require long term culture (i.e. to orient EC in the direction of flow ~24 hours) of the endothelial monolayer in shear flow, a syringe pump infusing fluid into the inlet of the VMMC drives flow. Cell media can be collected at the outlet and assayed for chemokines or other soluble agents released by the endothelium. The feasibility of this configuration is owed to the dimensions of our VMMC. A microchannel that is 75 μm in height and 2 mm wide requires less that 60 mL of fluid to apply 20 dyn/cm² at the endothelial membrane for 4 hours. Use of a peristaltic pump that circulates a constant volume of buffer over the monolayer can be used in place of the syringe pump. In order to simulate conditions associated with inflammation, TNF-α or other inflammatory stimuli is premixed with cell media. Following shear conditioning the outlet tubing is removed and a reservoir is inserted in its place. Running the syringe pump in withdraw
mode an assay measuring leukocyte adhesion efficiency on the monolayer is carried out.

PDMS is transparent so that cellular interactions within the VMMC can be imaged from below with an inverted DIC-fluorescence microscope coupled to a CCD camera and image analysis system. This system represents a virtual lab on a chip since it affords up to 8 separate experiments in which HAEC are preconditioned by defined shear regimes, activated by cytokines, and treated with inhibitors under static or shear flow conditions. Inflammatory responses are detected in situ by immunofluorescent imaging of CAM up-regulation or transcription factor activation or by adhesive interactions with leukocytes.98–31

4. Adhesion Molecule Expression on Cultured Aortic Endothelium Studied in a Linear Gradient of Shear Stress

Hemodynamics exerts a significant influence in the inflammatory response of vascular endothelium to cytokine.32,33 The relationship between the magnitude of shear stress and upregulation of endothelial CAMs has been widely studied using PFFC to produce a constant shear rate and defined average shear stress.34–37 In order to examine the focal nature by which atherosclerotic plaques form within defined regimes of shear stress in the vasculature, we produced a modified version of our VMMC that delivers a linear decrease in shear stress from the inlet to the outlet. This flow channel was used to study endothelial response along a channel consisting of a continuous human aortic endothelial cell (HAEC) monolayer. The design of the flow chamber was adapted from Hele-Shaw flow theory.38 A linear decrease in shear stress magnitude along the centerline of the channel, parallel to the longitudinal axis, was achieved by designing the sidewalls of the flow chamber to coincide with the streamlines of a two dimensional stagnation flow and making the end of the channel shaped to match the iso-potential lines. Using this design it was possible to expose the HAEC to a physiological range of shear stress with high spatial resolution while maintaining a constant shear gradient.31 The generated wall shear stress ($\tau_w$) along the channel centerline at the endothelial membrane is given by:

$$\tau_w = \frac{6\mu Q}{h^2 v_1} \left(1 - \frac{x}{L}\right), \quad (4.1)$$
where $\mu$ is the flow medium viscosity, $Q$ is the volumetric flow rate, $h$ is the channel height, $w_1$ is the entrance channel width, $L$ is the total channel length, and $x$ is the length measured from the entrance at any point down the channel. The dimension of the flow chamber used in this study consisted of the following parameters: $h = 100 \ \mu m$, $w_1 = 2 \ mm$, $L = 20 \ mm$. The linearity of shear stress with distance down the flow chamber, as defined by Equation 2, was previously validated experimentally based on streamline analysis.38

The Hele-Shaw chamber was applied to determine the relative importance of the magnitude versus the gradient of shear stress as endothelial cells respond to stimulation by TNF-$\alpha$. To gauge the response to shear stress we measured the change in expression of endothelial ICAM-1, VCAM-1 and E-selectin under static and flow conditions. Endothelial CAM expression was measured as a function of distance down the flow channel, in which shear stress decreased linearly from 16dyne/cm$^2$ at the inlet to essentially zero at the exit. This shear stress range was chosen to model the transition from atheroprotective (i.e. $\geq$12 dyne/cm$^2$) to athero-prone (i.e. $\leq$4dyne/cm$^2$) arterial regions as defined in previous studies.7 CAM expression after 4 hours of TNF-$\alpha$ in the presence of laminar shear stress was detected by immunofluorescence of antibody conjugated quantum dots and quantified by image analysis over distinct 0.1 mm$^2$ regions, corresponding to an area on the monolayer containing 50 HAEC.

The relative change in E-selectin and VCAM-1 expression as a function of the magnitude of shear stress over discrete areas is plotted in Fig. 2A and B as the percent of unstimulated endothelial MFI under static conditions. ICAM-1 was also presented in this study and found to increase to maximum level at $\sim$12 dyne/cm$^2$. Addition of TNF-$\alpha$ (0.3 ng/mL) under static conditions stimulated upregulation of VCAM-1 by 350%, and ICAM-1, and E-selectin by 150% and 250%, respectively. Shear alone elicited a 100% increase in ICAM-1 expression at high SS, whereas VCAM-1 and E-selectin expression was not significantly upregulated at any position along the gradient.

In the presence of shear stress and TNF-$\alpha$, ICAM-1 expression increased linearly with shear stress rising to 450% of the unstimulated static condition, before reaching a plateau. The greatest change in CAM expression on inflamed HAEC occurred within the low range in shear stress from 2–4 dyne/cm$^2$, correlating with shear stress values found within vascular regions prone to atherogenesis.7 In contrast, TNF-$\alpha$ stimulated
CAM expression was invariant at shear stress greater than 10 dyne/cm², which reflects the quiescence of inflammatory response within straight unperturbed arterial regions (i.e. 12–17 dyne/cm²). The rate of change of CAM expression was steepest between 2–8 dyne/cm², which suggests a mechanotransduction signaling pathway that modulates inflammation within this range of SS. Although the precise mechanisms by which shear stress superposes with cytokine to regulate inflammatory gene expression have yet to be determined, it is clear that they act through distinct, often converging pathways to modulate the activity of transcription factors associated with inflammation including NFκB, AP-1, GATA, specificity protein-1 (SP-1), and IFN regulatory factor-1 (IRF-1).32,39–43

5. Monocyte Recruitment on Vascular Mimetics

Monocyte activation and recruitment to lesions is a harbinger of atherosclerosis. For instance, monocytes from individuals with hypercholesterolemia exhibit upregulated expression of integrins44 and increased adhesion.45–47 In response to insult, endothelium and monocytes produce
cytokines and chemokines that in turn upregulate the expression of CAMs on adjacent endothelial cells. Adhesion is mediated by coordinate ligation of membrane receptors on monocytes and their cognate ligands on the inflamed endothelium. Direct observation of monocyte recruitment in microfluidic channels on TNF-α stimulated endothelium shear conditioned in the Hele-shaw chamber was performed to examine the importance of a linear gradient of shear stress on this multi-step process. The level cytokine stimulation and the magnitude of fluid shear stress tightly regulate monocyte recruitment. As depicted in Fig. 2 we examined monocyte arrest on a monolayer that was aligned parallel to the direction of shear stress preconditioning, in which HAEC were exposed to 0–16 dyne/cm². Monocytes were infused at a constant shear stress of 2 dyne/cm², and adhesive interactions were video recorded. These data reveal the effect of the shear stress gradient on CAM expression and in turn its significant impact on monocyte recruitment. Monocyte recruitment efficiency was found to vary as a function of position down the flow channel. This essentially provides a map of recruitment efficiency as a function of the differential expression of CAMs that are modulated by the superposition of cytokine activation and shear stress. A shear stress of 7 dyne/cm² emerged as a critical value for altering CAM expression and favoring monocyte arrest, as recruitment efficiency increased significantly below this threshold. This observation is consistent with the fact that both E-selectin and VCAM-1 expression begin to rise from the baseline at this shear stress. Significantly, E-selectin reached the greatest rate of change in expression between 6–9 dyne/cm². Thus, HAEC ligands that support monocyte capture and arrest are critical for optimum transition to stable adhesion and transmigration.

6. Lipid Primes Endothelium for an Enhanced Response to Inflammation and Increases Monocyte Recruitment

In a separate set of studies, we used the VMMC to examine the role of dietary lipids (triglyceride rich lipoprotein, TGRL consisting of vLDL and chylomicron remnants) and TNF-α stimulation on monocyte recruitment to HAEC. HAECs were treated with TGRL (2.5 mg/dL) for 2 hours a day for 1, 2 or 3 days, followed by a 4 hour TNF-α exposure (0.3 ng/mL) after the last TGRL treatment. This was meant to simulate the repetitive injury sustained by endothelium during consumption of a high fat diet. E-selectin
Fig. 3. Cellular adhesion molecule expression and monocyte recruitment on human aortic endothelium exposed to triglyceride rich lipoprotein (TGRL) for 3 days, followed by TNF-α treatment. TNF-α concentration for all panels is 0.3 ng/mL. TGRL concentration is 2.5 mg/dL. Adapted from Ting HJ et al. (2007) Circulation Research 100(3): 381–390.

expression was significantly higher after a single TGRL treatment followed by TNF-α compared to TNF-α alone (Fig. 3A). ICAM-1 expression did not change with lipid treatment (data not shown). VCAM-1 expression was significantly increased over TNF-α alone after 2 days of TGRL pretreatment, but 3 days of TGRL treatment in the absence of TNF-α did not affect VCAM-1 expression (Fig. 3B). Thus we find superposition of lipid and cytokine or low shear stress and cytokine (Fig. 2A, B) can potentiate expression of two endothelial CAMs obligatory for monocyte arrest.

Recruitment of monocytes to HAECs at 2 dyn/cm² was imaged by phase contrast microscopy in order to gauge the effect of the amplification of VCAM-1 over cytokine stimulation by TGRL. Monocytes were categorized as rolling, arrested, or transmigrated across the endothelial monolayer. The number of adherent monocytes increased over a range of TNF-α
stimulation and 0.3 ng/mL was a concentration at which 50% of maximum monocyte interaction was observed (data not shown). Pretreating HAECs with TGRL for 3 days and subsequently stimulating them for 4 hours with TNF-α effectively doubled monocyte recruitment efficiency over TNF-α alone, whereas neutrophil recruitment did not increase. Consistent with CAM upregulation, TGRL treatment alone did not support monocyte recruitment. We next examined the CAMs supporting increased monocyte recruitment. In the presence of TNF-α stimulation alone, monocyte arrest and transmigration was supported by β1 and β2 integrins, as blocking each with antibody added up to the control level of 6 monocytes per field (Fig. 3C). ICAM-1 and VCAM-1 supported firm adhesion; however, capture and rolling required expression of E-selectin, as determined by antibody inhibition. Monocyte recruitment on TGRL treated HAEC remained dependent on both β1 and β2 integrins (Fig. 3D). However, inhibition of β2 integrins blocked proportionally less monocyte arrest on TGRL-primed HAECs versus TNF-α alone, suggesting that β1 integrin is predominant. Interestingly, greater cooperativity between β1 and β2 integrins was apparent for the TGRL primed HAECs, as the total number of monocytes arrested was 30% greater than the sum of monocytes recruited when blocking integrins individually. These data reveal that priming with vLDL lipids increases monocyte recruitment to inflamed HAECs in a β1- and β2-integrin dependent manner.

Summary
Design and implementation of a custom microfluidic flow channel that reversibly bonds to a glass coverslip or plastic tissue culture plate containing a living endothelial monolayer we denote vascular mimic microvascular chambers (VMMC) was presented. This device allows the study of both acute exposure to inflammatory molecules that upregulate CAMs over hours, as well as more chronic studies using shear flow to mechanically modulate the endothelial response. The combination of a living substrate along with fine tune control of the shear rate and stress distribution throughout the channel allows a variety of biological studies to be performed. We examined how endothelial cells sense a linear gradient of fluid shear stress and transduce signals that regulate membrane expression of cell adhesion molecules and monocyte recruitment. We also demonstrated that repetitive exposure to native dietary lipids prime HAECs for an
amplified inflammatory response to TNF-α. Employing our VMMC we showed monocytes were preferentially recruited over neutrophils on HAECs inflamed by both cytokine and dietary vLDL in shear flow. These vascular mimetic models provide an opportunity to perform ex vivo studies of the contributions of hyperlipidemia, hemodynamics, and vascular inflammation in the etiology of atherosclerosis.

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References


