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ABSTRACT

The vascular endothelium responds to shear stress generated by blood flow and changes functions to regulate blood flow and maintain tissue homeostasis. Recently, we found that arteriolar high shear stress leads to increased expression of vasodilatory and antithrombotic genes in human retinal microvascular endothelial cells (HRMECs). However, it is unknown whether low shear stress, which is induced by hypoperfusion particularly in the retinal venules where leukocyte-endothelial interactions mainly occur, affects the retinal endothelial function. We studied the effect of low shear stress on proinflammatory gene expression in HRMECs. The cells were cultured on glass plates and exposed to laminar shear stresses of 0 (static), 1.5 (relatively low flow), and 15 dyne/cm^2 (relatively high flow) for 24 hours using parallel plate-type flow-loading devices. The mRNA expressions of adhesion molecules, cytokines and chemokines, and procoagulant factors were evaluated using real-time reverse-transcription polymerase chain reaction. HRMECs exposed to 1.5 dyne/cm^2 significantly up-regulated the mRNA expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin. The cells exposed to 1.5 dyne/cm^2 of stress also had increased cytokine/chemokine mRNA expression, i.e., interleukin (IL)-6, IL-8, platelet-derived

growth factor-B, and monocyte chemotactic protein-1. Procoagulant factors, i.e., tissue factor and plasminogen activator inhibitor-1 mRNA, increased significantly with exposure to 1.5 dyne/cm^2 of stress. Our results showed that relatively low shear stress causes up-regulation of proinflammatory genes in HRMECs, suggesting that decreased shear stress due to vascular hypoperfusion might change the phenotypic characterization of the retinal vascular endothelium and be associated with leukocyte-endothelial interactions.

Increasing evidence has suggested that shear stress generated by blood flow is a critical modulator of the vascular endothelial phenotype (Ando and Yamamoto, 2009). In the retinal circulation, blood flow changes dynamically in pathologic states, such as diabetic retinopathy (DR) (Bursell et al., 1996; Nagaoka et al., 2010). In recent decades, attraction and adhesion of leukocytes to the vascular endothelial wall have been shown to be important components of the inflammatory process in DR (Kern, 2007), and these leukocyte-endothelial interactions may contribute to vascular leakage and capillary nonperfusion (Miyamoto et al., 1999; Schroder et al., 1991). The changes in retinal blood flow may affect these inflammatory states, but there is little evidence regarding the relationship between the function of the retinal vascular endothelium and retinal blood flow. Recently, we found that long-term exposure to shear stress in the retinal arterioles (> 15 dyne/cm^2) significantly up-regulates endothelial nitric oxide synthase (eNOS) and thrombomodulin mRNA expression and down-regulates endothelin-1 mRNA expression in human retinal microvascular endothelial cells (HRMECs) (Ishibazawa et al., 2011). These results suggested that “high” shear stress might be associated with the vasodilatory and antithrombotic properties of retinal vessels under physiologic conditions present in

the retinal circulation. However, in the veins and venules, while the physiologic level of shear stress in the postcapillary venules was reported to be as high as 6 dyne/cm^2 (Kamiya et al., 1984; Malek et al., 1999), we previously reported that the first branch of the retinal venules in healthy humans had a shear stress level of about 20 dyne/cm^2 (Nagaoka and Yoshida, 2006). Moreover, we previously reported a mathematical model for the distribution of hemodynamic parameters in the human retinal microvascular network and showed that the shear stress of the retinal small veins (postcapillary venules) was about 15 dyne/cm^2 or less (Takahashi et al., 2009). A number of previous studies have reported that leukocyte-endothelial interactions occur mainly in the postcapillary venules but not the arterioles (Granger and Kubes, 1994; Ley, 1996; Tsujikawa and Ogura, 2012). Therefore, it is important to examine the effect of low shear stress at the level of the retinal venules on the vascular endothelial cells. Using cultured HRMECs, we recently observed that endothelin-1 mRNA expression increased in response to “low” shear stress of 1.5 dyne/cm^2 (Ishibazawa et al., 2011), which was assumed to be decreased shear stress because of hypoperfusion particularly in the postcapillary venules. Although *in vivo* experiments showed that reductions in shear stress induce leukocyte

rolling and adhesion in the postcapillary venules in the retina and mesentery (Suzuki et al., 1991; Xu et al., 2004), the manner in which this pathological “low” shear stress directly affects the phenotypic characterization of the retinal vascular endothelium remains to be determined. Therefore, we studied the effect of low shear stress especially on proinflammatory gene expression in HRMECs.

Primary HRMECs (ACBRI 181, Cell Systems Corporation, Kirkland, WA) were cultured in Medium-199 (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum, 50 μg/ml heparin, antibiotics, and 30 μg/ml endothelial cell growth factor. The cultured cells were routinely passaged with 0.05% trypsin/2 mM EDTA solution before reaching confluence. At the final stage, the cells were seeded at a density of 2.0×10^4 cells/cm^2 on 1% gelatin-coated glass plates. We previously described the shear stress experiments (Ishibazawa et al., 2011). Briefly, we used a parallel plate-type flow chamber. The closed circuit that included the flow chamber, a peristaltic pump, and a medium reservoir was connected using silicone tubes. These experiments were performed in an incubator at 37ºC with 5% CO_2. Based on the structure of the flow chamber, the shear stress (τ , dyne/cm^2) exerted on the cells was calculated using the formula:

$$\tau = \mu \cdot 6Q/(a^2b),$$

where μ is the viscosity of the perfused fluid (poise), Q the volumetric flow rate (mL/s), and a and b the height and width of the channel (in centimeters), respectively. In the current study, the HRMECs were exposed to the following magnitudes of laminar shear stress: 0 dyne/cm^2, static control; 1.5 dyne/cm^2, relatively low flow in the hypoperfused venules; and 15 dyne/cm^2, relatively high flow in the normal retinal venules. Total RNA samples were isolated from the cells using TRI Reagent (Sigma-Aldrich, St. Louis, MO). After reverse transcription, a real-time polymerase chain reaction was performed using the Smart Cycler II System and CYBR Premix EX Tag RR041A (TaKaRa Biochemicals, Tokyo, Japan). The specific primer pairs are shown in the Table. All values are expressed as the mean ± standard error of the mean (SEM). The data were assessed using one-way analysis of variance followed by the Bonferroni's post-hoc test. $P < 0.05$ was considered significant.

The HRMECs exposed to 1.5 dyne/cm^2 of stress had significantly up-regulated mRNA expression of intercellular adhesion molecule-1 (ICAM-1) (4.2-fold vs. static), vascular cell adhesion molecule-1 (VCAM-1) (2.5-fold vs. static, 4.3-fold vs. 15

dyne/cm^2 of stress), and E-selectin (3.7-fold vs. static, 2.4-fold vs. 15 dyne/cm^2 of stress) (Fig. 1). ICAM-1 mRNA expression also was significantly up-regulated with exposure to 15 dyne/cm^2 of stress compared to the static control. The cytokine/chemokine mRNA expression levels (> double vs. static and 15 dyne/cm^2 of stress), i.e., interleukin (IL)-6, IL-8, platelet-derived growth factor-B (PDGF-B), and monocyte chemotactic protein-1 (MCP-1), increased with exposure to 1.5 dyne/cm^2 of stress (Fig. 2). The expression of procoagulant factors, i.e., tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) mRNA, significantly increased with exposure to 1.5 dyne/cm^2 of stress (2.8-fold vs. static, and 1.5-fold vs. static, respectively).

The current results indicated that relatively low shear stress caused significant up-regulation of a number of pathophysiologically relevant genes, the products of which have proinflammatory and procoagulant functions in cultured retinal microvascular endothelium. Previous studies of aorta and large arteries have reported that atherosclerosis develops preferentially in regions of the arterial tree, especially at or near branch points, bifurcations, and curvature, where the blood flow is disrupted, i.e., non-uniform, irregular, and reciprocating (Cornhill and Roach, 1976; Glagov et al., 1988).

The disrupted flow exhibits low shear stress that up-regulates proatherosclerotic genes or proteins that promote development of atherosclerosis (Cunningham and Gotlieb, 2005). In the current study, because we focused on the venular level of shear stress, we used the parallel plate-type flow chamber that generates laminar shear stress even if the flow rate is low (Kosaki et al., 1998). Our results showed that laminar low shear stress of 1.5 dyne/cm^2 also up-regulated proinflammatory genes in cultured retinal vascular endothelium.

This level of low shear stress may represent markedly reduced blood flow particularly in the postcapillary venules, in which multiple steps of leukocyte-endothelial interactions (rolling, activation, and firm adhesion) mainly occur. Using acridine orange leukocyte angiography, Nishiwaki et al. (1995) reported that only a few leukocyte-endothelial interactions were present under physiologic conditions in the capillaries, whereas there were no rolling leukocytes in the main retinal arteries and veins. However, increased leukostasis was observed in the retinal venules of ischemia-reperfusion and diabetic rats, and it was accompanied by destruction of the retinal structures, leakage from the retinal vasculature, and blockage of the capillaries

(Miyamoto et al., 1999; Tsujikawa et al., 1998). Persistent low-grade inflammation, which causes up-regulation of inflammatory mediators and mechanical blockage of retinal capillaries due to plugged leukocytes, is thought to contribute to damage of the retinal endothelial cells, leading to development of early- and late-stage DR (Tang and Kern, 2011). Xu et al. (2004) reported that shear stress decreased during the inflammatory reaction in the retinal veins of mice and this reduction was correlated negatively with leukocyte rolling and adhesion in the postcapillary venules. Therefore, the current results of up-regulated gene expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin in HRMECs exposed to low shear stress may support the leukocyte-endothelial interactions particularly in hypoperfused venules. Furthermore, increased expression of cytokine/chemokine (IL-6, IL-8, PDGF-B, and MCP-1) mRNA in a low shear state may enhance recruitment of leukocytes to the endothelial surface. Therefore, these results might be related partly to chronic low-grade inflammation during disrupted blood flow, such as in DR.

We also focused on procoagulant gene expression in low shear stress, because high shear stress might contribute to the antithrombotic properties of retinal vessels

(up-regulation of eNOS and thrombomodulin genes) under physiologic conditions (Ishibazawa et al., 2011). Endothelial cells do not express TF except when exposed to inflammatory molecules such as tumor necrosis factor-alpha (Manly et al., 2011). The current observation of increased expression of TF mRNA in HRMECs exposed to low shear stress may support the phenomenon that low shear stress elicits inflammatory stimulation in HRMECs. Furthermore, the gene expression of PAI-1, an inhibitor of fibrinolysis, also was up-regulated under low shear conditions. Grant et al. (1996) reported that PAI-1 was overexpressed in the retinal capillaries of patients with diabetes with nonproliferative DR. Our findings of up-regulated expression of procoagulant genes suggested that low shear stress might contribute partly to the hypercoagulable state in the hypoperfused retina, resulting in capillary nonperfusion.

The current study had some limitations. First, we assumed that the shear stress level in the hypoperfused venules (especially the postcapillary venules) in retinal circulatory disorders was 1.5 dyne/cm^2. It is difficult to accurately measure shear stress especially in hypoperfused venules *in vivo*. Moreover, in preliminary experiments, we compared the effects of exposure to 0.75 dyne/cm^2 and 1.5 dyne/cm^2, and the latter had higher levels of

proinflammatory gene expression than the former (data not shown). Therefore, we used 1.5 dyne/cm^2, which was the predicted rate from the normal range of shear stress in small venules, as the decreased level of shear stress. Second, ICAM-1 mRNA also was up-regulated under relatively high-flow conditions (15 dyne/cm^2). This result corresponded to that of a previous study in which the shear stress (2.5-46 dyne/cm^2) increased ICAM-1 expression in a shear-force independent manner in other vascular endothelium (Nagel et al., 1994). Although ICAM-1 is widely known as a critical adhesion molecule in the retina in the presence of inflammation, ICAM-1 is expressed continuously on the endothelial surface at a certain level because the living body has continuous blood flow that generates continuous shear stress. In the current study, the ICAM-1 mRNA expression in the cells exposed to 1.5 dyne/cm^2 of stress increased slightly but not significantly more than in the cells exposed to 15 dyne/cm^2 of stress; therefore, this increase might be meaningful in the pathologic state in the retinal circulation. Further studies are essential for determining whether the decreased shear stress due to vascular hypoperfusion directly affects leukocyte-endothelial interactions in the retina. Third, we used commercially available HRMECs that had a mixed endothelial

population, although we discussed the phenotypic changes of the vascular endothelial cells especially in the retinal veins. The endothelia in the retinal vasculature are heterogeneous; there was clear evidence of differences in the endothelial cells between the retinal artery and vein in terms of morphometric characteristics (Kang et al., 2011) and the artery- or vein-specific gene expression in relation to such as the Notch pathway (Adams, 2003). However, the current results of the retinal endothelial response to relatively low shear stress might partly explain leukocyte recruitment and adhesion and the hypercoagulable state, especially in the impaired retinal circulation in the postcapillary venules.

In summary, the current results showed that low shear stress causes up-regulation of proinflammatory genes in HRMECs. This effect might be related to the pathophysiologic changes in retinal circulatory disorders.

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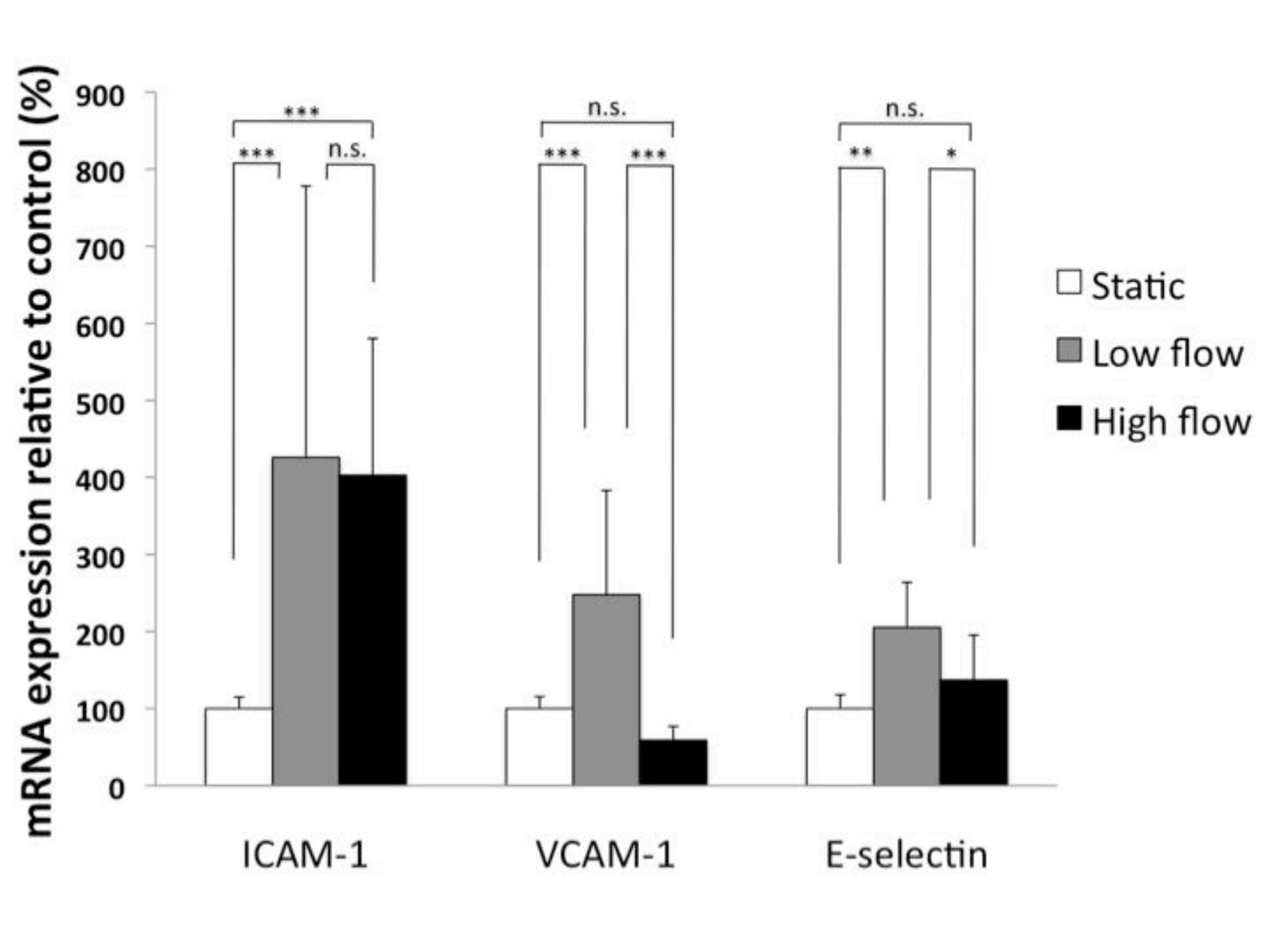
Legends

Fig. 1. Gene expression of adhesion molecules. The HRMECs were exposed to shear stress levels of 0 dyne/cm^2 (static control), 1.5 dyne/cm^2 (low flow), and 15 dyne/cm^2 (high flow). Values are expressed as the mean ± SEM (n=10). *P<0.05, **P<0.01, ***P<0.001.

Fig. 2. Gene expression of cytokines/chemokines and procoagulant factors. HRMECs were exposed to shear stress levels of 0 dyne/cm^2 (static control), 1.5 dyne/cm^2 (low flow), and 15 dyne/cm^2 (high flow). Values are expressed as the mean ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001.


mRNA expression relative to control (%)
900
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Static
Low flow
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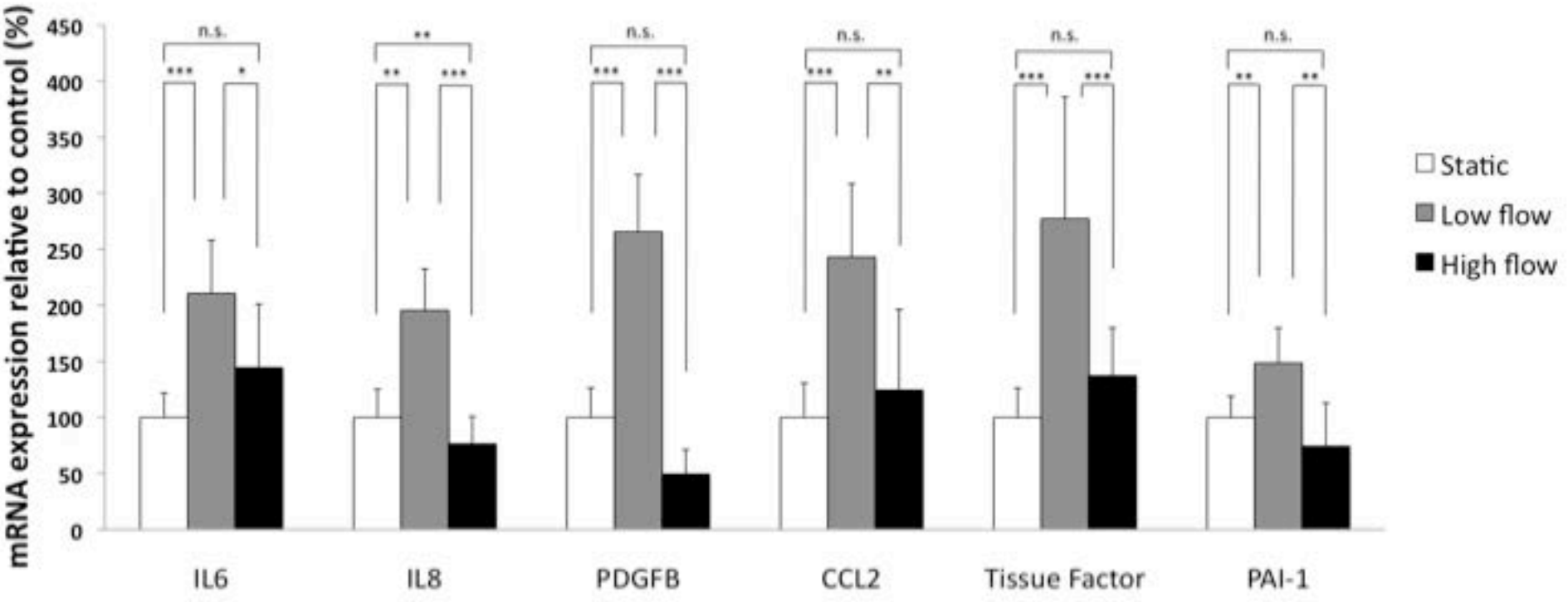
ICAM-1
VCAM-1
E-selectin


mRNA expression relative to control (%)
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IL6
IL8
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CCL2
Tissue Factor
PAI-1
Static
Low flow
High flow