A Novel High-Throughput In Vitro Model for Identifying Hemodynamic-Induced Inflammatory Mediators of Cerebral Aneurysm Formation

Kamil W. Nowicki, Koji Hosaka, Yong He, Peter S. McFetridge, Edward W. Scott, Brian L. Hoh

Abstract—Cerebral aneurysms are thought to develop at locations of hemodynamic shear stress, via an inflammatory process. The molecular mechanism that links shear stress to inflammation, however, is not completely understood. Progress in studying this disease is limited by a lack of a suitable in vitro model. To address this, we designed novel in vitro parallel-plate flow chamber models of a straight artery, a bifurcation, and a bifurcation aneurysm. We compared endothelial cell phenotypes across the 3 different models and among microenvironments within each flow model by cytokine array, ELISA, and relative immunofluorescence. Human aneurysms express interleukin-8 and chemokine (C-X-C motif) ligand 1 (CXCL1), whereas normal arteries do not. The bifurcation aneurysm model showed significantly higher interleukin-8 and CXCL1 levels than both the straight artery and bifurcation models. Within the bifurcation and bifurcation aneurysm models, endothelial cells near the bifurcation or within the aneurysm sac microenvironments have significantly higher expression of CXCL1, and interleukin-8 and CXCL1, respectively, than at the straight proximal segment or the limbs of the bifurcation. Murine aneurysms express CXCL1, and it is the primary ELR+ CXC chemokine expressed, whereas normal arteries do not. CXCL1 antibody blockade results in significantly fewer murine aneurysms (13.3 versus 66.7%; \( P=0.0078 \)), decreased neutrophil infiltration, and vascular cell adhesion molecule 1 expression than an immunoglobulin G control. We successfully designed and validated a novel hemodynamic model of cerebral aneurysms in vitro. We also show that shear stress–induced CXCL1 plays a critical role in cerebral aneurysm formation. (Hypertension. 2014;64:00-00.) ● Online Data Supplement

Key Words: aneurysm ■ endothelium ■ hemodynamics ■ inflammation

Cerebral aneurysms are focal dilations of cerebral arteries that can rupture and result in subarachnoid hemorrhage, leading to significant mortality and morbidity.\(^1\) Computational flow dynamic (CFD) studies have implicated high wall shear stress (WSS) and WSS gradients in initiating aneurysm development.\(^2–5\) Hemodynamic shear stress has been implicated in activation of the inflammatory pathway through nuclear factor-κB in the endothelium.\(^6\) Because animal studies are costly, time-consuming, and difficult to manipulate, an effective in vitro screening tool is needed to study the cell phenotypes and inflammatory mediators that occur in the context of cerebral aneurysm hemodynamic conditions.

To address this need, we designed novel in vitro parallel-plate flow chamber models of a straight artery, a bifurcation artery, and a bifurcation aneurysm, characterized by reproducible hemodynamic conditions to study the effect of shear stress on endothelial cell (EC) phenotypes at different microenvironments. Importantly, the model is simple, effective, and has high throughput in that it is low cost and only takes 24 to 48 hours to produce results. We propose that low shear stress and shear stress gradients at bifurcations and bifurcation aneurysms induce endothelial expression of specific inflammatory factors that promote aneurysm formation and growth.

Methods

Detailed descriptions of all methods are available in the expanded methods section of the online-only Data Supplement.

Parallel-Plate Flow Chamber Design

Uzarski et al.\(^7\) originally designed the parallel-plate chamber for a straight artery. The idealized 3-flow models of a straight artery, bifurcation, and bifurcation aneurysm were created based on previously published CFD studies\(^8–9\) and anatomic dimensions of human cerebral arteries.\(^9\)

Modeling Shear Stress

Mesh generation was performed in Gambit 2.0 (ANSYS, Canonsburg, PA). CFD simulation using measured flow rates (Figure S1 in the online-only Data Supplement) was done in ANSYS Fluent (ANSYS, Canonsburg, PA). Postprocessing and visualization of velocity streamlines, WSS, and WSS gradients were performed in Tecplot 360 (Tecplot, Bellevue, WA).
Shear Stress Experiments

Human umbilical vein ECs at passage 2 to 5 and pooled from 3 donors (Figure S2) were seeded on coverslips cut from tissue culture plastic dishes (Techno Plastic Products, Trasadingen, Switzerland) at a density of 20000 cells/cm² and grown to confluence. ECs were exposed to pulsatile shear stress >27 hours and ultimately exposed to shear stress at 10 dyne/cm² for 24 hours in all parallel-plate flow chambers at 5% CO₂ and 37°C.

Animals

All animal experimentation was performed under the Institutional Animal Care and Use Committee–approved protocol (#201303748).

Mouse Intracranial Aneurysm Model

Murine intracranial aneurysms were created in female, 8- to 12-week-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) using a method described previously. For the antibody blockade, 100 μg/mL anti-CXCL1 antibody (MAB453; R&D Systems) or 100 μg/mL IgG2A control antibody were injected retro-orbitally every 2 days.

Human Aneurysm and Superficial Temporal Artery Specimens

Collection of human cerebral aneurysm and superficial temporal artery specimens was performed under the institutional review board–approved protocols (#448–2007 and #201400190). Patients gave written informed institutional review board research consent.

Statistical Analysis

Statistical analyses were performed by a biostatistician (D.W.N.) and are described in detail in the online-only Data Supplement.

Results

Bifurcation and Bifurcation Aneurysm Parallel-Plate Flow Chamber Models Simulate WSS Patterns Found in Bifurcations and Cerebral Aneurysms

CFD studies of human and rabbit aneurysms have demonstrated that the average WSS within the aneurysm dome is lower than in the parent vessel. For the bifurcation aneurysm model, WSS was 8.6 dyne/cm² in the proximal straight segment, 5.2 dyne/cm² at the bifurcation, and 4.3 dyne/cm² in the branching limbs. For the bifurcation aneurysm flow chamber, WSS was 8.6 dyne/cm² in the proximal straight segment, 4.7 dyne/cm² at the bifurcation, 4.2 dyne/cm² in the branching limbs, and 0.8 dyne/cm² within the aneurysm sac.

Low Hemodynamic Shear Stress and Shear Stress Gradients Upregulate Endothelial Expression of Inflammatory Mediators

We compared the inflammatory conditions across the 3 different flow chamber models (straight artery, bifurcation aneurysm, and arterial bifurcation; n=3 each) by using cytokine arrays. Multiple markers were upregulated in the bifurcation and bifurcation aneurysm flow models compared with the straight artery. Interleukin-8 (IL-8) was the most differentially expressed cytokine (Figure 2A).

Low Hemodynamic Shear Stress and Shear Stress Gradients at the Aneurysm Sac Microenvironment Upregulate IL-8 Expression

ELISA of perfusate demonstrates that IL-8 secretion was significantly different across the 3 flow models (P<0.0238). Significantly more IL-8 was secreted in the bifurcation aneurysm model (269.5 pg/mL; n=5) than the bifurcation (146.8 pg/mL; n=5; P<0.0395) or straight model (147.5 pg/mL; n=5; P<0.0409; Figure 2B). The difference between the bifurcation and straight models was not significant (P=0.9998).

We investigated IL-8 expression by relative fluorescence–immunocytochemistry at different microenvironment sites within the bifurcation and the bifurcation aneurysm flow models to determine which microenvironment locales exposed to shear stress gradients are prone to inflammation. Within the bifurcation flow model, IL-8 expression was not significantly different among the bifurcation, proximal straight segment (1.12 versus 1.00 relative fluorescence unit [RFU]; P=0.55), or branching limb (1.12 versus 1.08; P=0.195) microenvironments (n=24 for each group; P=0.65; Figure 2C). The effect of microenvironment location on IL-8 protein expression in the bifurcation aneurysm model, however, was highly significant (n=24 for each group; P<0.0001; Figure 2D). IL-8 protein expression in the aneurysm sac was estimated to be 33% higher than at the proximal straight segment (1.28 versus 1.00 RFU; P<0.0001), 15% higher than at the branching limbs (1.28 versus 1.13 RFU; P=0.001), and not significantly different than at the bifurcation (1.28 versus 1.13 RFU; P=0.144). IL-8 expression at the proximal straight segment was estimated to be 26% lower than at the bifurcation (1.00 versus 1.12; P<0.001) and 18% lower than at the branching limbs (1.00 versus 1.12 RFU; P<0.001). IL-8 was 8% higher at the bifurcation than at the branching limbs (1.13 versus 1.12 RFU; P=0.009). Representative IL-8 immunohistochemistry is shown for the bifurcation aneurysm chamber (Figure S3A).

Figure 1. Bifurcation and bifurcation aneurysm flow chamber design and computational fluid dynamics. A, Three-dimensional model showing plastic coverslip, silicone gasket with the flow field, and polycarbonate bioreactor cell. B, Complete, sealed chambers with color-coded maps showing components of the flow field. C, Computational flow dynamic showing velocity, wall shear stress (WSS), and wall shear stress gradient (WSSG) within the designed flow chambers.
Figure 2. Continued
Low Hemodynamic Shear Stress and Shear Stress Gradients at the Bifurcation and Aneurysm Sac Microenvironments Upregulate Chemokine (C-X-C Motif) Ligand 1 Expression

Chemokine (C-X-C motif) ligand 1 (CXCL1) is the closest functional murine homologue of IL-8. Because CXCL1 was not present on the original cytokine array panel (Figure 2A), we performed a CXCL1 ELISA on the perfusate from the same experimental samples. ELISA of 20× concentrated perfusing medium from the 3 different flow models demonstrates that CXCL1 secretion is significantly different across the 3 flow models (P<0.001). Significantly more CXCL1 was secreted by ECs in the bifurcation flow model (34.3 pg/mL; n=7) and bifurcation aneurysm flow model (64.8 pg/mL; n=7) than in the straight flow model (14.4 pg/mL; n=9; P=0.003). The difference between CXCL1 expression in the bifurcation and bifurcation aneurysm flow models was not significant (P=0.477; Figure 2E).

CXCL1 protein expression at the 3 microenvironments within the bifurcation flow model was highly significant (n=24 for each group; P<0.0001 by mixed-effects linear model; Figure 2F). CXCL1 expression at the bifurcation was estimated to be 16% higher than at the proximal straight segment (1.16 versus 1.00 RFU; P<0.001) and 7% higher than at the branching limbs (1.16 versus 1.08; P=0.039). CXCL1 expression at the proximal straight segment was estimated to be 8% lower than at the branching limbs (1.00 versus 1.08 RFU; P=0.001).

CXCL1 protein expression at different microenvironments within the bifurcation aneurysm flow model was also highly significant (n=24 for each group; P<0.0001 by mixed-effects linear model). CXCL1 expression at the aneurysm sac was estimated to be 29% higher than at the proximal straight segment (1.44 versus 1.00 RFU; P<0.001), 16% higher than at the bifurcation (1.44 versus 1.23 RFU; P<0.001), and 10% higher than at the branching limbs (1.44 versus 1.25 RFU; P=0.002). CXCL1 expression at the proximal straight segment was estimated to be 14% lower than at the bifurcation (1.00 versus 1.23 RFU; P=0.001) and 19% lower than at the branching limbs (1.00 versus 1.25 RFU; P<0.001). No difference was found in CXCL1 expression between the bifurcation and the limbs (P=0.224). These data confirm that the aneurysm sac microenvironment is the primary contributor of increased CXCL1. Representative CXCL1 immunohistochemistry is shown for the bifurcation aneurysm chamber (Figure S3B).

ELR+ CXC Chemokines Are Expressed in Human and Murine Aneurysms

The in vitro data obtained from our novel model is supported by our in vivo studies. Human aneurysm specimens tested positive for IL-8 and CXCL1 (n=5/5), whereas control superficial temporal arteries did not (n=0/3; Figure 3A and 3B).

Our mouse aneurysm model serves as a tool to further investigate this process. We analyzed the expression of ELR+ CXC chemokines, CXCL1-1, -2, and -5 to 6/LIX in mouse 2-week aneurysms (Figure S4A and S4B). Reliable antibodies against CXCL3 are not available for use in mice. ELR+ CXC chemokines attract neutrophils and thus act as functional homologues of IL-8 in the mouse species. CXCL1 was expressed in the intima and the media of 2-week (5/5) aneurysms and aneurysm vessels (Figure S4B), whereas CXCL2 and CXCL5-6/LIX were not detected (0/5 for both). Furthermore, CXCL1 was expressed in 3-day- and 1-week aneurysmal tissues but not in control arteries from sham animals (Figure 3C). ECs were visualized with MEGA-32.

CXCL1 Blockade Reduces Murine Intracranial Aneurysm Formation by Preventing Inflammatory Cell Infiltration

Mice treated with anti-CXCL1 antibody every 48 hours (Figure 4A) developed significantly fewer saccular aneurysms than immunoglobulin G (IgG)–treated control mice at 2 weeks after aneurysm induction (13.3% versus 66.7%; P=0.0078; n=15 each; Figure 4B and 4C). The majority of aneurysms were localized to arterial branch points (Figure S5). The decrease in aneurysm formation in the anti-CXCL1 group was not attributable to changes in systemic systolic and diastolic blood pressure (Figure S6).

As expected, neutrophil infiltration in the anti-CXCL1–treated mice was significantly decreased at 2 weeks when compared with IgG control–treated mice (435 versus 2410 cells/mm²; n=15 for both; P=0.043; Figure 5A and 5B). Macrophage infiltration in the anti-CXCL1–treated mice was not significantly different at 2 weeks when compared with IgG control–treated mice (7163 versus 9496 cells/mm²; n=15 for both; P=0.056; Figure 5C and 5D). In addition to endothelial and mural cells, infiltrating neutrophils were found to be positive for CXCR2, a receptor for CXCL1.

Neutrophils and Macrophages Are Present in Human Aneurysms

All human aneurysm specimens analyzed were found to be positive for neutrophils and macrophages (n=5/5), whereas control superficial temporal arteries were negative (n=0/3; Figure 5E).

Discussion

Multiple studies have suggested a critical role for hemodynamics and inflammation in cerebral aneurysm formation.3,4,6,8,11

Figure 2. Continued ELR+ CXC chemokines interleukin-8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1) are the most abundantly expressed cytokines in the bifurcation aneurysm flow chamber. A, Chemokine profiles of human umbilical vein endothelial cells (HUVECs) exposed to pulsatile flow in different flow chambers. Out of 40 inflammatory cytokines analyzed, B, IL-8 was the most abundant differentially expressed cytokine in the aneurysm flow chamber, which was confirmed by IL-8 ELISA (P=0.0238). B indicates bifurcation; BL, branching limb; and PSS, proximal straight segment; C, IL-8 expression in the bifurcation flow chamber is not significantly different (P=0.065). RFU indicates relative fluorescence unit. D, HUVECs express more IL-8 in the aneurysm and bifurcation regions of the bifurcation aneurysm flow chamber (P<0.0001). AS indicates aneurysm sac. E, HUVECs express more CXCL1 in the bifurcation and bifurcation aneurysm models than in the straight flow model (P=0.0076). F, HUVECs express more CXCL1 in the bifurcation region of the bifurcation flow chamber (P<0.0001). G, HUVECs express more CXCL1 in the aneurysm sac of the bifurcation aneurysm flow chamber (P<0.0001). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Data are presented as mean±SEM.
yet current tools to study this link are limited. We present a novel, idealized flow model based on parallel-plate flow chamber geometry and validated by computational fluid dynamics (Figure 1) that replicates WSS patterns typically seen at bifurcations and aneurysms. The advantages of this in vitro model compared with animal models are many: it is easy to use, low cost, and produces results quickly. These characteristics make it ideal for initial high-throughput screening to identify putative hemodynamic-induced inflammatory mediators that can then be validated in vivo.

There are limitations to this in vitro approach because radial stretch and 3-dimensional matrices cannot be easily studied. The flow models represent idealized approximations of the hemodynamic shear stress patterns in bifurcations and bifurcation aneurysms and lack an impingement zone near the flow divider. Our goal was to study shear stress cytokines released only by ECs and avoid interactions with vascular smooth muscle cells; therefore, the model lacks the multicell layer characteristic of blood vessels. Despite these limitations, we were able to validate this powerful yet simple tool by identifying a novel role for IL-8 and CXCL1 in cerebral aneurysm formation (Figure 2).

The role of neutrophil-attracting ELR⁺ CXC chemokines, IL-8 and CXCL1, has not been studied in cerebral aneurysm formation. Within the CXC family, the chemokines with an ELR⁺ (Glu-Leu-Arg) motif attract neutrophils. Although mice do not express IL-8, CXCL1 has been described as a functional murine homologue of human IL-8. CXCL1 shares >60% of amino acid sequence with IL-8 and activates the same receptor, CXCR2. Murine CXCL1 functions in inflammation, neutrophil chemotaxis, angiogenesis, and response to prostaglandin E2 similar to human IL-8.

Figure 3. ELR⁺ CXC chemokine expression in human and murine aneurysms. A, Endothelial cells in human aneurysm tissues are positive for interleukin-8 (IL-8; arrows), whereas endothelial cells in control superficial temporal arteries (STAs) are negative (blue, DAPI; green, CD31; red, IL-8; scale bar, 10 μm). B, Endothelial cells in human aneurysms are positive for chemokine (C-X-C motif) ligand 1 (CXCL1; arrows), whereas endothelial cells in control STAs are negative (blue, DAPI; green, CD31; red, CXCL1; scale bar, 10 μm). C, CXCL1 expression in mouse aneurysmal vessels and aneurysm specimens is present in the media at 3 d and 1 and 2 wk, whereas arteries from sham mice are negative (blue, DAPI; green, CD31; red, CXCL1; scale bar, 10 μm).

Figure 4. Chemokine (C-X-C motif) ligand 1 (CXCL1) blockade prevents mouse intracranial aneurysm formation. A, Experimental scheme. Ang indicates angiotensin; and BAPN, β-aminoproprionitrile. B, Representative pictures of Circle of Willis from immunoglobulin G (IgG)- and anti-CXCL1-neutralizing antibody-treated mice (scale bar, 2 mm). C, Mice treated with CXCL1-neutralizing antibody ≥2 wk develop significantly less aneurysms compared with IgG2A-treated mice (P<0.0078). Data are presented as mean±SEM.
A low shear stress microenvironment is present within the aneurysm dome as soon as it forms.4,8,11 Within the aneurysm dome, the average WSS is lower than in the parent artery.3–5 Recently, aneurysm growth has been reported to occur in regions of low WSS of the aneurysm dome.8,11 Low-level shear stress can induce expression of ELR+ CXC chemokines, primarily IL-8 and CXCL1, in the endothelium,13 which upregulate intercellular adhesion molecule, vascular cell adhesion molecule 1, and selectins.13 Low or oscillatory shear stress in arterial bifurcations, stenosed, or atherosclerotic vessels results in proinflammatory phenotype26 through upregulation of nuclear factor-κB and IL-8 via mitogen-activated protein kinase.21 Aoki et al6 found that hemodynamic shear stress activates the proinflammatory pathway nuclear factor-κB via prostaglandin E2 in cerebral aneurysms. Activated platelets can release CXCL7, providing additional stimulus for neutrophil chemoattraction.27 Conversely, neutrophils can bind to damaged EC wall and initiate thrombus formation.28 In this work, we focused on cytokine profiling and neutrophil recruitment. The bifurcation and aneurysm sac flow model microenvironments exposed to either low average hemodynamic shear stress or shear stress gradients were characterized by higher expression of CXCL1, and IL-8 and CXCL1, respectively (Figure 2). Elevated IL-8 in the blood of cerebral aneurysm patients has been reported,29 and CXCL1, the proposed murine functional homologue of human IL-8,17 was the primary CXC

Figure 5. Effects of chemokine (C-X-C motif) ligand 1 (CXCL1) blockade on inflammatory cell infiltration. CXCL1 blockade >2 wk results in (A and B) significantly less neutrophil infiltration (P=0.043; blue, DAPI; green, NIMP-R14; red, CXCR2; scale bar, 10 μm). G and D, No significant differences in macrophage infiltration were found (P=0.056; blue, DAPI; green, F4/80; red, CXCR2; scale bar, 10 μm). E, Human aneurysm specimens are positive for infiltrating neutrophils (open arrows) and macrophages (full arrows), whereas control STAs are negative (blue, DAPI; green, neutrophil elastase; red, CD68; scale bar, 10 μm). Data are presented as mean±SEM.
chemokine expressed in murine aneurysms at 2 weeks after aneurysm induction (Figure S4). CXCL1 was also expressed at 3 days and 1 week (Figure 3C).

Murine cerebral aneurysm formation (Figure 4) and neutrophil infiltration (Figure 5) were significantly decreased after CXCL1 antibody blockade. Formation of aneurysms in our murine model is dependent on the combination of hypertensive and angiotoxic insults. Hypertension is created via high salt diet, carotid and renal artery ligations, and angiotensin II −releasing pump. Angiotoxic effects of β-aminoproprionitrile with damage to the elastin layer by elastase injection provide the final stimulus for aneurysm formation. Differences in aneurysm formation between the anti-CXCL1 and IgG groups could not be attributed to changes in systemic blood pressure (Figure S6). Rather, blockade of CXCL1 likely reduces aneurysm formation and neutrophil infiltration by discouraging cell-cell interactions between the endothelium and immune cells. By upregulating vascular cell adhesion molecule 1 on the cell surface, CXCL1 effectively arrests monocytes and neutrophil binding to the activated endothelium. Indeed, vascular cell adhesion molecule 1 was increased in the endothelium in the aneurysm sac of the bifurcation aneurysm flow chamber model when compared with other microenvironments and in the aneurysms of IgG-treated mice (Figures S7 and S8).

Perspectives

Using our novel model, we demonstrate, for the first time to our knowledge, that shear stress−mediated endothelial inflammation and CXCL1-dependent neutrophil recruitment are important factors in cerebral aneurysm formation. This is significant because neutrophils have been largely neglected in studies of cerebral aneurysms. We speculate that blocking shear stress−induced CXCL1 could be used to prevent new cerebral aneurysm formation and growth of established lesions. The novel methods and findings presented here will promote further insights into link between hemodynamics and inflammation in cerebral aneurysm development.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**
- In vitro approach can be used to identify hemodynamic-induced inflammatory mediators of cerebral aneurysm formation. Hemodynamic shear stress leads to increased endothelial secretion of interleukin-8 and chemokine (C-X-C motif) ligand 1 (CXCL1) in cerebral aneurysms. CXCL1 is the primary ELR+ CXC chemokine expressed in murine cerebral aneurysms. Anti-CXCL1 blockade significantly decreases murine cerebral aneurysm formation through decreased neutrophil recruitment.

**What Is Relevant?**
- Inflammation is a hallmark feature of cerebral aneurysms; however, early molecular events in aneurysm formation are still not fully understood. This study addressed the link between hemodynamic shear stress and early inflammatory mediators and, through a combination of in silico, in vitro, and in vivo approaches, demonstrates a critical role for shear stress–induced CXCL1-dependent neutrophil recruitment in cerebral aneurysm formation.

**Summary**
Cerebral aneurysm formation is an inflammatory mediated process driven by abnormal hemodynamic shear stress. Our study offers a new in vitro model for study of cerebral aneurysm formation. Our data suggest that CXCL1-dependent neutrophil infiltration may be a target for therapeutic intervention for cerebral aneurysms.
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A novel high-throughput in vitro model for identifying hemodynamic-induced inflammatory mediators of cerebral aneurysm formation

Kamil W. Nowicki, B.S.¹, Koji Hosaka, Ph.D.¹, Yong He, Ph.D.², Peter S. McFetridge, Ph.D.³, Edward W. Scott, Ph.D.⁴, Brian L. Hoh, M.D.¹

University of Florida¹Department of Neurosurgery, ²Department of Surgery, ³Department of Biomedical Engineering, and ⁴Department of Molecular Genetics and Microbiology.

Corresponding Author:

Brian L. Hoh, M.D.

PO Box 100265

Gainesville, FL 32610

Fax: 352-392-8413

Phone: 352-273-9000

Email: brian.hoh@neurosurgery.ufl.edu
EXPANDED METHODS

Parallel-Plate Flow Chamber Design

The parallel-plate flow chamber used for a straight artery flow model was a design recently published by Uzarski et al.\textsuperscript{1} The idealized parallel-plate flow chamber for flow models of a bifurcation and bifurcation aneurysm were created based on previously published CFD studies\textsuperscript{2-6} and anatomical dimensions of human cerebral arteries\textsuperscript{7}. We replicated the patterns of low to high WSS ratio in the aneurysm region to parent vessel, respectively, by designing the bifurcation and bifurcation aneurysm parallel-plate flow chambers to contain a region of uniform WSS of 10-dyne/cm² in the proximal straight segment, 5-dyne/cm² in the limbs of the bifurcation, and an average WSS of 1.5-dyne/cm² within the aneurysm sac (Fig 1A and B). For our design we used the equation $\tau = 6\mu Q / bh^2$, where $\tau$ is wall shear stress, $\mu$ is fluid viscosity, $Q$ is fluid flow, $b$ is channel width, and $h$ is channel height to arrive at the dimensions of the chamber.

Modeling Shear Stress

Flow characteristics in the flow chambers were obtained using Computational Fluid Dynamics (CFD) analyses. High-resolution mesh generation was performed in Gambit (ANSYS, Canonsburg, PA). Boundary layers were created near the wall. The height of the first layer was 0.01 mm. Only one fourth of the chamber volume was simulated using the symmetric nature of the chamber. Extensions were added to the inlet and outlet to eliminate the interference of the inlet and outlet. Approximately 1.1 million cells were created. The density and viscosity of the culture medium were set to 1000 kg/m³ and 0.001 Pa·s, respectively. Laminar flow was assumed. Lumen-average velocity waveform was applied to the inlet boundary. Flow rates were measured using a perivascular ultrasonic transducer (model 2SB) and a flow meter (model T106, Transonic Systems, Ithaca, NY). The measured flow rates in one cycle are shown in Figure S1. A pressure boundary condition was set at the outlet. Time-dependent terms were discretized in an implicit scheme with second-order accuracy. The momentum equations were discretized using a second-order upwind scheme. A segregated solver was used to solve the momentum and continuity equations. Pressure-velocity coupling was realized through the Semi-Implicit Method for Pressure-Linked Equations (SIMPLE) algorithm. The time-step size was 0.005 s. A residual of $10^{-5}$ was set as the convergence criterion. Simulation was performed in Fluent (ANSYS, Canonsburg, PA). Flow was converged in the second cycle and data from the second cycle were analyzed. Post-processing and visualization of velocity streamlines and wall shear stress (WSS) were performed in Tecplot 360 (Tecplot, Bellevue, WA).

Cell Culture

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from human umbilical vein by collagenase perfusion as described previously\textsuperscript{8}. Collection of human umbilical cord specimens was performed under an Institutional Review Board-approved protocol. HUVECs were pooled from 3 donors, and cultured in Vasculife VEGF medium prepared as per manufacturer's instructions (Lifeline Cell Technology, Frederick, MD) at 5% CO₂ and 37 °C. Primary cell culture purity was confirmed by CD31 (Dako) and Van Willebrandt (Dako) staining, and cell morphology (Figure S1).

Cell Culture Purity Verification By Immunohistochemistry
Cells at P1 were cultured for 2-3 days in Nunc Lab Tek II Tissue Culture slides (Thermo Fisher Scientific, Rochester, NY) until confluence and fixed in 4% PFA. Cells were then permeabilized by 15-min treatment with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO), blocked in 2% normal horse serum (S-2000, Vector Labs, Burlingame, CA) for 1 hour and stained with monoclonal mouse anti-human CD31 (M082301-2, DAKO) or monoclonal mouse anti-human von Willebrand Factor (vWF) antibody (M061601-2, DAKO) for 2 hours, washed, and incubated with donkey secondary anti-mouse antibody Alexa Fluor 488 (Life Technologies) for 1 hour. Cells were then washed and counterstained with DAPI. The whole field of each chamber was manually scanned to look for non-staining cells. Representative pictures are shown in Figure S2A.

Cell Culture Purity Verification By Flow Cytometry
Cells at P3 were cultured for 2-3 days in T25 flasks (TPP Techno Plastic Products) until 80% confluence, activated with 10 nM TNF-α for 4 hours, and then collected using 0.05% Gibco trypsin (15090-046, Life Technologies) in PBS. Cells were stained for endothelial cells markers 1) VE-Cadherin (FITC) and E-Selectin (PE) and 2) CD31 (FITC) and VCAM-1 (PE), and analyzed using BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and FACSDiva software (v.6.0). The antibodies used were PE-conjugated mouse anti-human E-selectin antibody (551145, BD Pharmingen), FITC-conjugated mouse anti-human VE-cadherin antibody (560411, BD Pharmingen), PE-conjugated mouse anti-human VCAM-1 antibody (555647, BD Pharmingen), and FITC-conjugated mouse anti-human CD31 antibody (555445, BD Pharmingen). After an appropriate cell staining, cells were suspended 500uL of PBS in a 5 mL round-bottom tube (BD Falcon 352052). Single cells were determined and gated using FSC and SSC. Results of analysis by flow cytometry are shown in Figure S2B and C.

Shear Stress Experiments
HUVECs (passage 2-5) pooled from 3 donors (Figure S1) were seeded on coverslips cut from tissue culture plastic dishes (Techno Plastic Products, Trasadingen, Switzerland) at a density of 20,000 cells/cm² and grown to confluence. For our experiments we used HUVECs due to their availability,9, 15 similarities to brain endothelial cells in COX-2 dynamics9 and miRNA profiling10, and physiological, low basal expression of COX-2.11 Endothelial cells (EC) were exposed to pulsatile shear stress over 27 hours. Recent studies have shown that shear stress - pre-conditioned ECs respond to hemodynamics changes in a more physiological manner than shear stress – naïve static cultures.12, 13 The system was pre-shared at increasing rate until reaching the final average magnitude of shear stress of 10-dyne/cm² in order to achieve a more physiologically-relevant cell phenotype. First, cells were pre-conditioned at 1-dyne/cm² for one hour, followed by 5-dyne/cm² for two hours, and finally exposed to shear stress at 10-dyne/cm², over a period of 24 hours in straight, bifurcation, and bifurcation aneurysm parallel-plate flow chambers at 5% CO₂ and 37 °C. The volume of perfusing medium used was normalized with respect to the total cell growth area for each chamber type. Fluid flow was delivered in a pulsatile manner at 58 pulses/min with a Masterflex L/S Digital Drive (Cole-Parmer, Vernon Hills, IL). Our flow models can be successfully used (data not shown) with a previously published EC-VSMC co-culture system.14 At the end of each experiment the chambers were disassembled and coverslips were removed under sterile conditions. Cells were fixed in ice-cold methanol (Sigma-Aldrich, St. Louis, MO) for 5 minutes, and then stored in PBS at 4 °C for immunocytochemistry. Conditioned medium from each system was collected and frozen at -80
oC for analysis.

**Cytokine Array and ELISAs**

Levels of 40 different human inflammatory factors in the perfusing medium from the straight, bifurcation and bifurcation aneurysm flow chambers were measured using the Human Inflammation Array – C3 (AAH-INF-3, RayBiotech, Norcross, GA). Quantification of intensity (integrated density) values was performed using the MicroArray Profile plugin for ImageJ. IL-8 levels in the perfusing medium were quantified using the Human CXCL8/IL-8 Quantikine ELISA Kit (D800C, R&D Systems, Minneapolis, MN). CXCL1/GRO-α levels were quantified using the Human CXCL1/GRO alpha Quantikine ELISA Kit (DGR00, R&D Systems, Minneapolis, MN).

**Relative Fluorescence Immunocytochemistry and Confocal Microscopy**

RF-ICC staining for CXCL8/IL-8, CXCL1/GRO-α or VCAM-1 was performed on cells from shear experiments and intensity values were quantified using confocal microscopy. Cells previously fixed in ice-cold methanol were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 15 minutes, blocked in 2% normal horse serum (S-2000, Vector Labs, Burlingame, CA) for 1 hour and then incubated with mouse anti-human IL-8 antibody (ab18672, Abcam, Cambridge, MA) or rabbit anti-CXCL1 antibody (ab86436, Abcam) or rabbit anti-VCAM-1 antibody (sc-8304, Santa Cruz Biotechnology, Dallas, TX) overnight, washed, and incubated with the donkey secondary anti-rabbit antibody Alexa Fluor 568 (Life Technologies, Grand Island, NY) for 1 hour at room temperature. Finally, nuclei were counterstained with DAPI (H-1200, Vector Labs, Burlingame, CA). As a negative control, and to quantify background fluorescence, coverslips with cells from shear stress experiments were stained with rabbit IgG antibody (I-1000, Vector Labs) in place of IL-8, CXCL-1 or VCAM-1 antibody. Stained slides were imaged immediately or stored at 4 °C up to 5 days. To quantify relative protein expression of IL-8, CXCL-1 or VCAM-1 via relative immunofluorescence we used a DSU Spinning Disk Confocal Scanner mounted on an Olympus IX81 inverted fluorescent microscope (Olympus, Center Valley, PA) with a x40 dry objective and C4742-80-12AG Monochrome CCD camera (Hamamatsu Photonics, Bridgewater, NJ). Eight random images were taken from each micro-environment of interest for each chamber. Each image was an average of a stack of 7 images taken at 1 µm apart in the z-direction. Z-stack images were analyzed using SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO) and only raw image file were used to quantify intensity values. For each of the slides stained from each flow chamber experiment, an additional static culture slide was stained to act as a reference point in case slides could not be imaged immediately. Any of the raw intensity values from flow chamber experiment slides were normalized with respect to a reference static culture slide. This protocol, once established, was strictly adhered to for each experiment. Lastly, the final recalculated intensity values from each measurement were normalized with respect to the intensity values from the proximal straight segment for each sample.

**Animals**

All animal experimentation was performed under an Institutional Animal Care and Use Committee-approved protocol #201303748.
Mouse Intracranial Aneurysm Model

Murine intracranial aneurysms were created in female 8-12 week-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) using a method described previously. Briefly, the left common carotid artery and the right renal artery are ligated to induce hypertension. One week later, an Alzet micro-osmotic pump model 1004 (DURECT Corp, Cupertino, CA) is implanted subdermally to deliver Angiotensin II (Bachem AG, Switzerland) at 1000ng/kg/min; and 10 uL of 0.8% porcine elastase (Worthington Biochemical Corp, Lakewood, NJ ) in normal saline is injected into the right basal cistern using stereotactic coordinates: 1.2 mm rostral of bregma, 0.7 mm lateral of midline and 5.3 mm ventral of the dorsal aspect of the skull. The animals are fed a hypertensive diet with 8% NaCl and 0.12% BAPN (Harlan Laboratories, Indianapolis, IN). For the antibody blockade, 100 µg/mL mouse anti-CXCL1/GRO alpha/KC/CINC-1 antibody (MAB453, R&D Systems, Minneapolis, MN) was injected retro-orbitally two days before, on the day of, and every two days after aneurysm induction. In control animals, 100 µg/mL rat IgG2A Isotype Control antibody (MAB006, R&D Systems, Minneapolis, MN) was injected retro-orbitally on the same schedule. For sham surgeries, the arteries were isolated but not ligated during the ligation procedure, and normal saline solution was injected intracranially instead of 0.8% elastase. Instead of Angiotensin II pump implant, normal saline was injected subdermally. Mice were euthanized 3 days, one week or two weeks after aneurysm induction.

Quantification of Aneurysm Formation, Inflammatory Cell Infiltration and VCAM-1 Expression in CXCL1 Blockade

At the end of 2-week period mice treated with IgG2A or anti-CXCL1 were euthanized by 3 mL 4% PFA in PBS cardiac perfusion into the left ventricle followed by injection of 1 mL brilliant blue in 20% gelatin in PBS. The brilliant blue in gelatin injection was used for visualization of blood vessels to allow a blinded observer to determine saccular aneurysm formation in Circle of Willis (COW) from each mouse. The blinded observer did not perform any surgeries or treatments for this experiment. Representative images were taken using Leica dissection microscope with Volocity 3D analysis software. After recording aneurysm formation, intact brains with COWs were fixed in 4% PFA for 24 hrs, and then transferred to 18% sucrose solution for 24 hrs. Tissues were then embedded in OCT and sectioned for staining and quantification of neutrophil and macrophage infiltration and VCAM-1 expression in endothelial cells. The sections were mounted on numbered slides that did not directly identify the treatment regimen. The master sheet identifying the slides and the treatment regimen for each specimen was kept in a separate file in the laboratory. The following stereology rules were used: for each aneurysm positive specimen, 3 sections 100 µm apart through the aneurysm were taken; for aneurysm negative specimens, 3 sections 100 µm apart were taken at the anterior cerebral artery (ACA) starting at the anterior commissure, the middle cerebral artery (MCA), and posterior cerebral artery (PCA). For both, aneurysm positive and negative specimens, the outcome measure was averaged from all sections taken for that specimen (either only aneurysm or total average of ACA and MCA and PCA sections) and representative images were taken. For each section, the number of inflammatory cells and VCAM-1 positive endothelial cells were counted. The observer performing inflammatory cell infiltration and VCAM-1 expression by endothelial cells did not have access to the master sheet during quantification. The outcome measure was quantified relative to vessel wall or aneurysm wall area. The visualization of immunohistochemistry slides and quantification of vessel wall or aneurysm wall area was
Blood Pressure Measurements in IgG- and Anti-CXCL1-Treated Mice

The murine intracranial aneurysm model is dependent on effects of both hypertension and elastase. We performed blood pressure measurements to verify that decreased aneurysm formation in the anti-CXCL1-treated group was not due to changes in systolic or diastolic blood pressure. Mice underwent ligation and elastase injection surgeries with pump implantation as described above and received 100 µg/mL mouse anti-CXCL1 antibody (MAB453, R&D) retro-orbitally two days before, on the day of, and every two days after aneurysm induction. In control animals, 100 µg/mL rat IgG2A antibody (MAB006, R&D Systems) was injected retro-orbitally on the same schedule. Blood pressure measurements were using CODA Non-invasive pressure system (Kent Scientific Corp., Torrington, CT) using the tail-cuff method. Five mice were used for each experimental group. For each mouse, five measurements were taken and were then averaged on days -9, -4, on the day of, and 3, 7, and 14 days post aneurysm induction via elastase injection. Mice were sacrificed 2 weeks post aneurysm induction as in the previous experiment. One mouse from the IgG group died 7 days post aneurysm induction. Similarly, one mouse died from the anti-CXCL1 group 13 days after aneurysm induction. Results are shown in Figure S6.

Human Aneurysm and Superficial Temporal Artery Specimens

Collection of human cerebral aneurysm and superficial temporal artery specimens was performed under an IRB-approved protocol #448-2007. Patients signed informed IRB research consent. All aneurysm and superficial temporal artery specimens were harvested from living patients at the time of craniotomy and aneurysm clipping surgery. Tissues were collected from aneurysm domes and included ruptured and unruptured aneurysms. Specimens were immediately fixed in 4% paraformaldehyde. Deidentified specimens were then used for immunohistochemistry under IRB-approved protocol #201400190. Details regarding physical characteristics of collected tissues are summarized in Table S1.

Immunohistochemistry of Mouse and Human Aneurysm Specimens

Murine aneurysm specimens were first fixed in 4% PFA for 24 hours, and then dehydrated in 18% sucrose solution. Tissues were mounted in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA) and sectioned at 5 µm. Heat mediated antigen retrieval in Dako Target Retrieval Solution (Dako, Carpinteria, CA) was performed for all murine immunohistochemistry studies. Following a block in 2% normal horse serum (S-2000, Vector Labs) for 1 hour, the specimens were incubated with rat anti-MECA-32 antibody (BD 550563, BD Biosciences, San Jose, CA) to visualize endothelial cells, rabbit anti-CXCL1/GRO-α antibody (ab86436, Abcam), goat anti-mouse CXCL2/GRO-β/MIP-2α antibody (AF-452-NA, R&D Systems), rabbit anti-mouse CXCL5-6/LIX antibody (500-P146, Peprotech, Rocky Hill, NJ), rat anti-neutrophil antibody [NIMP-R14] (ab2557, Abcam), rat anti-mouse F4/80 (MCA497R, AbD Serotec, Düsseldorf, Germany) to visualize macrophages, rabbit anti-CXCR2 (ab14935, Abcam) or rabbit VCAM-1 antibody (sc-8304, Santa Cruz, Dallas, TX) overnight at 4 °C, and washed. For immunohistochemistry of human aneurysm samples, the tissues were fixed in 4% PFA and embedded in paraffin. After tissue sectioning at 5 µm, the samples were de-paraffinized by xylene and ethanol. Heat mediated antigen retrieval in 10 µM Sodium Citrate buffer pH 6.5 for all samples was performed. The staining protocol for CXCL1/GRO-α, CXCR2
and VCAM-1 was then followed exactly as for immunohistochemistry of mouse aneurysm specimens except that mouse anti-human CD31 antibody (IR61061-2, Dako) was used in place of MECA-32 to visualize the endothelial cell layer, rabbit anti-neutrophil elastase antibody (ab68672, Abcam) was used to visualize neutrophils, and mouse anti-CD68 antibody [KP1] (ab955, Abcam) was used to visualize macrophages. To visualize IL-8, mouse anti-human IL-8 antibody (ab18672, Abcam) was used. The secondary antibodies used were Alexa Fluor 488 donkey anti-rat antibody (A-21208, Life Technologies), Alexa Fluor 488 donkey anti-rabbit antibody (A-21206, Life Technologies), Alexa Fluor 568 donkey anti-rabbit antibody (A-21206, Life Technologies), and Alexa Fluor 594 donkey anti-mouse antibody (A-21203, Life Technologies) and were incubated for 1 hour at room temperature. Finally, for both murine and human specimens, nuclei were counterstained with DAPI (H-1200, Vector Labs, Burlingame, CA).

**Statistical Analysis**

**IL-8 ELISA**

We used analysis of variance (ANOVA) to determine whether the outcome measure differed across the three types of flow chambers. We used Tukey’s method to control for the Type I error rate when making post-hoc pairwise comparisons. P-values <0.1 but >0.05 were defined as marginally significant. P-values <0.05 were defined as statistically significant.

**CXCL-1 ELISA**

We used a Kruskal-Wallis test to determine whether the outcome measure differed across the three types of flow chamber. Kruskal-Wallis test was used since the variation in the outcome variable was highly different across the three groups and did not meet assumptions for ANOVA. Bonferroni-corrected pairwise Mann-Whitney tests were then used to determine differences between the pairs. P-values <0.1 but >0.05 were defined as marginally significant. P-values <0.05 were defined as statistically significant.

**IL-8, CXCL1, and VCAM-1 Relative Fluorescence Immunocytochemistry**

We used a mixed effects linear model (The lme() function in the R package nlme) to determine whether intensity differed across the four locations. We took location as our fixed factor, and we considered chamber and experiment to be random factors. We assumed a variance components covariance structure. In data collection, a single chamber could be used only once per experiment, but it could be used again in subsequent experiments. However, information identifying each chamber was not recorded, so it is not known which, if any, chambers were reused. For analysis, we assumed no chambers were reused, so that chamber was nested within experiment. P-values <0.1 but >0.05 were defined as marginally significant. P-values <0.05 were defined as statistically significant.

**CXCL1 Blockade in the Mouse Intracranial Aneurysm Model**

We used Fischer’s Exact Test to determine whether aneurysm formation differed between IgG2A- and anti-CXCL1-treated mice. Each Circle of Willis was examined by a blinded observer who did not perform the surgeries or treatments for this experiment and aneurysm formation was recorded (yes/no). It was estimated that the IgG2A group has 11.7 times the odds of developing aneurysm (95% CI=[1.68, 148]). P-values <0.1 but >0.05 were defined as marginally significant. P-values <0.05 were defined as statistically significant.
Neutrophil and Macrophage Infiltration and VCAM-1 Expression in CXCL1 Blockade

We used a Mann-Whitney test to determine whether inflammatory cell infiltration (neutrophil or macrophage) and VCAM-1 expression by endothelial cells was different between IgG2A- and anti-CXCL1-treated mice. P-values <0.1 but >0.05 were defined as marginally significant. P-values <0.05 were defined as statistically significant.

REFERENCES


Table S1. Physical characteristics of human aneurysms and control arteries.

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Figure S1. The measured flow rate in one cycle for the designed flow chamber. Flow rate was measured using a perivascular ultrasonic transducer (model 2SB) and a flow meter (model T106, Transonic Systems, Ithaca, NY).
Figure S2. A) Representative immunohistochemistry images showing HUVEC culture morphology at P1 demonstrating the purity of cells isolated from human umbilical veins. Green: CD 31 or vWF, Blue: DAPI. Scalebar = 20 μm. B) Following activation with 10 nM TNF-α. FACS analysis showed that the presumed HUVECs were 99.7% double-positive for VE-Cadherin (FITC) and E-Selectin (PE) and C) 95.2% double-positive for CD31 (FITC) and VCAM-1 (PE) indicating that they were genuine endothelial cells.
Figure S3. Representative images from bifurcation aneurysm flow chamber experiments used to quantify A) IL-8 and B) CXCL1 relative-fluorescence for each environment. PSS: proximal straight segment, B: bifurcation, AS: aneurysm sac, BL: branching limbs. (scalebar=10 μm)
Figure S4. A) Two-week mouse aneurysm specimens are (scale bar=2 mm) B) positive for ELR+ chemokine CXCL1 but not CXCL2 or CXCL5-6. (*Blue: DAPI, Green: CD31, Red: CXCL1, CXCL2 or CXCL5-6, scalebar=100 μm)*
Figure S5. Location of cerebral aneurysm formation in IgG control and anti-CXCL1-treated mice.
Figure S6. Blood pressure measurements show that in the murine intracranial aneurysm model, there is no significant difference in systolic and diastolic blood pressures at 2 weeks between IgG-treated and anti-CXCL1-treated mice. Mean systolic BP: 123.0 vs 127.15 mm Hg (p=0.69, n=4) for IgG and anti-CXCL1-treated groups, respectively. Mean diastolic BP: 101.4 vs 104.9 mm Hg (p=0.71, n=4) for IgG and anti-CXCL1-treated groups, respectively. NS = no significance, *p<0.05.
Figure S7. A and B) VCAM-1 expression in mouse aneurysms is significantly decreased in anti-CXCL1 treated mice at two weeks (33.4% vs 76.4% positive cells/section, arrows, n=15 for both, p<0.001) (Blue: DAPI, Green: MECA32, Red: VCAM-1, scalebar=10 μm). C) HUVECs express more VCAM-1 in the aneurysm sac of the bifurcation aneurysm flow chamber than other regions (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, representative images for each environment are shown). D) Endothelial cells (CD31+) in human aneurysm tissues (arrows) are positive for VCAM-1 (arrows) whereas endothelial cells in control STAs are negative. (Blue: DAPI, Green: CD31, Red: VCAM-1, scalebar=10 μm). Data are presented as mean±s.e.m.
Figure S8. Hemodynamic shear stress and shear stress gradients cause initial endothelial dysfunction by increasing proliferation and apoptosis, which leads to degeneration of the IEL. Once an aneurysm develops, the hemodynamics change and the endothelial cells within the dome are then exposed to low shear stress leading to increased secretion of inflammatory mediators such as IL-8 (human) or CXCL1 (human and mouse). This leads to upregulation of VCAM-1 and causes inflammatory cell infiltration, primarily neutrophils, in the low shear stress region of the dome, further degeneration of the vascular wall and eventual rupture.